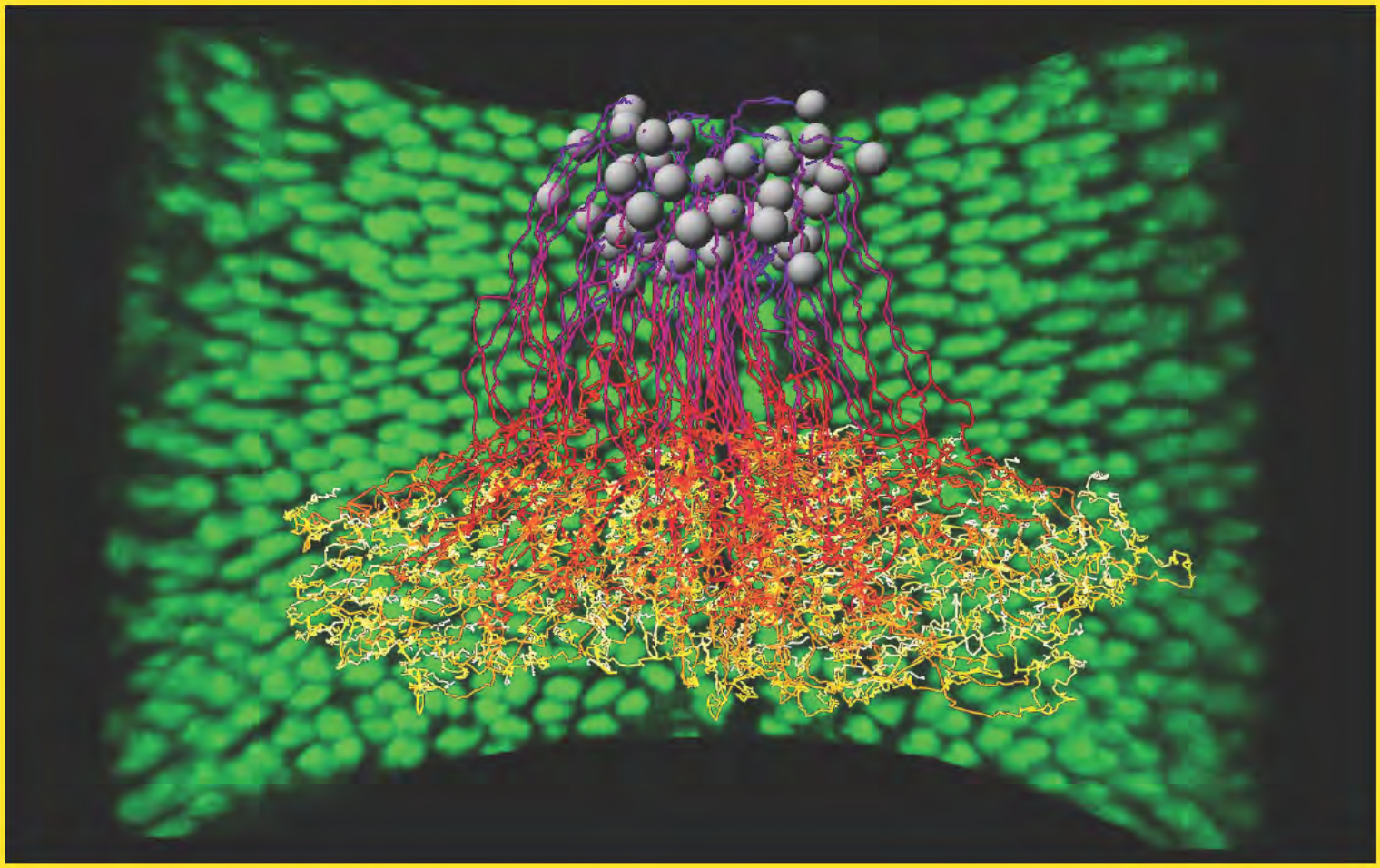

# CALTECH Biology Annual Report 2008

## Front Cover Illustration

The migratory path of mesoderm cells during gastrulation in *Drosophila* embryos. A dorsal view of mesoderm cell tracking data as they spread to form a monolayer during gastrulation. Cells are imaged live with a nuclear marker (Histone2A-gfp). The imaging data is then imported into data-processing software to follow the movement of each cell over time (colored lines, blue=early time points, yellow=late time points). The ectoderm, which is the substratum for mesoderm cell migration, is shown in the background for reference.

See abstract 376 by Amy McMahon, Willy Supatto, and Professors Scott E. Fraser and Angelike Stathopoulos.

(Angelike Stathopoulos lab)

## Back Cover Illustration

An intersegmental nerve in a third instar *Drosophila* larva. A single segment was duplicated and rotated to produce this hexagonal image. A confocal micrograph generated by Mutsuhito Kurusu and manipulated by Violana Nesterova.

See abstract 229 - Kai Zinn lab.

# Division of Biology

# California Institute of Technology

# Pasadena, California

# Annual Report
# 2007 - 2008

# BIOLOGY

Yolanda Duron, Annual Report Coordinator

**Research Reports**

Biological research summarized in this report covers the time period from June, 2007 through July, 2008. The annual report is not intended to serve as an official forum, since some portions of the research listed in this report have not yet been published. When referring to an individual abstract(s), special permission must be obtained from the investigator.

References to published papers cited throughout the report are listed at the end of each individual research report.

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**SEYMOUR BENZER, PH.D.**



**CHRISTOF KOCH, PH.D.**



**MASAKAZU KONISHI, PH.D.**



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# DEVELOPMENTAL AND REGULATORY BIOLOGY

**BRUCE A. HAY, PH.D.**



**SARKIS MAZMANIAN, PH.D.**



**ELLIOT M. MEYEROWITZ, PH.D.**

**ELLEN V. ROTHENBERG, PH.D.**



**ANGELIKE STATHOPOULOS, PH.D.**

# FACILITIES

# INTRODUCTION

**100 Years Ago: 1908**

Carl Spencer Millikan was appointed Professor of Biology at the Throop Polytechnic Institute, an early name for what is now Caltech. He replaced Joseph Grinnell, the noted mammalogist and ecologist, who departed from Throop to take the position of Founding Director of the Museum of Vertebrate Zoology at the University of California (Berkeley).

**75 Years Ago: 1933**

Thomas H. Morgan, founder and first Chair of the Biology Division was announced as the winner of the 1933 Nobel Prize in Physiology or Medicine for his pioneering work in genetics. He was Caltech's second Nobel Prize winner, and the first of the seven Biology Division professors who have so far received this honor.

**50 Years Ago: 1958**

From Biology 1958, the 1958 Annual Report:

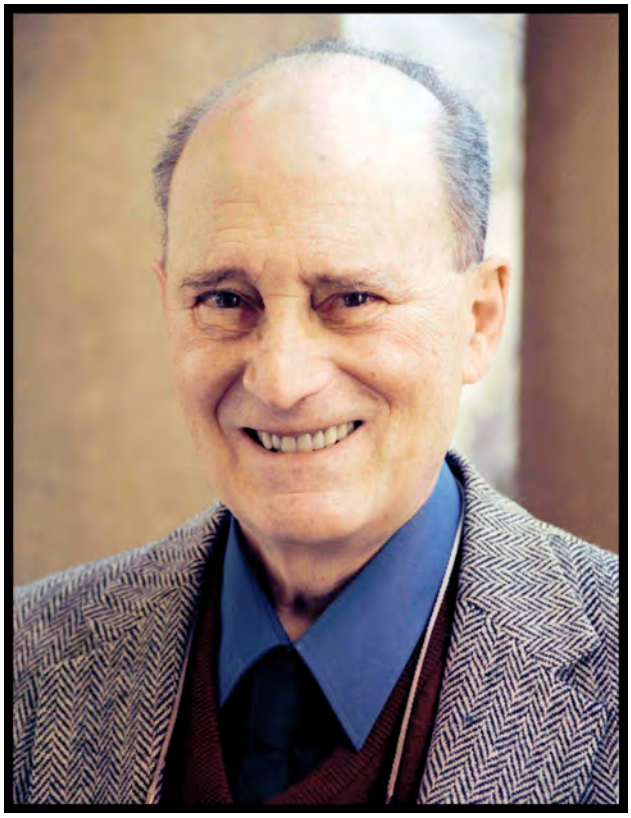
"During the past year Institute physiologists have discovered what appear to be new functions of glutamic acid. In dilutions of one part to 200,000 parts of water it causes contraction of crustacean muscle. It now seems possible that it may function as a 'neurohumor.'"

"In the Division reports of last year, a new method of characterizing large molecules was described. It is now clear that this method… will be widely useful. It is the so-called gradient-density method… During the past year the density-gradient method has been combined with isotope labeling by Institute Chemists and Biologists in a study of the manner in which DNA is replicated."

**25 Years Ago: 1983**

From the CALTECH Biology Annual Report 1983:

"Completion of the new Braun Laboratories… was marked by formal dedication ceremonies on December 13, 1982… In addition to the research groups of Professor Hood… the Biology half of Braun is now populated by research groups of our two new Professors, John Abelson and Melvin Simon. They will be joined in the fall of 1983 by a newly appointed Assistant Professor, Scott Emr."



**Grace C. Steele Professor of Molecular Biology, Giuseppe Attardi,** whose work linked degenerative diseases and aging to genetic mutations, died on April 5, 2008.

Attardi was among the first scientists to delve into the processes through which DNA's information is transferred. He identified all the genes of the DNA in human mitochondria--often called the powerhouses of biological cells. He then developed techniques for investigating genetic diseases, including Alzheimer's, and aging in general, which he discovered is associated with changes in mitochondrial DNA (mtDNA).

Born in 1923 in Vicari, Italy, a town of less than 3,000 people in the Province of Palermo, Attardi earned an MD from the University of Padua in 1947. He remained there for almost ten years as an assistant professor in the Institute for Histology and General Embryology. During those years, he also visited the Karolinska Institute in Stockholm, Sweden, as a research fellow in cell research and genetics, and the Washington University in St. Louis School of Medicine as a Fulbright Fellow.

Still on the Fulbright Fellowship, Attardi arrived at Caltech in 1959. He was appointed associate professor of molecular biology four years later. It was at Caltech that Attardi turned his interests to mitochondria, establishing that mtDNA is an active, working genome. This spurred research into the organelle's genetic machinery.

In recent years, researchers in Attardi's lab at Caltech have focused on how mtDNA replicates, and on detecting mutations that result from aging, and what effects those mutations have. The team discovered that older people carry a significantly greater number of genetic defects in a specific region of their mtDNA, suggesting that cell aging begins in the mitochondria.

During his career, Attardi garnered many distinctions. They include two Guggenheim Fellowships; election to the National Academy of Sciences; the Antonio Feltrinelli International Prize for Medicine from the Academia Nazionale dei Lincei; a degree of doctor *honoris causa* from the University of Zaragoza, Spain; the Passano Foundation Award in 2000; and the Gairdner Foundation International Prize.

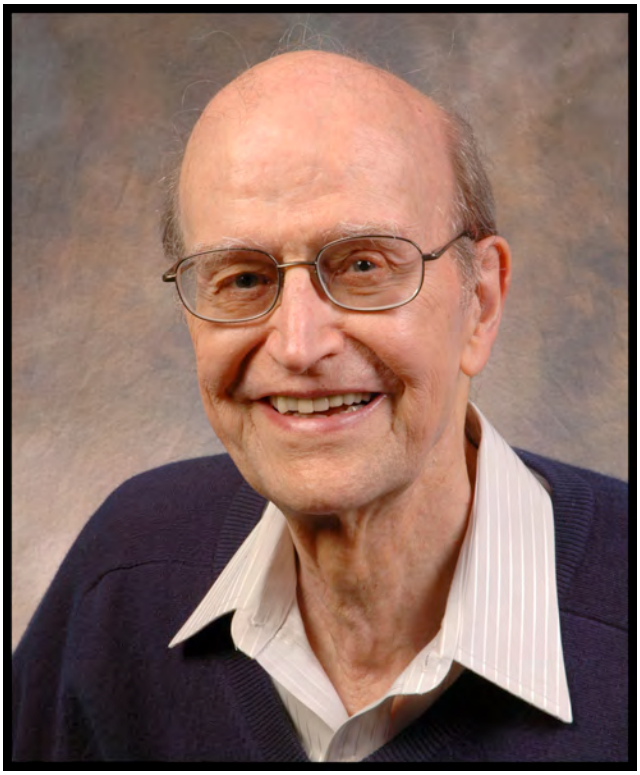
**James G. Boswell Professor of Neuroscience, Emeritus, Seymour Benzer**, a founder of the field of modern genetics, died on November 30, 2007.

Benzer's lasting impact on modern-day genetics can be seen in continuing work whose foundations he helped lay. Studies in gene mutations and regulation and in the genetic underpinnings of behavior can all be attributed to his groundbreaking research.

A native of New York City, Benzer attended Brooklyn College, earning a bachelor's degree in physics in 1942. After getting his Ph.D. in physics at Purdue University in 1947, he stayed on to teach the subject. A visit to Cold Spring Harbor Lab in 1948, followed by a two-year stint as a postdoctoral scholar at Caltech in the lab of Nobel Laureate Max Delbrück, introduced Benzer to the field of bacteriophage genetics, the study of viruses that infect bacteria. He immersed himself in it.

His work with bacteriophages led him to experiments with *Drosophila melanogaster*. He used mutants of this fruit fly to pioneer the field of neurogenetics, and his lab discovered the first circadian-rhythm mutants in a series of studies of how genes affect behavior. These experiments were replicated for other animal models and formed the foundation for the field of molecular biology of behavior. In his recent work, Benzer studied neurodegeneration in fruit flies in an attempt to find an approach for suppressing human diseases by modeling them, and for uncovering the genetics of aging.

Throughout a career that spanned physics, biophysics, molecular biology, and behavioral genetics, Benzer garnered many top honors. His memberships included the National Academy of Sciences, the Royal Society, and the American Academy of Arts and Sciences. He was awarded the National Medal of Science; the Wolf Prize in Medicine from Israel; the Crafoord Prize of the Royal Swedish Academy of Sciences; the International Prize for Biology from Japan; the Albert Lasker Award for Basic Medical Research; and the Albany Medical Center Prize. He was also one of the few two-time winners of the Gairdner International Award, which is often a precursor to the Nobel Prize.



# FERGUSON AWARD, 2008

**Koch Rutishauser Meyerowitz Mortazavi Wold**

**Dr. Ali Mortizavi** was co-recipient of the Ferguson prize for his thesis entitled: "Structure and Evolution of Mammalian Gene Networks." His thesis work is an investigation of the structure of mammalian gene networks and their evolution. He first used the neuronal transcriptional repressor NRSF/REST as his test case to work out new computational methods and functional methods that are needed in order to fully map transcription factor-target gene relationships in large mammalian genomes. His initial computational work showed how to leverage evolutionary conservation of binding motifs to successfully predict the vast majority of target binding sites in the genome for NRSF. Ali then developed the bioinformatics and computational underpinnings for the method called ChIP-Seq, and did all the analysis of the first ChIP-Seq study. This new functional genomics method uses ultra-high-throughput DNA sequencing to map all sites of interaction between a transcription factor and its *in vivo* targets. His approach differed from prior ones in its high resolution and in the ability to scale to full genome-wide dimension in the large human and mouse genomes. In that study, performed in collaboration the Myers lab at Stanford, Ali empirically defined the NRSF interactome. This validated the computationally predicted sites from his first study. He also defined new targets with novel variant binding motifs, a method which is now coming into wide use. Ali then worked with Brian Williams to develop RNA-Seq, which is a way to quantify the transcriptome, with particular sensitivity and specificity for low abundance RNAs and for mapping of RNA splicing isoforms. These RNA measurements, along with RNA polymerase ChIP-Seq data obtained by Ali, define the output of the networks of interest. With the new methods in hand, Ali applied them to study the evolution of entire NRSF interactome among multiple mammalian genomes. A key conclusion from that work is that there is a high level of plasticity in the interactome map of NRSF, as well as a highly conserved core set of target sites.

**Dr. Ueli Rutishauser** was co-winner of the Ferguson prize for his thesis entitled: "Learning and representation of declarative memories by single neurons in the human hippocampus and amygdala." Ueli Rutishauser's thesis work focused on a fundamental question in neuroscience - how is information stored in the brain when we learn and remember? Episodic memories allow us to remember not only that we have seen an item before but also where and when we have seen it (context). Neurons in the medial temporal lobe (MTL) of human and other animal brains are critically involved in the acquisition of such memories. Since events happen only once, the ability to distinguish novel from familiar stimuli is crucial to rapidly encode such events after a single exposure. Successful detection of novelty is necessary for many types of learning. To examine these various aspects of memory, Ueli conducted electrophysiological recordings of single neurons in the human hippocampus and amygdala, collaborating with Dr. Adam Mamelak, at Huntington Hospital to record from the brains of living human beings who are hospitalized prior to brain surgery. Ueli recorded the activity of individual neurons while subjects learned, remembered, and later retrieved, the identity and location (on the screen) of a set of images. Ueli made several fundamental discoveries. First, he found neurons that showed evidence of learning after exposure to a stimulus a single time (single-trial learning). While it is clear that humans and other animals can learn from a single experience, no one had ever seen neurons that changed their response properties this quickly. Ueli discovered two classes of neurons that exhibit single-trial learning: novelty and familiarity detectors, which show a selective increase in firing for new and old stimuli, respectively. The neurons retain memory for the stimulus for 24 h. During retrieval, these neurons distinguish stimuli that will be successfully recollected from stimuli that will not be recollected. Similarly, they distinguish between failed and successful recognition. Pictures that were forgotten by the patient still evoked a non-zero response. These data support a continuous strength of memory model: the stronger the neuronal response, the better the memory.

# PROFESSORIAL AWARDS, 2007 - 2008

**James G. Boswell Professor of Neuroscience, Richard A. Andersen**, presented the Notter Lecture at the University of Rochester, 2008.

**Max Delbrück Professor of Biology, Pamela J. Bjorkman, Investigator, Howard Hughes Medical Institute,** was elected a Fellow of the American Association for the Advancement of Science, 2007; and gave the Keynote Address, SBNet Structural Biology meeting, Tällberg, Sweden, 2007.

**Albert Billings Ruddock Professor of Biology, Marianne Bronner-Fraser,** received the following honors and awards: Honorary Professor, University College London; President-elect, Society for Developmental Biology; Board of Trustees, Gordon Research Conferences; NIDCR Council; Editor, *Developmental Biology*, *J. of Cell Biology*, *Molecular Biology of the Cell;* Scientific Advisory Board-Sontag Foundation; Scientific Advisory Board-March of Dimas Birth Defects Foundation; National Institute of Dental and Craniofacial Research Council; 2006-2009 Javits Award, NINDS.

**Associate Professor of Biology**, **Bren Scholar, David C. Chan**, Investigator, Howard Hughes Medical Institute, 2008.

**Professor of Biology, Investigator, Howard Hughes Medical Institute; Executive Officer for Biology, Raymond Deshaies,** was appointed a Fellow of the American Association for the Advancement of Science, 2007; received the Biology Undergraduate Student Advisory Committee (BUSAC) 2008 Biology Teaching Award.

**Esther M. and Abe M. Zarem Professor of Bioengineering, Michael H. Dickinson**, was inducted into the American Academy of Arts and Sciences (AAAS), 2008.

**Assistant Professor of Biology and Applied Physics; Bren Scholar, Michael Elowitz,** was awarded the MacArthur Fellowship in 2007; Investigator, Howard Hughes Medical Institute, 2008.

**Associate Professor of Biology, Grant J. Jensen**, was selected as one of the new 56 HHMI Investigators, 2008.

**George W. Beadle Professor of Biology and Division Chair Elliot M. Meyerowitz**, received an honorary doctoral degree (Docteur *Honoris Causa*) from the École Normale Supérieure, Lyon, France in June, 2007. He delivered a Plenary Lecture at the Joint Meeting of the British Genetics Society, British Society for Developmental Biology, and British Society for Cell Biology in Edinburgh on March 30, 2007; the Opening Lecture of the18th International *Arabidopsis* Congress in Beijing on June 21, 2007; the B.I.G. Lecture at the University of Lausanne on March 3, 2008 and the Mendel Lecture of the Academy of Science of the Czech Republic and Mendel Museum at the Mendel Museum in Brno on March 6, 2008.

**Professor of Biology, Shinsuke Shimojo,** was presented the "Most Creative Study Award" by the Japanese Society of Cognitive Science, June, 2008. He shared this award with his former collaborator, Professor Shin'ichi Ichikawa, University of Tokyo, for their work on counter-intuitiveness of Bayesian problems. He was awarded the "Nakayama Grand Prix" for his "significant contributions to the science of emotion," which was sponsored by the Nakayama Press, Japan, August, 2008.

**Howard and Gwen Laurie Smits Professor of Cell Biology, Alexander Varshavsky,** received the 2008 Gotham Prize in Cancer Research from the Gotham Foundation, New York, NY. Varshavsky also gave the 2008 Dulbecco Lecture at the Salk Institute for Biological Studies, La Jolla, CA.

### Other Awards

**Flora Hinz,** a Biology graduate student in Professor Erin Schuman's lab, received honorable mention as a Teaching Assistant this year during the Graduate Student Council (GSC) teaching and mentoring awards.

**Anna Maria Salazar**, received the Graduate Deans' Award for Outstanding Community Service, which is awarded to a Ph.D. candidate who, throughout his or her graduate years at the Institute, has made great contributions to graduate life and whose qualities of leadership and responsibility have been outstanding.

**The Biology Division hosted the following lecture:**

**Norman Davidson Lecture**
*Ron Davis*
May 7, 2008
**Genome Technology Center, Stanford University**
"Revolutionizing the practice of clinical medicine through innovations and cost effective technologies"

Photos from the Seymour Benzer Memorial, held March 14, 2008

Photos from the Giuseppe Attardi Memorial, held August 29, 2008

Photos from the Colloquium in honor of David Baltimore's 70th Birthday, held March 1, 2008

# BIOLOGY DIVISION STAFF

## INSTRUCTION AND RESEARCH

## ADMINISTRATION

# INSTRUCTION AND RESEARCH STAFF
# DIVISION OF BIOLOGY

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Raymond J. Deshaies, *Executive Officer for Biology*
Christof Koch, *Executive Officer for Neurobiology*

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*Professor of Biology*

John J. Hopfield, Ph.D.
*Roscoe G. Dickinson Professor of Chemistry and Biology*

Ray D. Owen, Ph.D., Sc.D.h.c.
*Professor of Biology*

Jean-Paul Revel, Ph.D.
*Albert Billings Ruddock Professor of Biology*

Melvin I. Simon, Ph.D.
*Anne P. and Benjamin F. Biaggini Professor of Biological Sciences*

James H. Strauss, Ph.D.
*Ethel Wilson Bowles and Robert Bowles Professor of Biology*

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*Professor of Biology*

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*Frank P. Hixon Professor of Neurobiology*

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*James G. Boswell Professor of Neuroscience*

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Investigator, Howard Hughes Medial Institute

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*Grace C. Steele Professor of Molecular Biology*

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**Support**: The work described in the following research reports has been supported by:
The Ellison Foundation
Bill and Melinda Gates Foundation
The Keck Foundation
National Institutes of Health
Skirball Foundation
The Waxman Foundation

**Summary**: The Baltimore laboratory has many different programs in progress. The largest is a program with the aim of providing a new method of therapy and potentially immunization against HIV/AIDS. This involves solving many independent problems. On the more basic side, we continue to investigate aspects of a key transcriptional regulatory protein we discovered more than 20 years ago, NF-κB. As part of our continuing interest in the immune system, we are also studying the roles of a series of microRNAs in innate and adaptive immune cell function and development. We finally have a few outlier studies that fall under the general rubric of infection and immunity: the evolution of influenza virus and the ability of a fetus to grow in a mother in spite of the immunologic differences between mother and fetus. Together, this work provides a rich set of objectives for an inventive group of postdoctoral fellows, graduate student and technical experts.

## 1. Engineering immunity to treat HIV and other dangerous pathogens

*Xin Luo, Ryan Michael O'Connell, Eun Mi Hur, Kenneth Yu, Alejandro Balazs, Anthony Walls, Emily Maarschalk, Christine Kivork, Sonal N. Patel, Jonathan Tsai, Angie Frausto, Lili Yang*[*]*, David Baltimore*

Supported by Bill and Melinda Gates Foundation through the Grand Challenges in Global Health Initiative, we are exploring a fundamentally new way of stimulating the immune system to fight off infectious diseases, focusing on HIV as a test of the concept. The premise of this project is that for some infections, including HIV, the immune system's natural responses are inherently inadequate, and therefore the traditional approach of using vaccines to stimulate and boost these responses is likely to be ineffective. As an alternative, we propose to genetically engineer immune cells that can produce adequate responses. Our work is intended to eventually lead to immunotherapy for people who are infected with HIV. It could also lead to new ways to prevent HIV infection.

Our strategy is to use gene therapy for expression of genes encoding neutralizing antibodies against HIV. Multiple problems must be solved to allow expression of designed antibody genes in the cells of infected people. We plan to implant genes in blood stem cells and allow the cells to give rise to B cells, the body's natural antibody-producing cells, requiring the solution of particular problems posed by the architecture of antibody genes. We must also design antibodies or antibody-like proteins that can efficiently neutralize the infectivity of HIV. This will be done by design methods or by selection. We must then prove the effectiveness of the design in human cells or in a mouse/human chimera. Finally, we have to drive the cost of the process to a low enough level to use in the less developed world. At low cost, it could even be a vaccine.

With the goal of developing this general method, captured by the phrase "Engineering Immunity" to treat infection by HIV and other dangerous pathogens, we have the following projects:

[*]*Lead Scientist and Project Manager*

**Project 1**
**To perfect lentiviral expression systems that can impart the anti-HIV specificities to the mouse antibody repertoire**
*Lili Yang, Anthony Walls*

The Baltimore laboratory has devised methods to generate functional T cells of defined antigen specificity in mice by retrovirus-mediated expression of T cell receptor genes in hematopoietic precursor cells (*Reference 1*). We will adapt these methods to generate functional B cells capable of secreting antibodies (Abs) or Ab-like proteins upon stimulation with a model antigen. For this purpose, we have constructed a retroviral vector that co-expresses a membrane-bound form of a hen egg lysozyme (HEL)-specific Ab, a secreting form of anti-HIV neutralizing Abs (Nab), and a GFP reporter gene. Upon transferring into HSCs, such a retrovector will direct the generation of "dual-specific" B cells in mouse that can undergo clonal expansion upon injection of HEL antigen, and secrete anti-HIV Abs. So far we have achieved the generation of HEL-specific B cells in mouse. These cells do undergo clonal expansion upon HEL stimulation *in vitro*, and when stimulated with HEL *in vivo*, can expand significantly, develop into plasma cells, and generate memory B cells. Most excitingly, repetitively immunizing the recipient mice with HEL antigen can bring up the serum anti-HIV Nab titer higher and higher. Currently we

are working to optimize the retrovector to achieve a therapeutic level of anti-HIV Nab.

**Project 2**

**To perfect lentiviral expression systems that can impart anti-HIV specificities to the human antibody repertoire**

*Kenneth Yu, Jonathan Tsai*

A natural B cell can produce both membrane-bound and secreted antibodies of a single specificity, and of a particular isotype, through molecular mechanisms of alternative splicing and isotype switching. The genetic machinery controlling these mechanisms is located within the 1Mb-length immunoglobulin heavy chain loci. We have demonstrated that it is possible to encompass these functions of the heavy chain locus in a compact 10kb lentiviral vector by taking advantage of the inefficient cleavage of virus-derived 2A peptides. We constructed fusion molecules of secretory IgG and IgM with the transmembrane domain of the IgM B-cell receptor (BCR) using different 2A peptides, chosen or designed for inefficiency. We hypothesized that inefficient cleavage at the 2A peptides will give rise to both secretory Ig and membrane bound BCR. We call this a "synthetic switch." When expressed in mammalian cell lines, these constructs produced functional secretory antibody and BCR. Using the engineering immunity approach, we expect to transduce human $CD34^+$ HSCs with a lentivector carrying this construct, and program the HSCs to develop into fully functional HIV-specific B cells that can respond to HIV or some other antigen and secrete a high titer of anti-HIV Nabs. The engineered lentivectors will be tested *in vitro* in a human B lymphopoiesis culture system (described in Project 3), and *in vivo* in a human immune system (HIS) mouse model (described in Project 4). Currently, we are testing the synthetic switch in inducible B cell lines and developing the virus production methodology that will enable high titer production of lentiviral vectors that contain RNA processing signals.

**Project 3**

**To program human B cells to produce an anti-HIV neutralizing antibody**

*Xin Luo, Emily Maarschalk*

It is widely accepted that the most effective method against the AIDS epidemic is a vaccine that can stimulate the production of anti-HIV antibodies. We hypothesize that, by infecting hematopoietic progenitor cells (HPCs) with lentiviral vectors encoding specific anti-HIV neutralizing antibodies, the immune system of individuals at risk for AIDS can be engineered to provide a life-long supply of such antibodies, so that infection and subsequent disease upon exposure to HIV can be prevented. To test this hypothesis, we have established a two-stage, *in vitro* culture system that supports normal human B-lineage development from HPCs to antibody-secreting plasma cells. By transducing human cord blood $CD34^+$ cells with lentiviral vectors encoding secretory b12 IgG, we were able to program human B cells to produce this anti-HIV neutralizing antibody. Our results suggest that HIV vaccination can be delivered by autologous transplantation of *in vitro*-programmed HPCs, which would develop into antibody-secreting B cells *in vivo* and provide a continuous supply of anti-HIV neutralizing antibodies.

**Project 4**

**To construct anti-HIV-producing lentiviral vectors and test *in vivo* using a HIS (Human Immune System) HIV infection mouse model**

*Ryan Michael O'Connell, Alejandro Balazs, Christine Kivork*

One major obstacle preventing the development of vaccines against HIV has been the lack of small animal models of infection to test novel approaches. Manz and co-workers have recently demonstrated that intrahepatic injection of human $CD34^+$ cord blood stem cells in newborn $RAG2^{-/-}g_c^{-/-}$ mice results in reconstitution of a human adaptive immune system in mice (Traggiai *et al*., 2004). The transplanted, or Human Immune System (HIS), mice develop human B, T and dendritic cells, generate structured primary and secondary lymphoid organs, and establish a functional immune response. Importantly, HIS mice are susceptible to infection by HIV.

We have successfully established the HIS mouse model in our lab. Methods to further enhance human cell engraftment are being explored. The first method is to deliver certain transgenes to CD34 cells to improve survival of human lymphocytes; along this line we have found that expression of BCL2 can lead to improved engraftment levels as assayed by luciferase expression. The second method is to use lentiviral vectors to systematically deliver specific growth factors and cytokines to HIS mice to assist the human graft development. As the cross-reactivity between human and mouse cytokines has its limitations, this approach will hopefully provide the necessary human cytokines to developing cells of human hematopoietic origin. Our initial pick is IL-7, and we are in the process of testing how the supply of this cytokine influences the human cell graft in HIS mice. Finally, we have challenged HIS mice with HIV, and have detected HIV infection and CD4 cell depletion in the infected mice. We are still in the process of analyzing the animals at various infection stages. On the other hand, we are continuously optimizing the lentivector expression system to achieve the best production of Nabs in the HIS mouse model, using strategies including codon optimization, promoter swapping and gene formatting. In addition, to facilitate the detection of individual neutralizing antibody, we created epitope-tagged neutralizing antibodies by fusing sequences encoding HA, Myc, and V5 tags to the C-terminus of the light chains of our four antibody vectors, and have shown that such modifications do not interfere with the function of the antibodies.

**Project 5**

**To construct lentiviral vectors encoding the IgA isotype of anti-HIV neutralizing antibodies and study their application to treat HIV infection**

*Eun Mi Hur, Sonal N. Patel*

Because mucosal transmission of virus is the major route of HIV infection in humans, establishing mucosal protection with effective neutralizing antibodies, such as IgA, secreted to mucosal sites is one of the objectives in this study. We constructed the human IgA form of anti-HIV neutralizing antibodies b12, 2F5, 2G12 in mammalian expression vector and lentivirus vector encoding heavy and light (lambda) chains of these antibodies and human immunoglobulin J chain and expressed them in human cell line. The interaction of b12 IgA and polymeric immunoglobulin receptor was tested *in vitro*. We also tested whether this engineered human IgA is efficiently transported through polymeric immunoglobulin receptor on mouse epithelium to mucosal sites such as gut and genital tract *in vivo*. Intravenously injected b12 IgA was detected in mucosal secretions collected in female genital tracts, small intestines and bronchial tracts in $Rag2^{-/-}\gamma C^{-/}$ mice. The efficiency of transport of b12 IgA varied but the antibody concentration was maintained stably at mucosal sites until 24 h post-injection *in vivo*. We also found oral administration of retinoic acid enhances homing of human T cells to mucosal sites of mice engrafted with human immune cells, suggesting that retinoic acid treatment can improve the efficiency of HIV-1 infection in humanized animal model. We are currently developing mice that stably produce b12 IgA *in vivo*, by injecting cells transduced with lentivirus vector expressing the antibody. The protective effect of the antibody from HIV-1 infection will be tested through mucosal challenge in humanized animal model.

**Project 6**

**To engineer bispecific and other designer anti-HIV antibodies and evaluate them for binding to and neutralizing HIV**

*Dr. Pamela Bjorkman's group at Caltech performs this objective.*

Using protein engineering and design methods, we are attempting to make protein reagents that will be more potent than existing neutralizing antibodies. This will allow them to be effective at lower concentrations in the blood or in secretions. We are pursuing several approaches, such as making bispecific Abs containing two different combining sites. We are expressing, purifying and determining affinities and an *in vitro* neutralization potencies of the following Abs and Ab-like proteins: chimeric proteins containing the gp120-binding domains of CD4 linked to a CD4-induced antibody specificity (in a variety of formats) and multimeric forms of neutralizing antibodies. For further information, see the Pamela Bjorkman lab abstracts, numbers 14 and 15.

**Project 7**

**To engineer the lentivector delivery system for the application of engineering immunity approach**

*This objective is performed by Leslie Bailey from Dr. Pin Wang's group at USC.*

We have identified an efficient and flexible method to target lentivirus-mediated gene transduction to a pre-determined cell type (Reference 2). It involves incorporation of a cell-specific binding determinant (CBD) and a fusogenic protein as two distinct entities into the lentiviral surface. The fusogen is modified so that it will not bind to its cognate receptor, so the CBD determines the targeting specificity. Using CD20 as a target antigen for human B cells, we have demonstrated that this targeting strategy is effective both *in vitro* and in intact animals. This methodology is flexible and can be extended to other forms of cell-type specific recognition to mediate targeting. The only requirement is that the antibody (or other binding protein) must be endocytosed after interaction with its cell-surface binding determinant. The technique allows for targeting of gene therapy by direct injection of a vector.

Hematopoietic stem cells (HSCs) are capable of differentiating into the entire blood system including cells important for the immune system and can provide a life-long source of a therapeutic gene. We have used our targeting method to target CD34, the presently most reliable marker for HSCs. By displaying an anti-CD34 antibody, as well as the fusogenic protein on the viral surface we were able to efficiently and selectively transduce CD34+ cells lines *in vitro* and *in vivo* in a xenografted tumor mouse model. We have performed a pilot study using the engineered lentivector that carries a luciferase reporter gene to target the engrafted human CD34+ HSCs in the HIS mouse model, and observed some promising results. Currently we are analyzing the experiment in details and will continue to perfect the targeting vector to achieve satisfactory gene delivery efficiency. Our future plan is to use the CD34-targeting lentivectors to delivery anti-HIV neutralizing antibody genes into HSCs to treat HIV infection.

*Note*: This project is a joint effort of three groups: David Baltimore and Pamela Bjorkman's groups at Caltech, and Pin Wang's group at USC.

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# 2. NF-κB

**Methylation of NF-κB**

*Chee-Kwee Ea, David Baltimore*

NF-κB undergoes several post-translational modifications upon activation, including phosphorylation, ubiquitination, nitrosylation, and acetylation. These regulatory modifications have distinct functional consequences. For example, acetylation of RelA at K218 and K221 inhibits IκBα binding and enhances DNA-binding; while acetylation of RelA at K122 and K123 inhibits its transcriptional activity. Protein methylation is another interesting post-translational modification. Protein can be post-translationally methylated at lysine, arginine, histidine and dicarboxylic amino acids by highly specific methyltransferases. The addition of methyl groups to the ε-amine of a lysine residue results in the formation of mono-, di-, and tri-methyl-lysines. This process can be reversed by demethylases. Histone is one of the best-studied proteins that undergo methylation. Specific sites of methylation on histones correlate with either activation or repression of transcription. Recently, several transcription factors, including p53, STAT1, RARα, and ERα , have been shown to be methylated and this modification affected their biological activities as transcription factors. Thus, we speculate that NF-κB may also be regulated by methylation.

The goal of this project is to: 1) to determine if NF-κB is methylated in response to stimulation; and 2) to understand the functions of NF-κB methylation. As a start, we simply asked if methylation has any role in the NF-κB pathway. We blocked methylation reactions in cells with a broad methylation inhibitor, 5´-deoxy-5´-methylthioadenosine (MTA), and found that it inhibited TNFα -induced NF-κB activation in a dose-dependent manner. We further found that MTA-treatment blocked neither degradation of IκBα nor nuclear translocation of RelA, indicating that MTA inhibits a step in NF-κB activation that is independent of the nuclear translocation process. Next, we tested if any methyltransferase methylates RelA *in vitro* by adopting an *in vitro* methylation assay reported by S. Chuikov *et al.* By using recombinant RelA fragments that cover all the lysine residues of RelA as substrates, we screened for methyltransferases that can methylate RelA *in vitro*. All the recombinant methyltransferases were expressed and purified from either *E. coli* or 293T cells. Up to this point, we have found that RelA is methylated by Smyd3 *in vitro.* Smyd3-mediated methylation was specific to RelA because p50 and TIFA were not methylated under the same experimental conditions. We further found that Smyd3-mediated methylation occurred within the N-terminal segment of the Rel-homology domain (RHD) of RelA (a.a. 1-110, p65N1). Smyd3 is a member of a subfamily of the methyltransferases that contains a SET domain (named for Su(var03-9), Enhancer of zeste, and Trithorax). This family of proteins is defined by a SET domain that is split into two segments by an MYND domain, followed by a cysteine-rich post SET domain. It has been shown that SMYD3 has histone H3-lysine 4 (H3-K4)-specific methyltransferase activity and it is overexpressed in colorectal carcinomas, hepatocellular carcinomas and breast cancer cell. In addition, we further observed that overexpression of wild-type Smyd3 in 293T cells, but not the methyltransferase-dead mutant Smyd3 (Y239F), enhanced TNFα-induced NF-κB-luciferase reporter. This result suggests that the methyltransferase activity is essential for Smyd3 function. On the other hand, knocking down expression of Smyd3 by RNA interference (RNAi) greatly reduced TNFα-mediated NF-κB-luciferase reporter. Moreover, the induction of several NF-κB regulated genes was greatly reduced in TNFα-treated Smyd3 knocked-down cells as determined by quantitative-PCR. These results demonstrate that Smyd3 is essential for TNFα-mediated NF-κB activation. To better understand the role of Smyd3 in TNFα-induced NF-κB activation, we checked IκBα degradation, nuclear translocation, and DNA binding of RelA after TNFα stimulation. Consistent with the effects of MTA, knockdown of the expression of Smyd3 affected DNA binding of RelA but neither degradation of IκBα nor nuclear translocation of RelA. Taken together, our results indicate that Smyd3 is not required for activation of IKK but is required for inducing DNA binding of NF-κB in the TNF signaling pathway. These results set the stage for a detailed study of the biological function of RelA methylation and we hypothesize that Smyd3 regulates DNA binding of NF-κB by promoting the methylation of RelA.

**Modulation of NF-κB action by O-GlcNAc glycosylation**

*Parameswaran Ramakrishnan, David Baltimore*

O-GlcNAc glycosylation is an abundant post-translational modification of serine or threonine residues occurring in nuclear and cytoplasmic proteins. Alterations in O-GlcNAcylation are linked to the pathology of diabetes and to neurological disorders through its influence on the activity and stability of proteins, as well as transcriptional regulation. NF-κB represents a family of transcription factors playing a pivotal role in diverse biological processes including inflammation and neuronal plasticity. Although NF-κB regulation by phosphorylation, ubiquitination and acetylation is well studied, much less is known about the O-GlcNAcylated forms of NF-κB. We are studying the modification of the NF-κB proteins by O-GlcNAc and its role in transcriptional regulation using cellular systems and animal models. In the initial phase, we will focus on identifying O-GlcNAcylated NF-κB proteins and map the sites of O-GlcNAc attachment by a glyco-proteomic approach. In the second phase, we will study the significance of O-GlcNAcylation of NF-κB by mutating the O-GlcNAc target site(s) and ascertaining the impact of these mutations on the stability, localization and activation of NF-κB. Thirdly, we will examine the influence of

O-GlcNAcylation on NF-κB transactivation and target gene specificity. These findings will significantly advance understanding the role of O-GlcNAcylated NF-κB in normal physiology, as well as in disease conditions like hyperglycemia and neuronal disorders, where both NF-κB and O-GlcNAc dysregulation have been implicated.

**mRNA stability is a internal timer of inflammatory events**

*Shengli Hao, David Baltimore*

The inflammatory response is a cellular response to infection and tissue injury that kills and removes invading microorganisms and restores tissue architecture. For long-lived animals, a rapidly initiated inflammatory response is critical for survival. This response involves a multitude of events that play out over time in a reproducible and organized manner and yet a single transcription factor, NF-κB, is mainly responsible for the activation. Understanding how such complexity can emerge from a seemingly simple transcriptional activation is challenging. Here we look at genes activated by proinflammatory materials in cultured cells and show that they can roughly be divided into three classes: I, rapidly induced and rapidly shut down genes; II, rapidly induced genes whose transcripts are maintained over time and III, genes whose transcripts accumulate slowly. The functions of the products of these three classes fall into three stages of inflammation: very early, somewhat later and quite late in the process. The patterns of gene activation are evident in various cell types. The classes are partly determined by the rapidity of transcriptional activation of the genes but a key determinant of the class of a particular gene is the stability of the mRNA for that gene. This is a consequence of the structure of the 3' untranslated segment of the mRNA. We propose that much of the orchestration of the inflammatory response is intrinsic to the genes that encode the different players in the response.

## 3. miRNA

**Molecular mechanisms of modulation of TNF receptor signaling by A20 and B94 proteins**

*Mark P. Boldin, Konstantin D. Taganov, David Baltimore*

Members of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily play a crucial role in activation, proliferation, survival and death of cells in the immune system. The prototype death receptor of the family is the type I TNF receptor (TNFR1). The biological functions triggered by the TNFR1 are characterized by a remarkable duality – infliction of tissue damage and cell death goes hand in hand with activation of tissue repair and expansion. The physiological reason for this duality lies in the ability of this receptor to trigger two kinds of intracellular signaling programs: a proteolytic cell death cascade and a number of kinase cascades leading to activation of gene expression, some of which may protect cells against cytotoxicity. TNF activates hundreds of genes at the transcriptional level of which a significant portion encodes intracellular modulators of the TNF receptor signaling or regulators of crosstalk between the TNF receptors and other signaling systems. Molecular mechanisms of action of two such regulatory molecules, called A20 and B94, are at the focus of our research.

While much knowledge has been accumulated in the past about activation of the core TNF signaling pathways, very little is known about their regulation and termination. A20 is a TNF-inducible gene that works in a negative feedback loop fashion and inhibits the TNFR1 signaling. We are currently trying to understand the molecular mechanism of A20 action and its target(s) in the TNF signaling pathways using a combination of proteomics and molecular biology approaches. We have recently discovered that the C-terminal part of A20 molecule, composed of seven zinc fingers, represents a novel type of ubiquitin-binding domain that can interact specifically with Lysine-63 (K63)-linked polyubiquitin chains. Polyubiquitin chains assembled through Lysine-48 (K48) of ubiquitin act as a signal for substrate proteolysis by 26S proteasomes, whereas chains assembled through K63 play a nonproteolytic role in activating NF-κB signaling, DNA repair and protein trafficking. We found that A20 molecule binds and sequesters K63-linked polyubiquitin chains on a key adapter molecule in TNF receptor signaling, RIP1 and thus, prevents the sequential activation of downstream IKK kinase complex.

B94 is an intracellular protein, which, like A20, is induced by TNF and other proinflammatory stimuli. Our preliminary findings indicate that B94 can act as a positive modulator in the NF-κB activation pathway. We plan to use B94 RNAi knockdown cell lines and B94 gene knockout mice to clearly establish the physiological role of this gene. We are also addressing the question of the molecular mechanism of action of B94 utilizing a combination of yeast two-hybrid and proteomics techniques.

**Analysis of regulation and function of microRNAs of miR-146 family**

*Konstantin D. Taganov, Mark P. Boldin, David Baltimore*

Inflammation is a complex, highly regulated defense reaction orchestrated by the host in response to an invading pathogen or injury. The conserved pathogen molecules are sensed by the innate immune cells such as macrophages and dendritic cells via a limited number of pattern-recognition receptors of the Toll-like receptor (TLR) family. Once activated through their TLRs, innate immune cells orchestrate the immune response aimed at the containment and destruction of the pathogen, as well as the activation of the adaptive immune system. TLR signaling has layers of negative regulation, since its overactivation can be deleterious to the organism and is the cause of multiple systemic and local inflammatory and autoimmune diseases.

MicroRNAs (miRNA) represent a class of tiny (~22nt) endogenous RNAs found in most metazoan organisms, as well as in viruses. Owing to their ability to

regulate expression of almost any protein-coding target gene, miRNAs are implicated in a variety of biological processes in animals. Recently, we have carried out a systematic effort to identify miRNAs that might play a role in the mammalian response to bacterial infection [*]. We identified three miRNAs that are sharply upregulated (miR-146, miR-132 and miR-155) in response to LPS treatment in monocytic cells, and performed a detailed analysis of miR-146 expression upon challenge with various microbial components and proinflammatory cytokines. Our findings suggest that miR-146 may function as a novel negative regulator of immune receptor signaling through post-transcriptional regulation of two key adapter molecules downstream of Toll-like and IL-1 receptors, TRAF6 and IRAK1.

Human genome, as well as most mammalian genomes, contains two mir-146 genes located on chromosome 5 (miR-146a) and 10 (miR-146b). We observed significant up-regulation of miR-146b primary transcript in response to LPS treatment, while its corresponding pre-miR and mature forms, in contrast to miR-146a, were below the detection level, suggesting that processing of this microRNA is controlled by different mechanism. Thus, mature miR-146a/b have distinct modes of regulation at levels of processing and transcription, and considering the nucleotide sequence difference between them, might contribute to the gene regulation in a very complex way. To advance our knowledge of the physiological role of miR-146 family in immune signaling, we are examining the contribution of these miRNAs to the development of various hematopoetic cell lineages, as well as to the immune response against bacterial pathogens in miR-146a knockout mice that we have recently generated. We plan to complement this work by gain-of-function studies using adoptive transfer experiments and transgenic mice overexpressing miR-146a in various hematopoietic compartments.

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**Investigating the impact of inflammation-induced microRNA-155 on hematopoietic cell development, function and disease**

*Ryan M. O'Connell, Dinesh S. Rao, Aadel A. Chaudhuri, Mark P. Boldin, Konstantin D. Taganov, John Nicoll, Ronald L. Paquette, David Baltimore*

Mammalian microRNAs (miRNAs) are emerging as key regulators of the development and function of the immune system. However, little is known regarding the role of miRNAs during inflammatory responses to infection. We have found that primary macrophages specifically upregulate miR-155 to high levels following exposure to a variety of microbial products and inflammatory cytokines. We also observe a strong but transient induction of miR-155 in mouse bone marrow after injection of bacterial LPS correlated with granulocyte/monocyte (GM) expansion. Demonstrating the sufficiency of miR-155 to drive GM expansion, enforced expression in mouse bone marrow cells causes GM proliferation in a manner reminiscent of LPS treatment. However, the miR-155-induced GM populations display pathological features characteristic of myeloid neoplasia. Extending possible relevance to human disease, miR-155 is overexpressed in the bone marrow of patients with acute myeloid leukemia (AML). Additionally, our results also find that miR-155 directly represses a subset of genes implicated in hematopoietic development and disease. Taken together, these data implicate miR-155 as a contributor to physiological GM expansion during inflammation and to certain pathological features associated with AML. Such a scenario emphasizes the importance of proper miR-155 regulation in developing myeloid cells during times of inflammatory stress.

**MicroRNA, B-cell development and B-cell neoplasia**

*Dinesh S. Rao, Ryan M. O'Connell, Aadel A. Chaudhuri, David Baltimore*

MicroRNAs are small non-coding RNAs that have important effects on hematopoiesis and in B-lymphopoiesis. B-cell development has been carefully studied and correlated with the proposed cell of origin in B-cell neoplasia. Interestingly, B-cell neoplasia most commonly occurs at stages of B-cell development that involve DNA rearrangement. We are interested in studying how microRNA-34 affects B-lymphoid development, and how it may be disrupted in cancer. miR-34 is a transcriptional target of the tumor suppressor protein Trp53, and analysis of the targets of miR-34 reveals a large number of targets involved in cancer initiation and progression. In addition, we have shown that miR-34a is induced during activation of B-lymphocytes, and that ectopic expression results in disrupted B-lymphopoiesis. We are continuing to characterize the defect in murine bone marrow transfer models and to determine which of the predicted targets results in this defect. Lastly, we will analyze whether miR-34 inhibits oncogenesis by B-cell oncogenes that it is predicted to target. These studies promise to reveal new and important connections between DNA damage pathways, B-cell development and B-cell neoplasia.

**Identification of an ultraviolet radiation-induced microRNAs**

*Thomas Su, David Baltimore*

Ultraviolet (UV) light causes many negative effects on the skin, including inflammation, pigment change, and skin cancer progression. On the cellular level, UV radiation is a major stressor to keratinocytes. An important consequence of cellular stress is the upregulation of specific microRNAs. MicroRNAs compose a family of non-coding RNAs recently found to provide a crucial post-transcriptional level of gene regulation in various cellular processes, including inflammation and carcinogenesis. Despite our knowledge of microRNAs and their response in cellular stress, it is not yet known if microRNAs play a

role in a very common stressor to the skin, UV radiation. Our current project aims to investigate the role of microRNAs in UV radiation. From an initial gene chip-based screen, we identified a few specific microRNAs that are upregulated upon UV irradiation. Currently, we are characterizing the regulation of these UV-induced microRNAs, as well as their functional role in the cell. Results from these experiments will provide a more complete understanding of the molecular changes following UV irradiation on the skin. Furthermore, an improved molecular characterization of the UV response may also provide novel therapeutic targets for a variety of UV dependent dermatologic conditions, from inflammation to skin carcinogenesis.

## 4. Outliers

**Immune regulation at the maternal - fetal interface**
*Daniel A. Kahn, David Baltimore*

For nearly 60 years, the immunologic paradox regarding the apparent tolerance of both the maternal and fetal immune systems during pregnancy has eluded understanding.

Recent understanding of the regulation of autoimmunity has found that naive T helper cells have the potential to become one of four well recognized subtypes: $T_H1$ (cellular immunity), $T_H2$ (humoral immunity), Treg (regulatory), and $T_H17$ (proinflammatory). Each subtype appears to cross regulate each other and the balance between Treg and $T_H17$ appears crucial for the development of autoimmune disease.

A major mystery in obstetrics is preeclampsia. This pregnancy specific disease can only cured by delivery and accounts for a significant amount of maternal and neonatal morbidity. The clinical risk factors (e.g., change in paternity) point to a potential immunologic etiology.

The central hypothesis that we are testing is that pregnancy activates immune regulatory cells in both the fetus and the mother as a result of antigenic differences.

In order to address this central hypothesis, three main sub-hypothesis are currently under investigation:

1. Pregnancy in the murine model induces an accumulation (in both quantity and function) of Tregs in both the fetal and maternal compartments.
2. Deviation toward $T_H17$ cells leads to adverse pregnancy outcome in the murine model.
3. Maternal and fetal immune regulatory cells are induced by single antigen differences such as the maternal awareness of male fetuses.

Early results have found an accumulation of Tregs in the pelvic lymph nodes compared with spleen and blood with advancing gestation. Additionally, there is demonstrable recall response to male antigens *in vitro* in female mice that have previously carried pregnancies, that is not seen in virgin mice.

**Anti-retroviral therapy mechanism determines stochasticity in HIV evolution**
*Alex Sigal, David Baltimore*

In the absence of a way to clear HIV completely, current anti-retroviral therapy (ART) relies on drugs to contain the virus to at times undetectable levels[1]. However, HIV infection is able to escape the suppressive effects of drugs by the evolution of drug resistance[2-4]. Understanding this evolutionary process is important in order to optimize drug regimens to delay the onset of resistance. Anti-retroviral therapy takes two predominant forms. One is reduction of the frequency of productive infection. This is the mechanism of reverse transcriptase inhibitors. The other is reduction of effective viral burst size once a cell has been infected. This is the mechanism of protease inhibitors[5]. Other current and future therapies are also expected to work by one of these mechanisms. The therapeutic agents are used in combination, and different combinations may give a similar reduction in the number of infected cells[6], but may use varying ratios of reduction of HIV infection frequency relative to reduction of HIV burst size. Here we investigate the rate of evolution of drug resistance, when infection frequency is decreased, relative to when burst size is decreased using both quantitative experiments and modeling approaches. This study should enable the design of more optimal drug combinations that would delay the onset of drug resistance in HIV infected patients.

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**Biophysical underpinnings of the antigenic evolvability of influenza hemagglutinin**
*Jesse D. Bloom, David Baltimore*

Our research focuses on understanding the remarkable ability of influenza to evade humoral immunity by rapidly evolving its hemagglutinin glycoprotein. We are working to define the biophysical properties that underly hemagglutinin's antigenic evolvability. To this end, one initial goal has been to develop a system for rapidly and quantitatively assaying antigenic evolvability. The basic idea is to generate a library of viral mutants, and then accurately quantify the fraction of these mutants that

evade antibody neutralization. We can then observe how this fraction of escape mutants (which can be thought of as a measure of antigenic evolvability) changes upon perturbations to the virus. We have successfully expressed an anti-hemagglutinin antibody and demonstrated that it potently neutralizes the x31 influenza strain. We have also been working on developing a high-throughput fluorescence-based assay for viral infection in tissue culture.

Our second initial goal has been to develop a method for predicting mutations to influenza hemagglutinin that enhance viral evolvability. The hypothesis that has guided this work is that mutations that stabilize hemagglutinin protein will enable antigenic escape by "buffering" other escape mutation. In order to predict which mutations will be buffering, we have used the idea that stabilizing mutations should be fixed more frequently by evolution. This approach reverses the usual tactic taken in phylogenetic tree reconstruction. In those approaches, an average substitution model $\boldsymbol{S}$ (typically based on a BLOSUM, PAM, or DAYHOFF matrix) is assumed, and the phylogenetic tree $\boldsymbol{T}$ is chosen that maximizes the probability of the observed sequence alignment $\boldsymbol{A}$, $\max_T \Pr(\boldsymbol{A} \mid \boldsymbol{T}, \boldsymbol{S}) \cdot \Pr(\boldsymbol{T})$ where some (often uniform) prior is chosen for $\boldsymbol{T}$. In contrast, we have used established methods to infer the phylogenetic tree and then written a computer program to find the position-specific probabilities that solve $\max_S \Pr(\boldsymbol{A} \mid \boldsymbol{T}, \boldsymbol{S}) \cdot \Pr(\boldsymbol{S})$ where the prior over $\boldsymbol{S}$ is chosen to provide structure specific information. These position-specific substitution probabilities can then be related to the stability effects of the mutation.

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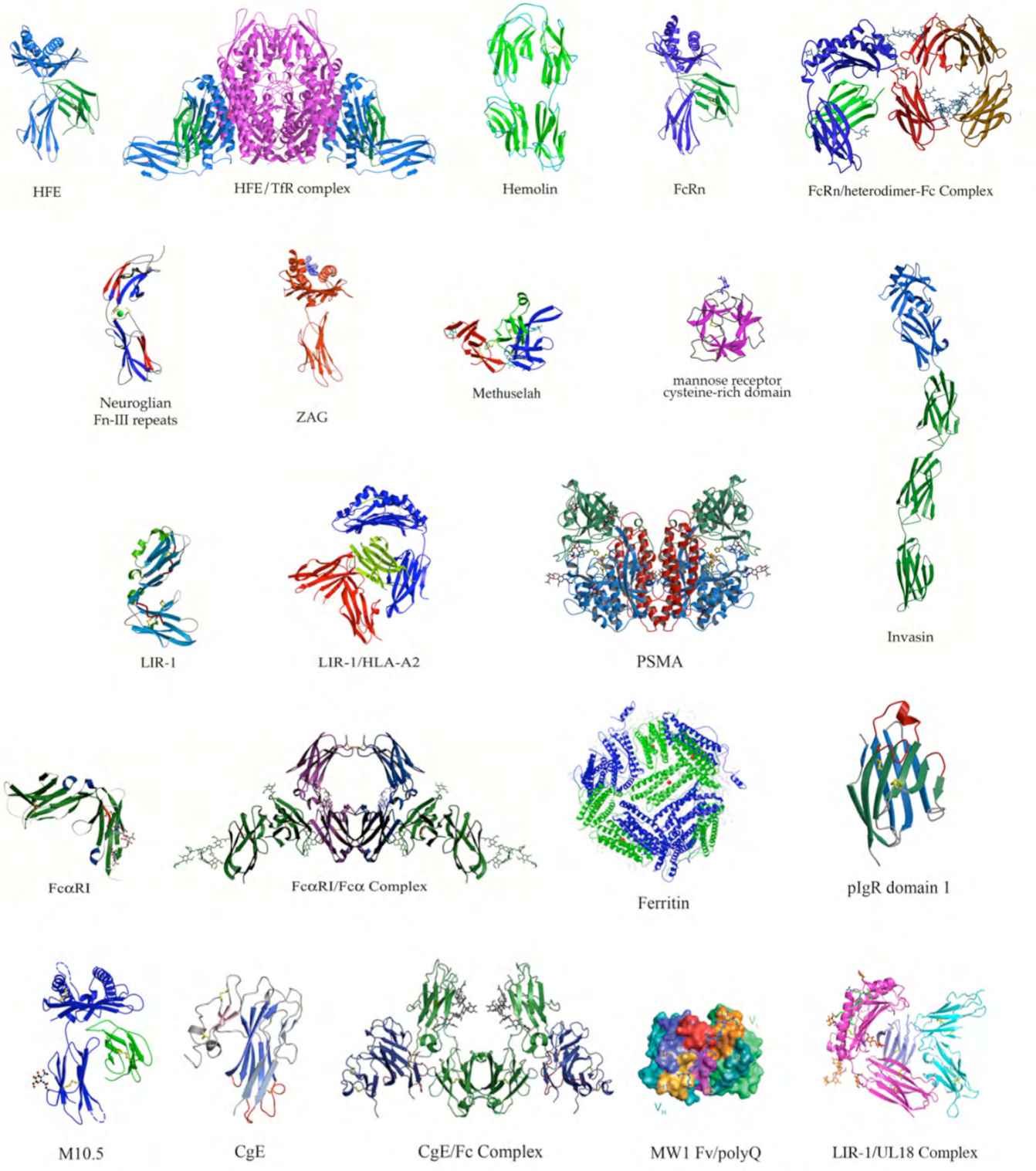
**Support:** The work described in the following research reports has been supported by:
Arnold and Mabel Beckman Fellowship (fellowship to Rich Olson)
European Molecular Biology Organization Fellowship (fellowship to Ron Diskin)
Bill and Melinda Gates Foundation: Collaboration for AIDS Vaccine Development Center
Bill and Melinda Gates Foundation: Grand Challenges in Global Health Initiative
Howard Hughes Medical Institute
Jane Coffin Childs Memorial Fund (fellowship to Tal Arnon)
Max Planck Research Award for International Cooperation
NIH
Technology Transfer Grubstake Award

**Summary:** My laboratory is interested in protein-protein interactions, particularly those mediating immune recognition. We use X-ray crystallography and biochemistry to study purified proteins. Examples of crystal structures determined by our laboratory are shown in Figure 1. Some of our work focuses upon homologs and mimics of class I major histocompatibility complex (MHC) proteins. Classical class I MHC proteins present peptides derived from self and non-self proteins to T cells during immune surveillance. MHC homologs share similar three-dimensional structures with classical MHC molecules but have different functions, including immune functions (IgG transport by FcRn, the neonatal Fc receptor, and evasion of the immune response by viral MHC mimics) and non-immune functions (regulation of iron metabolism by HFE, and serving as a chaperone for pheromone receptors in the case of M10 proteins). We are also comparing the structures and functions of host and viral Fc receptors with FcRn.

We have extended our characterizations of FcRn, an MHC-related receptor for IgG antibodies, to include cell biological studies of intracellular trafficking. FcRn is the receptor that transfers maternal IgG to the bloodstream of fetal and newborn mammals, thereby passively immunizing the neonate against pathogens likely to be encountered prior to development of its own fully-functional immune system. Transfer of IgG involves trafficking of FcRn-IgG complexes in acidic intracellular vesicles across an epithelial cell barrier in the placenta (for pre-natal transfer) or the intestine (for post-natal transfer). A general question exemplified by FcRn trafficking is how cargo-containing intracellular vesicles are transported to their correct ultimate locations – for example, how does the cell know that FcRn-IgG complexes should be transported across the cell for eventual release of IgG into the blood, whereas other receptor-ligand pairs should be transferred to degradative compartments? To study the process by which FcRn-IgG complexes are correctly trafficked across cells, we are using electron tomography, a form of electron microscopy, to derive three-dimensional maps of transport vesicles in neonatal rat intestinal epithelial cells at resolutions of 4–6 nm. To facilitate these studies, we developed gold-labeling and enhancement methods to locate individual IgG fragments bound to FcRn inside intracellular vesicles. Our three dimensional images of IgG transport reveal tangled webs of interlocking IgG-containing transport vesicles, some of which are associated with microtubule tracks to allow movement via motor proteins. Other IgG-containing vesicles include multivesicular bodies, normally associated with degradative functions, but apparently functioning in IgG transport in the specialized proximal small intestinal cells of a neonate. To complement these high-resolution, but static, studies, we are doing fluorescence imaging in live cells, which allows tracking in real time of labeled vesicles and quantification of the velocities and directions of FcRn-positive vesicles.

In addition to studying antibody receptors, we have begun a new project to improve upon the binding and neutralization properties of antibodies themselves. This work is part of a collaboration with David Baltimore's laboratory to "Engineer Immunity" against HIV. The idea is to direct life-long production of specified antibodies or antibody-like proteins with desired properties; for example, neutralizing antibodies or designed antibodies engineered to bind more tightly to a pathogen or to recruit immune effector cells. The antibodies would be produced *in vivo* by infecting autologous hematopoietic stem cells with lentiviral vectors bearing specific antibody genes, thus allowing life-long production of anti-HIV proteins. Our portion of the project involves designing, producing, and testing novel anti-HIV protein reagents in an effort to find proteins with increased efficacy in HIV neutralization. Although HIV has evolved to evade most/all antibodies (hence the difficulty of finding an immunogen capable of eliciting a strong neutralizing antibody response in vaccine development efforts), an attractive feature of the Engineering Immunity approach is that we are not limited to the traditional architecture of an antibody. Hence we can produce and express antibody-like proteins of different sizes (to facilitate access to hidden epitopes) and valencies (i.e., with different numbers of combining sites) and/or link antibodies to HIV-binding proteins such as the host receptor CD4.

**FIGURE 1**


HFE
HFE/TfR complex
Hemolin
FcRn
FcRn/heterodimer-Fc Complex
Neuroglian
Fn-III repeats
ZAG
Methuselah
mannose receptor
cysteine-rich domain
Invasin
LIR-1
LIR-1/HLA-A2
PSMA
FcαRI
FcαRI/Fcα Complex
Ferritin
pIgR domain 1
M10.5
CgE
CgE/Fc Complex
MW1 Fv/polyQ
LIR-1/UL18 Complex

**5. Structure of UL18, a peptide-binding viral MHC mimic, bound to a host inhibitory receptor**

*Zhiru (Jenny) Yang*

HCMV affects 70-90% of all human populations and can be life threatening to immunocompromised individuals, such as cancer, transplant, and AID patients. HCMV achieves a lifelong infection in host cells by adopting multiple mechanisms to evade the primed immune system, including down-regulation of host class I MHC (MHCI) molecules. Perhaps as a means of avoiding host immune responses triggered by low levels of MHCI, HCMV encodes a class I homolog called UL18, which unlike other viral and host MHC homologs, associates with endogenous peptides resembling those binding to host MHCI. Unlike classical class I molecules, however, UL18 is heavily glycosylated (13 potential N-linked glycosylation sites). The host cell receptor for UL18 is LIR-1 (ILT-4), an inhibitory receptor similar to KIR family members, but expressed on B cells, monocytes, macrophages, dendritic cells and a subset of natural killer cells. In addition to UL18, LIR-1 binds a broad range of host MHCI molecules, but with an affinity that is over 1000 times reduced compared to its affinity for UL18. We solved the 2.2Å crystal structure of a LIR-1/UL18/peptide complex, which reveals increased contacts and optimal surface complementarity in the LIR-1/UL18 interface compared to LIR/MHCI interfaces, explaining the >1000-fold higher affinity for LIR-1/UL18 interaction. Despite sharing only ~25% sequence identity, UL18's structure and peptide binding are surprisingly similar to host MHCI. The crystal structure suggests that most of the UL18 surface, except where LIR-1 and the host-derived light-chain bind, is covered by carbohydrates attached to 13 potential N-linked glycosylation sites, thereby preventing access to bound peptide and association with most MHCI-binding proteins. The LIR-1/UL18 structure demonstrates how a viral protein evolves from its host ancestor to impede unwanted interactions, while preserving and improving its receptor-binding site.

**6. The crystal structure of CHIR-AB1, a primordial avian classical Fc receptor**

*Tal I. Arnon[1], Jens T. Kaiser[2], Anthony P. West Jr., Rich Olson, Ron Diskin, Birgit C. Viertlboeck[3], Thomas W. Göbel[3]*

CHIR-AB1 is a newly identified avian immunoglobulin receptor that includes both activating and inhibitory motifs and was therefore, classified as a potentially bifunctional receptor. Recently, CHIR-AB1 was shown to bind the Fc region of chicken IgY and induce calcium mobilization via association with the common γ-chain, a subunit that transmits signals upon ligation of many different immunoreceptors. Here we describe the 1.8Å resolution crystal structure of the CHIR-AB1 ectodomain. The receptor ectodomain consists of a single C2-type immunoglobulin (Ig) domain resembling the Ig-like domains found in mammalian Fc receptors such as FcγRs and FcαRI. Unlike these receptors and other monomeric IgSF members, CHIR-AB1 crystallized as a two-fold symmetric homodimer that bears no resemblance to variable or constant region dimers in an antibody. Analytical ultracentrifugation demonstrated that CHIR-AB1 exists as a mixture of monomers and dimers in solution, and equilibrium gel filtration revealed a 2:1 receptor-ligand binding stoichiometry. Measurement of the 1:1 CHIR-AB1/IgY interaction affinity indicates a relatively low affinity complex, but a 2:1 CHIR-AB1/IgY interaction allows an increase in apparent affinity due to avidity effects when the receptor is tethered to a surface. Taken together, these results add to the structural understanding of Fc receptors and their functional mechanisms.

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**7. FcRn-mediated antibody transport across epithelial cells revealed by electron tomography**

*Wanzhong He[1], Mark S. Ladinsky[2], Kathryn E. Huey-Tubman, Grant J. Jensen[3], J. Richard McIntosh[2]*

The neonatal Fc receptor (FcRn) transports maternal IgG across epithelial barriers, thereby providing the fetus or newborn with humoral immunity before its immune system is fully functional. In newborn rodents, FcRn transfers IgG from milk to blood by apical-to-basolateral transcytosis across intestinal epithelial cells. The pH difference between the apical (pH 6.0-6.5) and basolateral (pH 7.4) sides of intestinal epithelial cells facilitate efficient unidirectional transport of IgG, since FcRn binds IgG at pH 6.0-6.5 but not pH ≥7. As milk passes through the neonatal intestine, maternal IgG is removed by FcRn-expressing cells in the proximal small intestine (duodenum, jejunum); remaining proteins are absorbed and degraded by FcRn-negative cells in the distal small intestine (ileum). We used electron tomography to directly visualize jejunal transcytosis in space and time, developing new labeling and detection methods to map individual nanogold-labeled Fc within transport vesicles and to simultaneously characterize these vesicles by immunolabeling. Combining electron tomography with a non-perturbing endocytic label allowed us to conclusively identify receptor-bound ligands, resolve interconnecting vesicles, determine if a vesicle was microtubule-associated, and accurately trace FcRn-mediated transport of IgG. Our results present a complex picture in which Fc moved through networks of entangled tubular and irregular vesicles, only some of which were microtubule-associated, as it migrated to the basolateral surface. New features of

transcytosis were elucidated, including transport involving multivesicular body inner vesicles/tubules and exocytosis via clathrin-coated pits. Markers for early, late, and recycling endosomes each labeled vesicles in different and overlapping morphological classes, revealing unexpected spatial complexity in endo-lysosomal trafficking.

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**8. A freeze substitution fixation-based gold enlarging technique for EM studies of endocytosed nanogold-labeled molecules**

*Wanzhong He[1], Christine Kivork[2], Suman Machinani[3], Mary K. Morphew[4], Anna M. Gail[5], Devin B. Tesar, Noreen E. Tiangco, J. Richard McIntosh[4]*

We have developed methods to locate individual ligands that can be used for electron microscopy studies of dynamic events during endocytosis and subsequent intracellular trafficking. The methods are based on enlargement of 1.4 nm Nanogold attached to an endocytosed ligand. Nanogold, a small label that does not induce misdirection of ligand-receptor complexes, is ideal for labeling ligands endocytosed by live cells, but is too small to be routinely located in cells by electron microscopy. Traditional pre-embedding enhancement protocols to enlarge Nanogold are not compatible with high-pressure freezing/freeze substitution fixation (HPF/FSF), the most accurate method to preserve ultrastructure and dynamic events during trafficking. We have developed an improved enhancement procedure for chemically fixed samples that reduced autonucleation, and a new pre-embedding gold-enlarging technique for HPF/FSF samples that preserved contrast and ultrastructure and can be used for high-resolution tomography. We evaluated our methods using labeled Fc as a ligand for the neonatal Fc receptor. Attachment of Nanogold to Fc did not interfere with receptor binding or uptake, and gold-labeled Fc could be specifically enlarged to allow identification in 2D projections and in tomograms. These methods should be broadly applicable to many endocytosis and transcytosis studies.

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**9. Characterization of FcRn-mediated transport pathways via confocal microscopy**

*Devin B. Tesar, Galina Jerdeva*

Movement of specific protein molecules across epithelial cell barriers by their cognate receptors is achieved via a multivesicular transport pathway known as transcytosis. Discrete steps in the procession of a receptor-bound ligand through the transcytotic network are characterized by association with distinct subpopulations of endosomal compartments. These subpopulations of endosomes can be identified by confocal microscopy using fluorescent markers (such as fluorescently-labeled transferrin) or antibodies against such markers (such as anti-Rab antibodies). We are currently working to decipher the transcytotic itinerary of FcRn, with or without its IgG ligand, by colocalizing FcRn and IgG with different endosomal markers. To achieve this, FcRn can be visualized by staining with an anti-FcRn monoclonal antibody that is directly conjugated to Alexa488. Rat IgG or Fc fragment can be directly labeled with fluorescent dyes (such as the Alexa 488 or 568), and antibodies against specific endosomal markers can be viewed using secondary antibodies conjugated to a far-red dye (such as Alexa 647). This allows for three-color confocal analysis to determine the intracellular localization of FcRn, it ligand, and marker proteins for particular compartments at different stages within the transport process. More recently, we have been working to develop techniques for live cell imaging using fast confocal imaging and have acquired an UltraVIEW ERS spinning disk confocal system. Spinning disk technology is specifically suited for fast acquisition of events in living cells that happen beyond the resolution of conventional confocal microscopes. The use of a highly-sensitive CCD detector allows for fast acquisition under conditions of limited laser exposure, greatly increasing fluorophore lifetime over the course of an experiment. The use of a Piezo-controlled objective turret allows for fast and precise Z-stepping in increments of 50 nm, making fast acquisition of Z-stacks possible. Standard microscopes, despite having exceptional resolution for imaging of fixed specimens, require approximately 7-15 seconds to scan a single channel at a resolution comparable to what a spinning disc system can accomplish in 20-50 milliseconds of exposure. These data can then be compared to the transport pathways of more well-characterized receptors such as the polymeric Ig receptor (pIgR) to evaluate which steps might be unique to, or particularly important during FcRn-mediated transport of IgG.

**10. Quantitative imaging of the FcRn-dependent transcytotic pathway using high-resolution fluorescence confocal microscopy**

*Galina V. Jerdeva, Devin B. Tesar, Scott E. Fraser[*]*

Specific delivery of proteins across polarized epithelia is controlled by receptor-mediated transcytosis. The pathways of a model receptor, the polymeric immunoglobulin receptor (pIgR), mediating transport of

dimeric and polymeric IgA(dIgA/pIgA) in the basolateral to apical direction are relatively well understood. The neonatal Fc receptor (FcRn) transports maternal immunoglobulin G (IgG) across intestinal or placental epithelial barriers in the apical to basolateral direction to provide immunity to fetal or newborn mammals and serves as a protection receptor for IgG. To investigate FcRn-mediated transport of IgG and to compare it to pIgR-mediated transport of dIgA/pIgA we are using Madin-Darby Canine Kidney (MDCK) cells expressing either one or both receptors FcRn (MDCK-FcRn, MDCK-FcRn-pIgR). The transfected cells specifically transcytose IgG or Fc and dIgA across polarized cell monolayers when applied apically and basolaterally, respectively. A spinning disk Confocal Imaging System (UltraVIEW ERS) with a sensitive EMCCD Camera allows acquisition of high-resolution 3D imaging volumes of cells after internalization of fluorescently-labeled ligands co-stained with endogenous intracellular markers with minimal bleaching of fluorescent probes. We are interested to identify at what stages of endocytosis / transcytosis the trafficking routes of Fc and pIgA intermix, and to learn the identities of endosomal compartments involved in receptor trafficking. Quantitative colocalization analysis of 3D volumes by Coloc Module of Imaris software (Bitplane) revealed significant colocalization of apically internalized Fc and basolaterally internalized pIgA after 20 min of chase but not after 5 min as revealed by calculations of the amount of colocalizations between two channels (13.95% $\pm$ 0.99% at 20 min chase; 1.13% $\pm$ 0.3% at 5 min chase) and Pearson's correlation coefficients (P) ($p=0.38 \pm 0.01$ at 20 min chase; $p=0.05 \pm 0.007$ at 5 min chase). A significant subpopulation of ligand-positive endosomes at the 20 min chase were also positive for the early endosomal marker EEA1 (10.9% + 1.30% for Fc; 9.66% + 1.67 for pIgA). P values for Fc /EEA1 and pIgA / EEA1 in regions of interest (ROI) containing endosomes were $0.33 \pm 0.2$ and $0.30 \pm 0.03$ respectively, indicating significant special overlap in internalized ligands and the marker. Studies of colocalization with other intracellular markers such as rab proteins will clarify the identity of common and unique endosomal compartments.

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**11. The chicken yolk sac IgY receptor, a mammalian mannose receptor family member, transcytoses IgY across polarized epithelial cells**

*Devin B. Tesar, Evelyn J. Cheung*[*]

In mammals the transfer of passive immunity from mother to young is mediated by the MHC-related receptor FcRn, which transports maternal IgG across epithelial cell barriers. In birds, maternal IgY in egg yolk is transferred across the yolk sac to passively immunize chicks during gestation and early independent life. The chicken yolk sac IgY receptor (FcRY) is the ortholog of the mammalian phospholipase A2 receptor, a mannose receptor family member, rather than an FcRn or MHC homolog. FcRn and FcRY both exhibit ligand-binding at the acidic pH of endosomes and ligand release at the slightly basic pH of blood. Here we show that FcRY expressed in polarized mammalian epithelial cells functioned in endocytosis, bi-directional transcytosis, and recycling of chicken FcY/IgY. Confocal immunofluorescence studies demonstrated that IgY-binding and endocytosis occurred at acidic but not basic pH, mimicking pH-dependent uptake of IgG by FcRn. Co-localization studies showed FcRY-mediated internalization via clathrin-coated pits and transport involving early and recycling endosomes. Disruption of microtubules partially inhibited apical-to-basolateral and basolateral-to-apical transcytosis, but not recycling, suggesting the use of different trafficking machinery. Our results represent the first cell biological evidence of functional equivalence between FcRY and FcRn and provide an intriguing example of how evolution can give rise to systems in which similar biological requirements in different species are satisfied utilizing distinct protein folds.

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**12. Intracellular trafficking of an antibody bipolar bridged complex of an HSV-1 Fc receptor bound to antibody/antigen complex**

*Alex Farley*

Herpes Simplex Virus (HSV) is a member of the alphaherpesvirus family, which also includes Varicella-Zoster Virus (ZVZ) and Pseudorabies Virus (PrV). Alphaherpesviruses are characterized by a relatively short replicative cycle in epithelial tissues and egression to and latent infection of the sensory neurons. HSV encodes two type 1 membrane-bound glycoproteins that together function as a receptor for the Fc portion of IgG. The heterodimer is composed of glycoprotein E (gE) and glycoprotein (gI) and it is found on the surface of virions and infected cells. The Fc receptor function of gE-gI is thought to provide a mechanism for immune evasion by blocking the effector function of host Fc recognition proteins in both the adaptive and innate immune systems. The gE-gI heterodimer is thought to bind to antigen-bound IgG in a process called bipolar bridging in which the antigen-binding fragments (Fabs) bind to an antigen, and gE-gI binds to the Fc. This process could provide a mechanism for HSV-1 to evade antibody-mediated immune responses via Fc receptors. The binding affinity of gE-gI for Fc is pH-dependent, whereby gE-gI binds IgG with high affinity at the slightly basic pH of the cell surface (~7.4) and releases IgG at acidic pH ($\leq$6.4). The sharply pH-dependent binding suggests that IgG that is endocytosed by gE-gI dissociates from gE-gI at the low pH of endosomal compartments, where it could be degraded, whereas gE-gI is recycled back to the cell surface. Our hypothesis is that Fc binding to gE-gI results in endocytosis of the antibody/antigen complex and its consequent degradation possibly in lysosomes. We have shown that the gE-gI heterodimer can internalize the Fc domain of IgG. Future work will include experiments to

address the intracellular trafficking patterns of gE-gI and the fates of gE-gI/IgG and gE-gI/IgG/antigen complexes.

**13. Crystallographic studies of the ternary complex of transferrin receptor with transferrin and HFE**

*Kathryn E. Huey-Tubman, Fan Yang, Matt Thornton, Anne B. Mason*[*]

Iron is transported in the serum of vertebrates bound to transferrin (Tf). Transferrin receptor (TfR) assists iron uptake into vertebrate cells through a cycle of endo- and exocytosis of Tf. TfR binds iron-loaded Tf (Fe-Tf) at the cell surface (pH 7.4) and carries it to the endosome. Iron dissociates from Tf at the acidic pH of the endosome (pH 6.0), but Apo-Tf remains tightly bound to TfR. The Apo-Tf/TfR complex then returns to the cell surface and at extracellular pH, Apo-Tf dissociates and is replaced by diferric Tf from the serum. TfR also interacts with HFE, an MHC class I-related protein that is mutated in patients with the iron overload disorder, hereditary hemochromatosis. HFE binds to TfR, decreasing its ability to bind iron-loaded Tf. Previous crystallographic studies have revealed the structures of the ectodomain of TfR alone (Lawrence *et al.*, 1999) and in complex with HFE (Bennett *et al.*, 2000), as well as several Tf forms and family members. A low-resolution (7.5 Å) structure of the ternary complex, TfR bound to HFE and Tf, has been produced from cryoelectron microscopy (Cheng *et al.*, 2004). To further understand the mechanisms of TfR-facilitated iron release from Tf and how HFE affects iron uptake, we want to solve the structure of the Fe-Tf/TfR/HFE ternary complex. We have designed and expressed in insect cells a heterodimeric mutant of TfR where one chain of the dimer binds Tf and the other chain HFE. This along with various forms of wild-type and mutant iron-loaded and iron-free Tfs (expressed both in insect cells and BHK cells) and HFE (expressed in CHO cells) will allow us to set up numerous crystallization trials.

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**14. Biochemical and structural studies of ferroportin**

*Adrian E. Rice, Douglas C. Rees*[*]

All known organisms, save two bacteria, require iron for survival. Despite its importance, iron in overabundance is toxic. In order to maintain a balance of iron levels, organisms have developed a highly specialized network of molecules designed to monitor and maintain iron homeostasis. When the fidelity of these networks is compromised, diseases such as iron deficiency and iron overload result. Mammals lack any regulated mode of iron excretion and therefore, must have highly regulated mechanisms for controlling the acquisition of iron from the diet. The primary site of iron absorption is the duodenum of the small intestine. This process can be categorized by two phases: 1) iron uptake across the brush border into the cytoplasm of duodenal enterocytes; and 2) iron export across the basolateral membrane of these cells into the blood. Iron from the diet is reduced from Fe3+ to Fe2+ by the membrane-bound iron reductase Dcytb and is transported across the apical brush border by an integral membrane protein called divalent metal transporter 1 (DMT1; also known as DCT1 and NRAMP2). Iron is then transported across the cell to the basolateral side where it is exported by the basolateral integral membrane iron transporter ferroportin (Fpn; also known as IREG1 and MTP1). Fpn is the only identified iron exporter in vertebrates and is an integral membrane protein containing 9-12 predicted alpha-helical transmembrane segments. Point mutations in Fpn lead to an autosomal dominant iron overload disease called ferroportin disease. My project aims to characterize Fpn from both a structural and biochemical standpoint. I have expressed and purified Fpn for crystallization trials and characterized the effects of mutations in the localization of Fpn in transfected cells.

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**15. Crystal structure of the hemojuvelin-binding fragment of neogenin**

*Fan Yang, Anthony P. West Jr.*

Hemojuvelin (HJV), a recently discovered GPI-anchored membrane protein, is known to be the upstream regulator of the key iron-regulatory hormone hepcidin, and loss of function mutations cause severe iron overload disease. HJV has been reported to interact with neogenin, a cell surface immunoglobulin (Ig) superfamily protein. Understanding how this interaction occurs on the molecular level may be helpful for gaining insights into mechanism of iron homeostasis. Previously, by expressing soluble forms of HJV and various truncations of neogenin and determining their interactions by surface plasmon resonance (SPR), we located the HJV-binding epitope on neogenin to be the two membrane-proximal fibronectin type III-like domains (sFNIII 5-6) with a minor contribution from the ectodomain tail[*]. We have now determined the 1.8Å crystal structure of the HJV-binding fragment of neogenin. The two FNIII domains are arranged on top of each other in a straight manner – the fact that only a few residues are between them may explain this seemingly rigid arrangement. This structure will aid our efforts to determine the structure of the HJV/sFNIII 5-6 complex.

**Publication**

[*]Yang, F., West Jr., A.P., Allendorph, G., Choe, S. and Bjorkman, P.J. (2008) Neogenin interacts with hemojuvelin through its two membrane-proximal fibronectin type III domains. *Biochemistry* **47**:4237-4245.

**16. Structural studies of class C GPCRs**

*Rich Olson*

I am employing structural techniques to investigate members of the class C family of G-protein coupled receptors (GPRCs). This diverse collection of small ligand-activated proteins is characterized by a large extracellular ligand-binding domain in addition to the canonical seven-transmembrane helical domain common to all GPCRs. Members of this family include mammalian V2R pheromone receptors, calcium-sensing receptors, $GABA_B$ receptors, metabotropic glutamate receptors, taste receptors, and fish odorant receptors.

A green-fluorescent protein (GFP)-based system for screening the expression and solution behavior of detergent-solubilized and soluble ectodomain constructs of different representative class C GPCR genes was used to identify candidates for further structural studies. My recent work has focused on an amino-acid sensory receptor identified by the expression screen. The ectodomain of this receptor was expressed and purified from a baculovirus expression system and structural studies are ongoing. In particular, methods for deglycosylating the extensively sugar-coated protein have been underway using the GFP-based screening process. I have also been investigating methods for expressing the full-length receptor in human embryonic kidney (HEK) and baculovirus-infected SF9 cells. All of these studies aim to understand the structure and mechanism of this unique and important family of GPCRs.

**17. Cryoelectron tomography of homophilic adhesion mediated by the neural cell adhesion molecule L1**

*Yongning He, Grant Jensen*[*]

The neural cell adhesion molecule L1 participates in homophilic interactions that are important for axon guidance and neuronal development. The structural details of homophilic adhesion mediated by L1 and other immunoglobulin superfamily members containing an N-terminal horseshoe arrangement of four immunoglobulin-like domains are unknown. Here we used cryoelectron tomography to study liposomes to which intact or truncated forms of the L1 ectodomain were attached. Tomographic reconstructions revealed an adhesion interface with a regular and repeating pattern consistent with interactions between paired horseshoes contributed by L1 proteins from neighboring liposomes. The characteristics of the pattern, such as the space between horseshoe pairs and/or between adjacent membranes, changed when N-linked carbohydrates were altered by removing sialic acids or converting from complex to high mannose or oligomannose glycans, suggesting a regulatory role for carbohydrates in L1-mediated homophilic adhesion. Using the results from tomograms and crystal structures of L1-related molecules, we present a structural model for L1-mediated homophilic adhesion that depends on protein-protein, protein-carbohydrate, and carbohydrate-carbohydrate interactions.

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**18. Improved neutralizing antibodies against HIV**

*Anthony P. West Jr., Joshua Klein, Priyanthi Gnanapragasam, Chris Foglesong, Rachel Galimidi, Noreen Tiangco, Maria Suzuki, Lili Yang*[1]*, David Baltimore*[2]

Together with David Baltimore's laboratory, we are attempting to develop a new "Engineering Immunity" approach to treating HIV/AIDS. This strategy envisions using lentiviral vectors to program a patient's immune system to produce designed anti-HIV molecules. The Bjorkman lab component of this project entails developing an improved set of antibody reagents that neutralize HIV. Although the vast majority of natural anti-HIV antibodies are highly strain-specific, a small number of broadly neutralizing anti-HIV antibodies has been identified. We are attempting to take these antibodies as a starting point to develop antibodies or antibody-like proteins engineered to bind more tightly to HIV or to recruit immune effector cells

One set of molecules we are investigating are the CD4/CD4i Ab reagents. When gp120 binds CD4, a conformational change occurs exposing the coreceptor-binding site. Antibodies targeting this CD4-induced site (so called CD4i Abs) often are broadly cross-reactive. Normally, they have little neutralization potency *in vivo* due to limited steric accessibility when gp120 is bound to cell surface CD4. Several years ago, to overcome this problem, Ed Berger's laboratory designed a bispecific reagent containing part of CD4 linked to the scFv form of 17b, a CD4i Ab. We are extensively exploring the potential of CD4/CD4i reagents since they target two conserved functional sites on gp120. These reagents have several components: domains 1 and 2 from CD4, a CD4i Ab combining site (scFv or Fab), linkers, and potentially an Fc domain to provide bivalency and an increased serum half-life. We are exploring different ways of combining these components, as well as comparing the effectiveness of several CD4i Abs. We hope to determine the reagent(s) with the most optimal combination of neutralization potency, expression efficiency, and half-life. The most promising reagent to date has an IgG-like architecture with CD4 attached to the CD4i Ab E51.

During purification of the neutralizing Ab 2G12, we observed that a small fraction of this Ab exists in a dimeric form. Our *in vitro* neutralization experiments demonstrate that this dimeric form of 2G12 is 20 to 30-fold more potent than the monomer. Based on a structural hypothesis on the nature of the dimer, we have designed a mutated form of 2G12 that yields a ~2-fold higher fraction of the dimer.

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**19. Steric occlusion limits the potency of the broadly neutralizing HIV-1 antibody 4E10**

*Joshua S. Klein, Priyanthi Gnanapragasam, Rachel Galimidi, Noreen Tiangco, Chris Foglesong, Maria Suzuki, Anthony P. West Jr.*

Recently, we observed that virus neutralization by monoclonal antibody 4E10, the most broadly neutralizing anti-HIV antibody characterized to date, to be subject to steric constraint. 4E10 binds a highly conserved linear epitope in the membrane proximal external region of gp41. In an *in vitro* neutralization assay with a panel of eight pseudotyped primary HIV isolates, we observed that scFv 4E10 was, on average, only 2-fold less potent than IgG 4E10 on a molar basis, whereas Fab 4E10 was 7-fold less potent. This effect was not observed for a scFv derived from monoclonal antibody b12, which is unlikely to suffer from any steric restriction because its epitope on gp120 probably faces out into the extracellular environment. We further observed that the range of differences in neutralization potency between scFv 4E10 and IgG 4E10 varied directly with the length of the gp120 variable loop V5 (R-squared = 0.73). Our data suggest that B cells expressing 4E10-like B-cell receptors may be difficult to activate because 4E10-like antibodies are sterically prohibited from accessing the 4E10 epitope on gp41 in its native membrane-bound state. In light of a report suggesting that the 4E10 epitope is present in a transient state following docking to HIV's receptors on target cells, we are currently exploring the hypothesis that shortening the length of the V5 loop could abrogate these steric effects and lead to immunogens capable of eliciting a broadly neutralizing gp41-specific response.

**20. Structural studies of the HIV spike recognition by neutralizing antibodies**

*Ron Diskin, Rachel Galimidi, Chris Foglesong, Noreen Tiangco, Maria Suzuki, Anthony P. West Jr.*

HIV is a retrovirus that cause AIDS, a devastating disease especially prevalent in developing countries. Low awareness for prevention and the inability to purchase modern drug therapies result in the fast spreading of the epidemic and a substantial decrease in life expectancy. Infection by HIV can be prevented in the presence of broadly reactive neutralizing antibodies (NAbs), which target the functional envelope spike protein of the virus. This spike mediates fusion of HIV to CD4 positive cells using a well-masked conserved core. HIV spikes consist of three gp120-gp41 proteins, which are the cleavage products of the gp160 precursor. This protein complex is the only viral-derived moiety presented on its surface. Attempts to trigger the immune system to produce NAbs using a vaccine have failed so far. One of the main obstacles impairing these trials is the lack of accurate structural data on the functional form of the envelope spike complex and the mechanism that NAbs exploit to recognize it. Our research is aimed at studying the mechanism that governs the recognition of the functional HIV spike complex by NAbs. To achieve this goal we plan to solve the crystal structure of the functional spike complex (the gp120-gp41 heterotrimer) or a related substructure (gp120 homotrimer) with or without various known and newly designed NAbs. As the crystallization of the functional heterotrimer may present many difficulties, we are also researching toward solving the crystal structures of the gp120 monomers in complex with novel NAbs. To enhance our chances for succeeding, we are utilizing a broad array of proteins derived from many different HIV clones thus, introducing sequence variability to the crystallization trials. The resulting information from our research could bridge the existing gap in our knowledge concerning the neutralization of HIV by NAbs. This may facilitate the production of artificial epitopes that would trigger the immune system to produce effective NAbs. If successful, such epitopes will serve as a much needed vaccine against HIV.

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**Professor Emeritus**: Charles J. Brokaw

**Summary**: Motor enzymes — dyneins, kinesins, and myosins — convert energy from ATP dephosphorylation into most of the movements performed by eukaryotic cells. We think that myosin and kinesin are reasonably well understood, although new experimental results from time to time surprise us. On the other hand, we have very little knowledge or understanding of the functioning of the axonemal dyneins that power the movements of flagella and cilia; these molecular complexes are a major challenge for the future. My current work uses computer simulation methods to explore ideas about motor enzyme function in situations ranging from experimental studies on individual motors to an intact flagellum containing tens of thousands of dyneins. Some of the simulation programs, as Macintosh applications, are available at: www.cco.caltech.edu/~brokawc/software.html

**21. Thinking about flagellar oscillation**

*Charles J. Brokaw*

It has been well established that the bending of cilia and flagella results from sliding between the microtubular outer doublets, driven by dynein motor enzymes. The mechanism responsible for oscillation in this system has not been established. Most theorizing has had to assume two distinct modes for dynein operation: on and off, or possibly forward and reverse. However, only the on or forward mode has been characterized. Theoretical mechanisms for oscillation, examined by computer simulations, have assumed that switching between modes might be controlled by the curvature of the flagellum, or by the direction of sliding. Neither idea has been shown to be capable of explaining oscillation in all situations. Oscillation in arrays of cilia becomes self-organized to produce metachronal waves. Oscillatory capability on individual flagellar doublets can become self-organized to produce doublet metachronism, the circumferential propagation of activity in helical bending waves. Similarly, it has been suggested that oscillatory capability distributed along a flagellum can become self-organized to produce propagated bending waves, but the mechanism that introduces metachronal phase differences and suppresses synchronous oscillation has not been established. Similar interactions between the mechanochemical cycling of adjacent dynein motors are likely to be strong enough to establish metachronism or some other form of coordination, but this possibility remains for future investigation.

**22. Mechanical properties of the passive sea urchin sperm flagellum**

*Charles J. Brokaw, Charles B. Lindemann**

The passive bending resistance of a flagellum results from a bending resistance of the microtubular outer doublets (EB) and a shear resistance contributed by interdoublet linkages (ES). Measurements of flagellar bending resistance normally give a value that combines these two components in an unknown manner. We have been attempting to obtain the ratio ES/EB by examining the shapes of bent flagella. Data are available from quiescent sperm, bent into a static "hook" shape, assumed to be the result of uniform dynein activity along the length of some of the outer doublets. New data has been obtained when dyneins are put into a "relaxed" state by vanadate inhibition, and bent with a microneedle. Beyond the position of the microneedle, a "counterbend" appears with a shape that is determined by equilibrium between EB and ES. Both situations give similar results for ES/EB. We are now trying to understand why ES behaves as a linear resistance, rather than in the nonlinear manner that would be expected for elastic linkages between outer doublets.

**Oakland Univ., Rochester MI*

**23. Oscillation resulting from doublet separation and reannealling**

*Charles J. Brokaw*

When ATP is supplied to partially disintegrated, demembranated flagella, where the outer doublets remain attached at the basal end, sliding of a doublet towards the basal end causes it to bulge out and separate away from the adjacent doublet. Further sliding in the distal region is then accommodated by bending of the sliding doublet into a large loop, and finally by complete separation. In some cases, this is followed by reannealling of the separated doublet, starting from the basal end where it is held close to its partner. The sequence can then be repeated. This situation, and other examples where doublets separate and reanneal, suggest that dyneins are not only the flagellar motors, but are also responsible for maintaining the proper operating distance between doublets. Although obviously this is a pathological situation, it is the only example of flagellar oscillation that can be explained by obvious on and off states, resulting from proximity to or separation from, the partner doublet. A program is being developed to simulate these experiments and obtain more understanding of how dynein functions as an adhesive.

**Publication**

Brokaw, C.J. (2009) Thinking about flagellar oscillation. *Cell Motil. Cytoskel.* In press.

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**Associate**: Elizabeth Bertani
**Member of the Professional Staff**: Martin Budd, Piotr Polaczek
**Research Fellows**: Huizian Liu, Taro Masuda-Sasa
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**Support**: The work described in the following research reports has been approved by:
CDMRP Breast Cancer
CIRM
Ellison Foundation
NIH

**Summary**: A hallmark of cancer cells, in addition to uncontrolled proliferation, is genomic instability, which appears in the form of chromosome loss or gain, gross chromosomal rearrangements, deletions, or amplifications. The mechanisms that suppress such instability are of the utmost interest in understanding the pathogenesis of cancer. Our lab studies the components of the DNA replication apparatus that promote genomic stability, primarily using yeast genetics and biochemistry.

Several years ago, Rajiv Dua in the laboratory discovered that DNA polymerase ε, one of four essential DNA polymerases in yeast, had not one, but two essential functions. Deletion of the polymerase domain left the cells viable because another polymerase activity could substitute. Conversely, deletion of the remaining, non-catalytic half of the protein was lethal. Shaune Edwards in the laboratory carried out a two-hybrid screen for proteins that interact with the enigmatic C-terminal region of pol ε in order to discover its function. She found that pol ε interacts with MRC1, the ortholog of mammalian claspin, a protein involved in mediating the signal from a stalled replication fork to the cell cycle checkpoint apparatus. Huiqiang Lou in the lab has recently shown that the interaction between Mrc1 and ε is regulated by phosphorylation by the master checkpoint kinase, ATM (Mec1 in yeast).

At least seven human diseases characterized by cancer predisposition and/or premature aging are correlated with defects in genes encoding DNA helicases. The yeast genome contains 134 open reading frames with helicase motifs, only eight of which have been characterized. Martin Budd in our laboratory identified the first eukaryotic helicase essential for DNA replication, Dna2. He showed by interaction studies that it was a component of the machine that is required for accurate processing of Okazaki fragments during lagging-strand DNA replication. Enzymatic studies to elucidate the sequential action of the DNA polymerase, helicase, and nuclease required for this processing form an ongoing mechanistic biochemistry project in the laboratory.

Stimulated by various reports in the literature implicating Dna2 in telomere biogenesis and structure, Wonchae Choe made the interesting observation that the bulk of Dna2 is localized to telomeres and that this localization is dynamic. During G1 and G2 phases of the cell cycle, Dna2 is at telomeres. During S phase Dna2 is present on the replicating chromatin. Current studies are aimed at defining the genes that regulate the localization. In addition to defects in replication, *dna2* mutants are also very sensitive to agents that induce double-strand breaks (DSBs). Osamu Imamura has shown that Dna2 is mobilized from telomeres in response to the induction of double-strand breaks. We are carrying out experiments to test the model that Dna2 delocalization from telomeres is part of the signaling system that induces the DNA damage and S phase checkpoints, as has also been suggested for yKu, a protein involved in non-homologous end joining and in stabilizing telomeres. One set of experiments used global synthetic lethal screening to identify a network of 822 genetic interactions that together account for the pathways protecting the eukaryotic genome from rearrangements due to DNA damage.

One model of cellular aging suggests that accumulation of DNA damage leads to replicative senescence. Most endogenous damage occurs during S phase and leads to replication fork stress. At least three human diseases of premature aging or cancer predisposition - Werner, Bloom, and Rothmund-Thompson - are caused by defects in helicases similar to Dna2. Martin Budd and Laura Hoopes found that *dna2* mutants have a significantly reduced life span. Microarray analysis by Isabelle Lesur shows that the *dna2* mutants age by the same pathway as wildtype cells; they just age faster. Interestingly, the human Bloom and Werner genes complements the replication defect of *dna2* mutants, suggesting that Dna2 works in the same pathway with these genes. We have now shown that the Dna2 helicase works with the yeast BLM ortholog, Sgs1 in the major pathway of double-strand break repair in yeast and are studying the same process in human cells. Dna2 is involved in an early event that results in the production of a single-stranded 3' end that has two functions. It is involved in strand invasion of the homolog and thus the initiation of strand exchange. Perhaps even more important the single-stranded DNA is a key intermediate in the activation of the cell cycle checkpoint that protects the cell from genome instability in the presence of a double-strand break arising from replication fork failure.

### 24. Shape control of bacterial magnetite crystals

*Daniel Poon[1], Molly Davis[2], Elizabeth Bertani*

Magnetic bacteria synthesize crystals of the iron ore, magnetite, by pumping environmental iron into preformed organelles called magnetosomes. The role of the magnetosomes in the life cycle of the bacteria is not clear, but each species synthesizes magnetite crystals of a different shape, which can be distinguished by microscopy. The crystals of *Magnetospirillum magnetotacticum*, strain AMB-1, are cubo octohedral, whereas, those of the *Magnetococcus*, MC-1, are elongate hexagonal. The C-terminal ends of four different AMB-1 proteins have been found tightly bound to magnetite crystals and the genes producing these proteins identified. The genes

appear to be clustered in the AMB-1 genome. The mature proteins are apparently cleaved during crystallization, leaving the C-terminal portion bound to the crystal. The C-terminal end of one such protein, the product of gene *mms6*, has been reported (Arakaki *et al*., 2003) to influence the shape of crystals when it is added during their formation in an *in vitro* system. In order to establish the importance of this observation, we have begun a project to clone similar genes from MC-1, overexpress the C-terminal fragments of the proteins, and test them singly or in groups for their possible effect on shape during crystal formation in an *in vitro* system. In addition, we propose to carry out genetic experiments where we replace the AMB-1 genes with their MC-1 equivalents, to study their effects on crystal shape *in vivo*. In preparation, we have cloned three of the genes from AMB-1 and MC-1 and confirmed that magnetite crystals are produced by the *in vitro* system.

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**25. DNA2 helicase/nuclease phosphorylation by Mec1 is important for the DNA damage response in *Saccharomyces cerevisiae***

*Barbara Kraatz Fortini, Judith L. Campbell*

DNA2 is an essential ATPase, nuclease, helicase, ssDNA annealing and strand exchange enzyme with a well-characterized role in Okazaki fragment processing. Further, DNA2 is involved in telomere maintenance, rDNA stability, DNA repair, and the DNA replication checkpoint. The nuclear localization of Dna2 throughout the cell cycle is dynamic; Dna2 is localized to telomeres during G1 and G2 but is released to internal chromosome sites during S phase. Interestingly, Dna2 is also released from telomeres in a DNA damage dependant fashion.

In *Saccharomyces cerevisiae*, Mec1, a member of the PI-3 kinase-related kinase (PIKK) family, orchestrates the DNA damage response pathway by phosphorylating a variety of proteins. Dna2 is now known to be a target of Mec1, both *in vitro* and *in vivo* after DNA damage. Non-phosphorylatable Dna2 mutants are highly sensitive to DNA damaging agents including MMS, hydroxyurea, and phleomycin. Creation of a phospho-specific antibody to the modification site shows phosphorylation of Dna2 within minutes after treatment with DNA damaging agents. These data implicate DNA2 as an important player in the DNA damage response.

**26. Multiple nucleases and helicases participate in telomere maintenance and DBS repair**

*Martin Budd, Judith L. Campbell*

Multiple nucleases (Rad27, Dna2, Mre11, Sae2, and Exo1) with overlapping functions participate in multiple pathways in DNA replication, DNA repair, and telomere maintenance thus, making it difficult to determine the distinct roles of each nuclease. Dna2 is an essential helicase/nuclease, whose best-understood role is processing of Okazaki fragments in conjunction with Rad27/FEN1 nuclease. However, *dna2* mutants are also sensitive to x-rays, and this sensitivity is not suppressed by overexpression of RAD27, suggesting the mechanism of Dna2 in repair of x-ray damage, presumably double-strand breaks, is different from that in DNA replication. Furthermore, Dna2 localizes to telomeres and is required for telomere biogenesis and stability in yeast. Telomeres and DSBs each require 5' to 3' resection in their repair, but the precise nuclease involved is not known. We have investigated possible participation of Dna2 in such resection. One participant is clearly the MRX complex. Mre11 is a 3'->5' nuclease that resides in a complex with Rad50 and Xrs2 (MRX) and is required for both of these types of processing. *mre11Δ* strains have short telomeres and are very sensitive to x-rays. However, the nuclease activity of Mre11 is not required for 5' to 3' exonuclease processing at telomeres or chromosomal breaks. Since Dna2 is a 5' to 3' exo-endonuclease/helicase, we have tested the hypothesis that Dna2 may participate in the generation of the 3' G-rich overhang at telomeres and the 3' single-strand ends at chromosomal breaks in the absence of Mre11 nuclease.

We do not find a defect in end resection in *dna2 mre11-D56N* or *dna2 mre11-H125N* double mutants, as measured by appearance of single-stranded G tails. In fact, there may be an increase in single-stranded G-tails, perhaps due to defects in Okazaki fragment processing.

We do find a defect in x-ray repair in double mutants. Deletion of Pif1, another 5' to 3' helicase, suppresses both the inviability and the x-ray sensitivity of *dna2Δ* mutants. However, *pif1 dna2 mre11-D56N* and *pif1 dna2 mre11-H125N* were extremely sensitive to x-rays, although neither *pif1 dna2* nor *pif1 mre11D56N* nor *pif1 mrell-H125N* was sensitive. Therefore, in x-ray repair, Dna2 may be an Mre11-compensating nuclease. Mechanistically, we show that the nuclease activity of Dna2 is tightly coupled to the helicase activity during growth and x-ray repair. The behavior of *dna2Δ pif1Δ* strains with plasmids containing Dna2 nuclease minus-helicase minus, Dna2 nuclease minus-helicase plus, or Dna2 nuclease plus-helicase mutants, suggests that the Dna2 helicase creates potentially toxic structures in DNA replication and x-ray repair which are processed into non-toxic structures by Dna2 nuclease.

The processing of Okazaki fragments at telomeres requires Rad27. *rad27Δ* strains exhibit extensive 3' G rich single-stranded ends. *dna2-2 exo1Δ* strains also exhibit extensive G-rich 3' single-stranded DNA at the ends of telomeres at 37$^{o}$C. Therefore, Rad27 is not sufficient for

Okazaki fragment processing at the telomere, and either a functional Exo1 or Dna2 is also required.

**27. Bimodal interaction between Mrc1 and ε**

*Huiqiang Lou, Judith L. Campbell*

Complicated replication machines are sequentially assembled and precisely programmed during the M/G1 phases of the cell cycle. In addition, the replication machines must be protected during S phase, because progress of the replication machine along the template, while sequential, is not continuous. Replication forks stall periodically, either in difficult-to-replicate regions of the genome or due to exogenous environmental interference. How to prevent replication forks from irreversibly collapsing in S phase, when *de novo* assembly and programming are no longer permitted, becomes a fascinating question. Our finding that the Pol2 C-terminal half (Pol2C, 1265-2222) interacts with Mrc1 might shed light on these functions. Yeast Mrc1 is a homolog of vertebrate Claspin, a mediator in the S phase checkpoint pathway. It is also required for the proper assembly of the replication fork at origins (similar timing as Pol ε) and for normal fork progression. But how does Mrc1 work at replication forks? Mrc1, ortholog of Claspin, is both a central component of normal DNA replication forks and a mediator of the S phase checkpoint. We report that Mrc1 interacts with Pol2, the catalytic subunit of DNA polymerase ε, essential for leading-strand DNA replication and for the S phase checkpoint. Since Mrc1 also interacts with the putative MCM helicase, Mrc1 may modulate coupling of polymerization and unwinding. The N terminus of Mrc1 interacts with the catalytic N-terminal half of Pol2 (Pol2N), suggesting that Mrc1 may be a polymerase accessory factor. The Mrc1 C terminus interacts with the structural C-terminal half (Pol2C) and is required for normal replication. During the S phase checkpoint, Mrc1N becomes phosphorylated, abolishing Mrc1N/Pol2N binding but not Mrc1C/Pol2C interaction. The bimodal interaction between Mrc1 and Pol2 may identify a novel step in converting DNA damage on the leading strand into a molecular signal that activates the S phase checkpoint.

**28. Processing of G4 DNA by Dna2 helicase/nuclease and RPA**

*Taro Masuda-Sasa, Piotr Polaczek*

The polyguanine-rich DNA sequences commonly found at telomeres and in rDNA arrays have been shown to assemble into structures known as G-quadruplexes, or G4 DNA, stabilized by base-stacked G quartets, an arrangement of four hydrogen bonded guanines. G4 DNA structures are resistant to the many helicases and nucleases that process intermediates arising in the course of DNA replication and repair. The lagging-strand DNA replication protein, Dna2, has demonstrated a unique and cell cycle-dependent localization to telomeres and a role in *de novo* telomere biogenesis, prompting us to study the activities of Dna2 on G4 DNA-containing substrates. We find that yeast Dna2 binds with 25-fold higher affinity to G4 DNA formed from yeast telomere repeats than to single-stranded DNA of the same sequence. Human Dna2 also binds G4 DNAs. The helicase activities of both yeast and human Dna2 are effective in unwinding G4 DNAs. On the other hand, the nuclease activities of both yeast and human Dna2 are attenuated by the formation of G4 DNA, with the extent of inhibition depending on the topology of the G4 structure. This inhibition can be overcome by RPA. RPA is known to stimulate the 5' to 3' nuclease activity of Dna2; however, we go on to show that this same protein inhibits the 3' to 5' exo/endonuclease activity of Dna2. These observations are discussed in terms of possible roles for Dna2 in resolving G4 secondary structures that arise during Okazaki fragment processing and telomere lengthening.

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**Support:** The work described in the following research reports has been supported by:
Donald Bren Foundation
Ellison Medical Foundation
National Institutes of Health

**Summary:** The primary focus of our lab is to understand the role of mitochondrial dynamics in normal cellular function and human disease. Mitochondria are remarkably dynamic organelles that undergo continual cycles of fusion and fission. The equilibrium between these two opposing processes determines the overall morphology of mitochondria in cells and has important consequences for mitochondrial function.

Our research falls into several broad areas:

(1) What are the molecular mechanisms that mediate fusion and fission of the mitochondrial outer and inner membranes?
(2) How do fusion and fission effect the mitochondrial population, and do they regulate functions such as respiration, calcium homeostasis, and apoptosis?
(3) What role do mitochondrial dynamics play in human diseases, particularly mitochondrial encephalomyopathies and neurodegenerative disorders?

Because of the complexity of these issues, we use a diverse set of strategies and multiple experimental systems to investigate the mechanism and physiology of mitochondrial dynamics. To understand the mechanisms of mitochondrial fusion and fission, we use biochemistry and structural biology, approaches that have historically yielded insights in other well-studied cases of membrane remodeling. We complement these approaches with yeast and mammalian cell biology. For example, we have used mouse molecular genetics to derive a large set of genetically defined cell lines that enable systematic structure-function analysis of proteins involved in mitochondrial dynamics. To understand the role of mitochondrial dynamics in mammalian development and disease, we construct and analyze murine animal models. By using this diverse set of approaches, we hope to develop an integrated understanding of mitochondrial dynamics.

**Molecular mechanism of membrane fusion and fission**

From a mechanistic perspective, mitochondrial fusion is a unique process. It has features that are distinct from the best-understood examples of membrane fusion-virus entry and vesicular fusion. First, because mitochondria are double-membrane organelles, their fusion is a more complicated process requiring the coordinated merger of four lipid bilayers. Second, in contrast to vesicular fusion, mitochondrial fusion does not require SNARE molecules or AAA-ATPases. We would like to understand mitochondrial fusion and fission at a structural/mechanistic level and to determine whether there are common features in diverse forms of membrane trafficking.

Mitochondrial fusion requires three large GTPases: the mitofusins Mfn1 and Mfn2, and the dynamin-related protein OPA1 (**Fig. 1**). The mitofusins are located in the mitochondrial outer membrane, whereas OPA1 is located in the intermembrane space. Mfn2 is mutated in the peripheral neuropathy Charcot-Marie-Tooth (CMT) type 2A, and OPA1 is mutated in dominant optic atrophy.

We are using structural biology to understand the mechanisms of mitochondrial fusion and fission. A few years ago, we identified and solved the structure of a region of Mfn1 involved in the tethering of mitochondria during fusion. Current work is focused on gaining further structural insights into mitofusins and OPA1 (Yan Zhang, Huu Ngo, and Tadato Ban).

Mitochondrial fission requires the recruitment and assembly of the dynamin-related GTPase Dnm1 onto mitochondria (**Fig. 1**). A key issue, therefore, is how Dnm1 is recruited onto mitochondria. The resolution of this issue is particularly pressing, because Drp1 (the mammalian ortholog of Dnm1) recruitment is an early apoptotic event that is important for the efficient execution of death pathways. Using affinity purification and mass spectrometry, we identified Caf4 as a new component of the yeast mitochondrial fission machinery. We discovered that the mitochondrial outer membrane protein Fis1 uses the molecular adaptors Mdv1 and Caf4 to mediate Dnm1 recruitment to mitochondria. These findings led to the current model for Dnm1 recruitment.

We recently developed methods to produce recombinant mitochondrial fission complexes. With these methods, we solved the structure of a portion of the mitochondrial fission apparatus composed of Fis1 and the adaptor proteins (Yan Zhang). Our long-term goal is to reconstitute and solve structures of larger fission complexes. A mechanistic understanding of fission is crucial, because recent evidence indicates that mitochondrial fission is an important component of apoptosis.

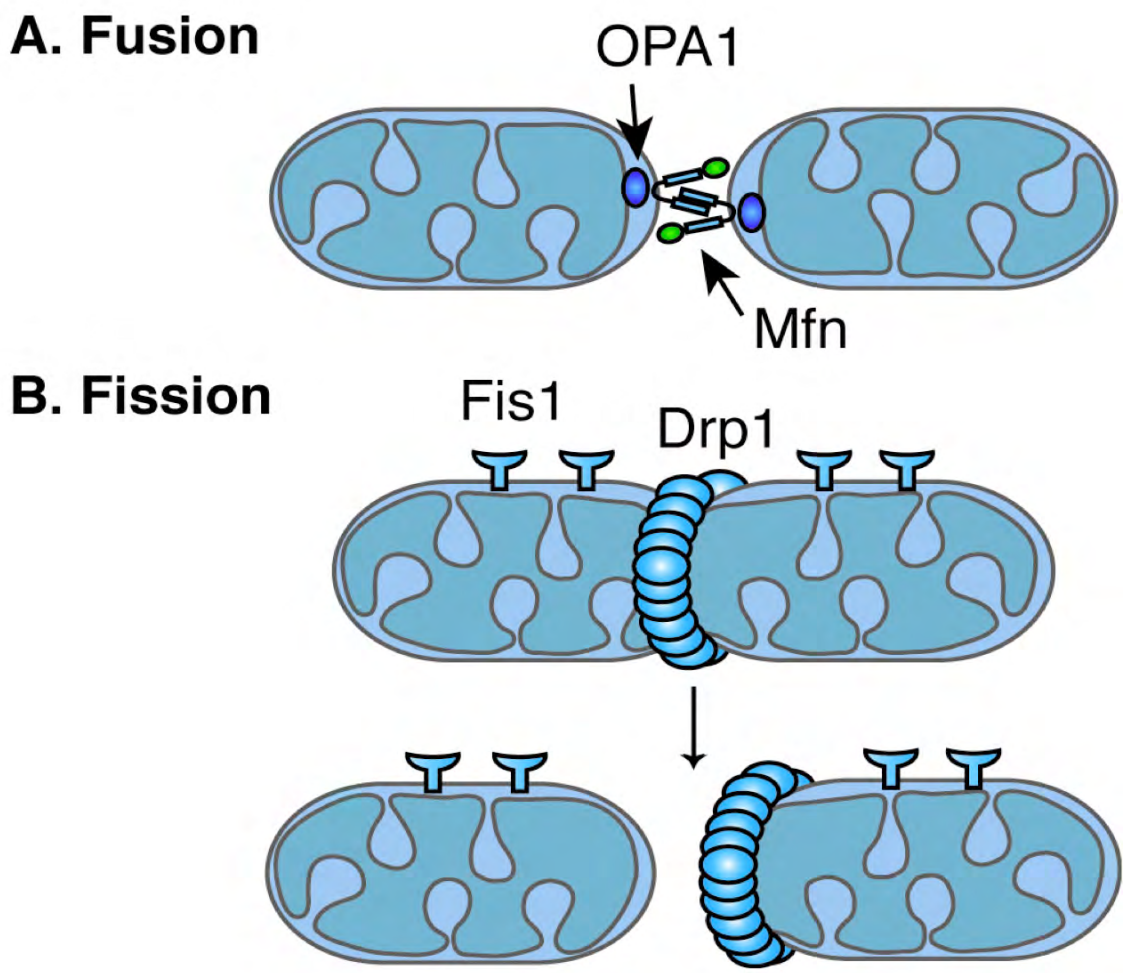


**Fig. 1:** The mitochondrial fusion and fission machinery

**(A)** The fusion machinery. Mitofusins (Mfn) are outer membrane proteins with a GTPase domain (green oval) and helical regions (blue rods). OPA1 (blue ovals) resides in the intermembrane space. In the absence of OPA1 or both Mfn1 and Mfn2, fusion cannot proceed. **(B)** The fission machinery. Fis1 is an outer membrane protein uniformly localized to mitochondria. Drp1 resides in punctate spots, and some of these spots undergo constriction, leading to fission. In yeast, Mdv1 and Caf4 (not shown) act as molecular adaptors linking Fis1 to Dnm1 (Dnm1 and Drp1 are orthologs).

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**Cellular and physiological functions of mitochondrial fusion and fission**

At the cellular level, a major issue is why cells maintain their mitochondria in a dynamic state. One clearly established reason is that a dynamic equilibrium between fusion and fission regulates the morphology of mitochondria. However, cells do not require high levels of fusion and fission to maintain tubular mitochondria, suggesting that there are additional reasons why mitochondria are such dynamic organelles.

A typical mammalian cell can have hundreds of mitochondria. However, each mitochondrion is not autonomous, because fusion and fission events mix mitochondrial membranes and contents. We found that mitochondrial fusion is necessary to maintain uniform function within a population of mitochondria. Fusion-deficient cells have greatly diminished respiratory capacity and reduced cell growth. In addition, the mitochondrial population shows heterogeneous properties, including wide variations in membrane potential. Based on these observations, we proposed that mitochondria do not function well as autonomous organelles and that the dynamic property of mitochondria is inherently important for organelle integrity. In normal cells, high rates of fusion and fission enable mitochondria to cooperate with each other through continual exchange of contents. Individual mitochondria can stochastically lose essential components, but such defects are short-lived, because mitochondrial fusion will restore the missing components from neighboring mitochondria. In cells lacking

mitochondrial fusion, such restoration of activity cannot occur, and defective mitochondria accumulate. Importantly, we have recently discovered that, in the absence of fusion, a large population of mitochondria lack mitochondrial DNA (mtDNA) nucleoids. Therefore, mitochondrial fusion is essential to allow defective mitochondria a pathway to recover mtDNA. This defect explains the respiratory and membrane potential aberrations found in fusion-deficient cells. We are interested in understanding additional roles of mitochondrial dynamics at the cell and tissue level.

Much of our work focuses on proteins called mitofusins (Mfn1 and Mfn2), which are transmembrane GTPases embedded in the outer membrane of mitochondria. These proteins are essential for fusion of mitochondria. To understand the role of mitochondrial fusion in vertebrates, we have constructed mice deficient in either Mfn1 or Mfn2 (Hsiuchen Chen). We find that mice deficient in either Mfn1 or Mfn2 die in mid-gestation. Mfn2 mutant embryos have a specific and severe disruption of a layer of the placenta called the trophoblast giant cell layer. These findings indicate that mitochondrial fusion is essential for embryonic development due to a cell type-specific dependence on mitochondrial fusion.

Embryonic fibroblasts lacking Mfn1 or Mfn2 display fragmented mitochondria, a phenotype due to a severe reduction in mitochondrial fusion. Cells lacking both Mfn1 and Mfn2 have completely fragmented mitochondria and show no detectable mitochondrial fusion activity. Our analysis indicates that mitochondrial fusion is important not only for maintenance of mitochondrial morphology, but also for cell growth, mitochondrial membrane potential, and respiration. Our results indicate that dynamics, by enabling content exchange between mitochondria, provides a pathway to maintain essential components within mitochondria.

**Mitochondrial dynamics in human disease**

Human genetic studies indicate that neurons are particularly sensitive to defects in mitochondrial dynamics. Mutations in the mitochondrial fusion genes Mfn2 and OPA1 cause the neurodegenerative diseases CMT2A and dominant optic atrophy. Both diseases are caused by selective loss of a specific class of neurons. One of our major goals, therefore, is to identify the cellular mechanisms leading to neurodegeneration when mitochondrial fusion is perturbed.

To address this issue, we have generated mice containing conditional null alleles of Mfn1 and Mfn2. Animals lacking Mfn2 have severe cerebellar ataxia. We found that Mfn2 protects against neurodegeneration in both developing and mature Purkinje neurons in the cerebellum (Hsiuchen Chen). Purkinje cells have the most extensive dendritic arbors of any neuron in the brain, and normally their dendritic processes are filled with abundant, tubular mitochondria. In the absence of Mfn2, however, Purkinje cells show poor dendritic arborization, few dendritic spines, and ultimately cell degeneration. We have traced this defect to mitochondrial fragmentation, respiratory dysfunction, and improper mitochondrial distribution within the dendrites. The respiratory defect likely results from loss of mtDNA nucleoids from a subset of mitochondria, as discussed above. Importantly, similar progressive defects in mutant Purkinje cells are recapitulated in cerebellar cell cultures. These results indicate that dendritic outgrowth is highly dependent on mitochondrial dynamics. With these animal and cell culture models, we can now further dissect the mechanistic link between mitochondrial dynamics and neurodegenerative disease.

The most common inherited form of optic neuropathy (autosomal dominant optic atrophy) is caused by mutations in OPA1. This mitochondrial protein is localized to the intermembrane space and is essential for mitochondrial fusion. Little is known about the mechanism of OPA1 action. In particular, there has been no good cell culture system to perform basic structure-function analysis of OPA1. To develop such a system, we have collaborated with Christiane Alexander (Max-Delbrück-Center for Molecular Medicine) to isolate mouse cell lines lacking OPA1 (Hsiuchen Chen). Using these cell lines, we have clarified the role of OPA1 in mitochondrial fusion and have identified the i-AAA protease Yme1L as a regulator of OPA1 processing (Zhiyin Song). Ultimately, a clear understanding of OPA1 will come from biochemical analysis, and we have developed expression systems that will enable such studies (Tadato Ban).

Finally, an understanding of mitochondrial dynamics will be essential for understanding a large collection of diseases termed mitochondrial encephalomyopathies. Many mitochondrial encephalomyopathies result from mutations in mitochondrial DNA (mtDNA). In mtDNA diseases, tissues maintain their mitochondrial function until pathogenic mtDNA levels exceed a critical threshold. Experiments with cell hybrids indicate that mitochondrial fusion, by enabling cooperation between mitochondria, can protect respiration even when >50% of mtDNAs are mutant. To understand the pathogenesis of mtDNA diseases, it is critical to explore how mitochondria can be functionally distinct and yet cooperate as a population within a cell. We anticipate that our studies with mice lacking mitochondrial fusion will help to shed light on this group of often devastating diseases (Hsiuchen Chen).

**29. Biochemical and structural studies of OPA1, a dynamin-related GTPase essential for mitochondrial fusion**

*Tadato Ban*

OPA1, dynamin-related GTPase, has been reported to play a central role in mitochondrial membrane fusion. Several dynamin family proteins have well-known functions in tubulation and constriction of lipid membranes in diverse cellular contexts, including endocytosis, vesicular trafficking, and mitochondrial division. Dynamin-related proteins assemble into higher order filamentous structures and interact with lipid membranes.

However, it is unknown if OPA1 is similarly capable of constricting and tubulating mitochondrial membranes, and how such activities might contribute to mitochondrial membrane fusion. To address such issues, we have developed methods to purify large amounts of recombinant OPA1. We are developing *in vitro* assays to explore the assembly properties of OPA1 oligomers and whether they interact with lipid bilayers. In addition, we are attempting crystallization trials to determine an atomic structure.

**30. Mitochondrial fusion and aging**

*Hsiuchen Chen*

Mitochondria possess a unique genome (mtDNA) that encodes essential components of the electron transport chain. The electron transport chain generates ATP, maintain the mitochondrial membrane potential, and produce reactive oxygen species (ROS). One theory of aging hypothesizes that accumulation of mtDNA mutations leads to mitochondrial and thus, cellular dysfunction. In support of this theory, mice carrying mutations in the proofreading subunit of mtDNA polymerase prematurely display a multitude of characteristics associated with aging, including reduced weight, hair loss, osteoporosis, anemia, and hearing loss. Most strikingly, these mice (termed POLG mice) also show reduced life spans.

Mitochondrial dynamics is crucial to preserving mitochondrial function. Loss of fusion leads to reduction in cellular respiration and membrane potential. In addition, disruption of fusion causes fragmentation of the mitochondria, resulting in a much greater number of mitochondria per cell, each with a significantly smaller mass. Anti-DNA stains show that whereas nearly all wild-type mitochondria possess mtDNA nucleoids, the majority of fusion-deficient mitochondria lack mtDNA. Therefore, there is sequestration of mtDNA into a much smaller mitochondrial mass. This mass may be insufficient to support adequate electron transport activity, thereby explaining the reduction of cellular respiration in the presence of mtDNA. It is important to note that with normal fusion, mitochondria can rapidly replenish stochastic loss of essential components. However, in the absence of fusion, mitochondria lacking mtDNA have no pathway by which to regain either the mtDNA or the proteins they encode, and are therefore, permanently dysfunctional.

An important issue is how the two pathways described above intersect. If fusion does indeed protect against mtDNA loss, lack of fusion may exacerbate the aging phenotype seen in the POLG mice. To test this hypothesis, we have mated the POLG mice to our fusion-deficient mice. Preliminary results indicate a genetic interaction between the processes of mitochondrial mtDNA mutation and organellar fusion. We are currently performing aging studies on these mice and have derived cell cultures to explore the cell biological consequences of increased mtDNA mutations in the absence of fusion.

**31. Mitochondrial fusion in muscle development**

*Hsiuchen Chen*

Our previous knockout mouse studies clearly indicate that mitochondrial fusion is developmentally and physiologically important. Genetic targeting of any of the three known fusion genes, Mfn1, Mfn2, and OPA1, leads to embryonic lethality. The importance of mitochondrial fusion is further underscored by the fact that two neurodegenerative diseases, Charcot-Marie-Tooth disease type 2A (CMT) and autosomal dominant optic atrophy (DOA), are caused by mutations in Mfn2 and OPA1, respectively.

To further explore the physiological requirements for mitochondrial fusion, we have created conditional knockout mice for both Mfn1 and Mfn2. Disruption of both Mfn1 and Mfn2 in skeletal muscle results in severely runted mice that survive to only two months of age. Muscle mass is greatly reduced in these mice, and histological analysis reveals significantly decreased muscle fiber diameters. In addition, all muscle fibers have greatly increased succinate-dehydrogenase (SDH) activity, as assessed by enzymatic assays. Increased SDH activity is a hallmark of many mitochondrial diseases, suggesting a common link between deficiencies in mitochondrial fusion and classical mitochondrial diseases.

**32. Structural studies of proteins mediating mitochondrial fusion**

*Huu Ngo*

Mitochondria are highly dynamic organelles that play crucial roles in energy metabolism, apoptosis, calcium, and iron signaling. To maintain their dynamic nature, mitochondria continuously fuse and divide. Mitochondrial fusion requires the coordinated merger of the outer and inner membranes. In yeast, the fusion event is mediated by the mitofusin Fzo1p and the inner membrane dynamin-related protein Mgm1p. The mammalian orthologs of Fzo1p are the mitofusins Mfn1 and Mfn2, whereas the ortholog of Mgm1p is OPA1. Fzo1p, Mfn1, and Mfn2 contain a GTPase domain and two or three hydrophobic heptad repeat (HR) regions. Although most of the key components of the machinery required for fusion have been identified, the mechanism of how these proteins interact is largely unknown. Our lab previously showed that the HR2 region of Mfn1 forms an anti-parallel, dimeric coiled-coil in *trans* to tether mitochondria. In this study, we attempt to elucidate the structures of full-length Fzo1p and Mfn2. To accomplish this goal, we have engineered several constructs to make soluble recombinant Fzo1p and Mfn2 by replacing a transmembrane domain of each protein with a flexible linker, and then expressing these proteins both in bacterial and baculovirus expression systems. Our results show that these new recombinant Fzo1p and Mfn2 are somewhat more soluble than their native proteins, but more optimization will be necessary to improve their yield.

**33. Mechanistic investigation of mitochondrial transcription factor A**

*Huu Ngo*

Mitochondrial transcription factor A (TFAM) is essential for both initiation of transcription and maintenance of mitochondrial DNA (mtDNA). TFAM can bind DNA specifically at sequences associated with mitochondrial replication origins. It can also bind DNA in a non-sequence-specific manner. TFAM contains two high-mobility group (HMG) regions, motifs found in DNA-binding proteins that distort DNA upon binding. The two HMG domains are followed by a C-terminal tail, which is believed to play an important role in transcriptional activation. Indeed, a deletion of the C-terminal region of human TFAM inhibits transcription. The yeast TFAM ortholog, Abf2p, lacks the carboxyl-terminal region and is only required for maintenance and packaging of mtDNA.

A large body of biochemical evidence indicates that TFAM compacts mtDNA upon binding and is present in sufficient quantities to entirely cover mtDNA, suggesting that TFAM may package mtDNA in a manner analogous to histones and nuclear DNA. However, the biochemical basis of this function is not well understood. We are investigating the mechanistic details of the interactions between TFAM/Abf2p and mtDNA by employing biochemical assays and X-ray crystallography. We have made mature forms, full-length constructs, and constructs lacking of the C-terminal tail of human TFAM, mouse TFAM, and yeast Abf2p. The interactions between these purified recombinant proteins and mtDNA will be examined.

**34. The role of OPA1 in mitochondrial fusion**

*Zhiyin Song*

Mitochondria in living cells form dynamic tubular networks that undergo continual fission and fusion. Some core factors of mitochondrial fission and fusion have been identified, including Fis1, Drp1, mitofusions, and OPA1. It has been reported that the yeast OPA1 orthologue Mgm1 is required for mitochondria inner membrane fusion but not outer membrane fusion *in vitro*. To elucidate whether OPA1 has a similar, selective role in inner membrane fusion, we have developed imaging assays to distinguish outer membrane fusion from inner membrane fusion.

To check the role of OPA1 in mitochondria outer membrane fusion, we marked the outer membrane of mitochondria with a photo-activatable GFP (PA-GFP). We found that this outer membrane marker is exchanged between mitochondria in OPA1-null cells but not in Mfn-null cells. We are currently examining this issue with electron microscopy. Our results indicate that OPA1, but not mitofusins, is dispensible for outer membrane fusion. Moreover, they suggest although CMT2A and DOA both affect mitochondrial fusion, they disrupt distinct steps in the process.

**35. Structural and functional studies of the fission apparatus of mitochondria**

*Yan Zhang*

Mitochondrial fission is an inherent feature of mitochondria, and it controls the morphology and functions of mitochondria. Several functions of mitochondrial fission have been implicated, including facilitation of cell death during apoptosis, mitochondrial distribution in neurons, and promotion of cellular senescence. Therefore, it is necessary to understand the molecular mechanism of mitochondrial fission. Budding yeast is an excellent model to study mitochondrial fission. Dnm1p, a dynamin-related protein, is recruited to mitochondria by mitochondrial outer membrane protein Fis1 and two adaptor proteins, Mdv1p and Caf4p. The latter are homologous proteins containing an NH2-terminal extension (NTE), a coiled-coil region, and a COOH-terminal seven-WD40 repeat domain. Serving as molecular bridges, these proteins bind to Fis1p through the NTE region and to Dnm1p through the WD40 region. Fis1p forms a six-helix bundle with the central four helices adopting two-tandem tetratricopeptide repeat (TPR)-like motifs, a well-characterized protein-protein interaction module. By analogy to other TPR proteins, the prominent hydrophobic groove on the concave side of TRP fold has been widely assumed to bind to Fis1 ligands. To elucidate the mechanism of the recruitment of mitochondrial fission complexes, I have solved the structures of Fis1p in complex with a fragment of Mdv1p or Caf4p NTE domain. The crystal structures reveal that either adaptor protein packs against a different region adjacent to the predicated TPR groove on the concave side of Fis1p. In addition, the longer Caf4p fragment also interacts with the convex surface of the Fis1 helical bundle, therefore forming a U-shaped structure and wrapping around Fis1p. Biochemical and cell biological experiments confirmed the importance of these interactions for fission activity *in vivo*. To extend these findings, I have been working on larger complexes, in which the adaptor proteins contain the coiled-coil domain. I am able to express and purify these protein complexes, and they are being screened for crystallization. In addition, I have also successfully expressed the Mdv1p WD40 domain and Dnm1p individually in the baculovirus system on small scale. This will lead to large-scale expression, purification, and future biochemical and structural characterization of the physical interactions between Dnm1p and the adapter proteins.

**36. Structural study of i-AAA protease Yme1L**

*Yan Zhang*

AAA+ proteases are ATP-dependent proteases. They usually consist of multimeric peptidases and AAA+ ATPases, which can reside on separate polypeptide chains or a single chain. In mitochondria, AAA+ proteases are located either in the matrix or in the mitochondrial inner membrane and play an important role in mitochondrial protein quality control and protein processing. The membrane anchored AAA+ proteases are classified based on the location of their catalytic domains: i-AAA

proteases face the mitochondrial intermembrane space and m-AAA proteases are exposed to the matrix. The i-AAA protease is composed of the Yme1L subunit. Yme1L has been thought to be involved in degrading misfolded mitochondrial proteins in the intermembrane space. Recently, our laboratory found that Yme1L is involved in cleavage of OPA1 at the S2 site to yield a short form of OPA1. OPA1, a dynamin-related large GTPase associated with mitochondrial inner membrane, is essential for mammalian mitochondrial fusion and protects cells from apoptosis. The linkage of Yme1L to OPA1 raises many questions: how does Yme1L perform protein processing at specific sites versus degradation of whole proteins? What are the roles of Yme1L during apoptosis? To seek answers to these questions, we are using a structural biological approach to understand the proteolytic mechanism of Yme1L. Using a bacteria expression system, I have successfully expressed, purified, and crystallized human Yme1L protease domain. The crystals diffract to 3 Å and belongs to the P6 spacegroup with a=b=114.4 Å and c=58.0 Å. Molecular replacement was carried out using a bacteria homologue FtsH protease domain as a search model. A solution was found, which contains two molecules in one asymmetric unit. The model is currently being refined. In the meantime, I have also expressed, purified and crystallized a mouse Yme1L fragment containing both the ATPase and the protease domains. Molecular replacement using various models did not produce good phasing. Attempt to obtain heavy metal derivatives by soaking native crystals in heavy metal solutions also failed due to the fragility of the Yme1L crystals. I have been working on producing a selenomethionine-substituted protein, which can be used to obtain *de novo* phasing using MAD or SAD.

**Publications**

Chen, H., McCaffery, J.M. and Chan, D.C. (2007) Mitochondrial fusion protects against neurodegeneration in the cerebellum. *Cell* **130**:548-562.

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**Support:** The work described in the following research reports has been supported by:
Beckman Institute
Gordon and Betty Moore Foundation
Howard Hughes Medical Institute
National Institutes of Health
Weston Havens Foundation

**Summary:** The Deshaies lab works on two basic biological processes: Control of cell division, and regulation of cell function by attachment of ubiquitin or ubiquitin-like proteins to target polypeptides. We are particularly interested in how attachment of ubiquitin to proteins enables their degradation, and how protein degradation via this mechanism is used to regulate cell division.

Defective control of cell division can result in disease, as when unrestrained cell proliferation leads to cancer. Defects of the ubiquitin system can also lead to cancer, as well as neurodegenerative diseases. An understanding of how the cell division machinery and the ubiquitin system operate will thus provide insight into basic cellular processes essential to the life of eukaryotic cells, and may suggest cures for diseases that affect millions of people.

We are using biochemical, molecular, and genetic approaches in baker's yeast and mammalian cells to investigate cell proliferation and the ubiquitin system. Our long-term goal is to understand how these processes work and how they are controlled. Baker's yeast is an excellent organism for basic cell biological studies because it is easy to work with, and many studies have confirmed that yeast and animal cells largely use the same proteins to regulate basic cellular processes.

The next section contains a brief description of the four major areas of investigation in the lab, followed by thumbnail descriptions of all current projects.

**SCF ubiquitin ligases: Mechanism, regulation, and physiology**

Cellular proteins are marked for degradation by attachment of the polypeptide ubiquitin. Ubiquitin is attached to substrates by a cascade comprising ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes. Ubiquitination occurs when an E3 enzyme binds to both substrate and E2 ubiquitin conjugating enzyme, bringing them into proximity so that ubiquitin is transferred from the E2 to substrate. Specificity and regulation of ubiquitination are typically imparted by E3s, which are the most diverse components of the system. Once ubiquitin is attached to a substrate, the reaction can either terminate (in which case the ubiquitin serves as a regulatory signal to modulate protein function or localization) or continue, leading to the assembly of a multiubiquitin chain. A chain of four ubiquitins suffices to specify destruction of the substrate by the proteasome.

*Mechanism of action of SCF ligases*: In 1999, we, and others, reported that RING domains underlie ubiquitin ligase activity (Seol *et al*., 1999). This discovery revealed what is now thought to be the largest class of ubiquitin ligases, with up to 385 members. The progenitor of the RING-based ubiquitin ligases, SCF (Feldman *et al*., 1997), defines a subfamily of multisubunit cullin–RING ligases that may number as many as 300-350 members, due to combinatorial mixing of subunits. Thus, there may be as many as 700 distinct RING ligase complexes, which would make it the largest-known family of enzymes in human cells (Petroski and Deshaies, 2005). As befits such a large family, the cullin–RING ligases have been implicated in a dazzling array of cellular and organismal processes, ranging from circadian rhythms to sulfur assimilation. However, despite the biological import of these enzymes, the mechanism of how they work remains unknown.

Over the past few years, we have made substantial progress towards understanding how SCF enzymes work. A key step was to develop a reconstituted system in which a physiological substrate (budding yeast Cdk inhibitor Sic1 assembled into cyclin–Cdk complexes) is ubiquitinated by a complex of SCF and the ubiquitin-conjugating enzyme Cdc34, and subsequently is degraded by the proteasome – work that was carried out by Renny Feldman, Craig Correll, and Rati Verma (Feldman *et al*., 1997; Verma *et al*., 2001). Matt Petroski then constructed Sic1 substrates bearing single ubiquitin acceptor lysines, and used these substrates to characterize the impact of ubiquitin chain position on substrate recognition and degradation by the proteasome (Petroski and Deshaies, 2003). Matt went on to use his single-lysine substrate to show that assembly of a ubiquitin chain can be broken down into distinguishable initiation and elongation reactions (Petroski and Deshaies, 2005).

We believe that the system that we have developed is the best available for studying biochemical mechanisms of ubiquitination, because our substrate is multiubiquitinated and degraded at rates that approach those that must occur *in vivo*, and the nature of the reaction products is defined due to the substrate having only a single lysine acceptor. We are now in an excellent position to exploit our *in vitro* systems to address basic questions that are of central importance to understanding the mechanisms that underlie the operation of the ubiquitin-proteasome system (UPS) (see abstracts by G. Kleiger, N. Pierce, and A. Saha). For example, how does a RING domain activate ubiquitin transfer from ubiquitin-conjugating enzyme to substrate? What is the basis for the

synthesis of the lysine 48-linked ubiquitin chains that signal proteolysis? How is processive ubiquitination of Sic1 achieved, and how does it relate to the dynamics of substrate and Cdc34 (E2) association with SCF? How does ubiquitin transfer occur across an ~50 Å gap that is thought to exist between the substrate and E2 enzyme bound to SCF? How do unique sequence elements of Cdc34 – including its tail and the acidic loop insertion sequence near the catalytic site – contribute to its function with SCF? None of these questions are resolved for any RING E3, and thus, illuminating the answers will establish paradigms that inform our understanding of how hundreds of ubiquitin ligase enzymes work. The insights that emerge from this effort may also provide clues to the development of drugs that modulate the activity of RING-based ligases.

*Regulation of SCF ubiquitin ligases*: It was originally thought that SCF ubiquitin ligases are constitutively active, and substrate turnover is regulated by phosphorylation of the substrate. Subsequently, it was shown that the Cul1 subunit of SCF is modified covalently by the ubiquitin-like protein Nedd8 thus, raising the possibility that SCF might be regulated post-translationally. In 2001, two students from the lab, Svetlana Lyapina and Greg Cope, reported that a poorly understood protein complex known as COP9 Signalosome (CSN) binds SCF in animal cells, and promotes the cleavage of Nedd8 from Cul1 via a tightly-associated Nedd8 isopeptidase activity (Lyapina *et al.*, 2001). This was the first biochemical function ascribed to CSN, and opened the door to the study of SCF regulation by reversible cycles of 'neddylation.' Subsequently, Greg Cope discovered that the Csn5 subunit harbors a motif that we named 'JAMM' (for JAb1/Mpn domain Metalloenzyme) (Cope *et al*., 2002). We predicted that JAMM comprises a novel metalloprotease active site. Later, Xavier Ambroggio, who was a joint student with Doug Rees in Chemistry, substantiated this prediction by employing X-ray crystallography to show that the conserved residues of the JAMM motif coordinate a zinc ion in an active site-like cleft of the protein AF2198 from *Archaeglobulus fulgidis* (Ambroggio *et al.*, 2004). We continue to investigate the regulation of SCF by CSN (see abstract by E. Emberley). We hope to understand how CSN itself is controlled, and what role CSN plays in sustaining active SCF complexes and sculpting the repertoire of SCF complexes in a cell.

**Mechanism of action and regulation of the 26S proteasome**

Once substrates are ubiquitinated by E3s, they are degraded by the 26S proteasome. The 26S proteasome is a large protein machine that comprises two major subcomplexes: the 20S 'core' proteasome and the 19S regulatory 'cap.' The 20S core forms a cylindrical structure that houses the protease active sites of the proteasome. Each end of the 20S cylinder is decorated with a 19S cap. The 19S cap can be further subdivided into the 'lid' and the 'base.' The base contains six ATPases that are thought to form a ring that abuts the end of the 20S cylinder. The lid, in turn, sits upon the base. The base is thought to control access of substrates into the 20S proteolytic chamber, whereas the lid confers ubiquitin dependence. The 26S proteasome degrades proteins that are linked to a chain of at least four ubiquitins. The tetraubiquitin chain mediates binding of the attached substrate to the proteasome, after which it is disengaged from bound partners, unfolded, deubiquitinated, and translocated into the proteolytic chamber of the proteasome where the denuded substrate is degraded.

*A fully reconstituted system to study Sic1 degradation*: To harness the power of yeast molecular genetics to enable dissection of the mechanism-of-action of the proteasome, we developed a system wherein ubiquitinated Sic1 generated *in vitro* with recombinant $SCF^{Cdc4}$ is degraded by affinity-purified yeast proteasomes (Verma *et al*., 2001). Remarkably, purified proteasomes can extract ubiquitinated Sic1 from complexes with S phase cyclin-Cdk, degrade the Sic1 and release active S phase cyclin-Cdk protein kinase. This result emphasized that the proteasome has the intrinsic ability to disassemble protein complexes to selectively degrade ubiquitinated substrates, and set the stage for our subsequent studies on substrate targeting and deubiquitination.

*Role of deubiquitination in the degradation of Sic1*: In the course of characterizing the degradation of ubiquitinated Sic1, we noticed that when the 20S protease inhibitor epoxomicin was present, ubiquitinated Sic1 was converted to a completely deubiquitinated species (Verma *et al*., 2002). Fortuitously, at the same time as this we observed that the CSN – which is related to the lid subcomplex of the proteasome 19S cap – cleaves the ubiquitin-like protein Nedd8 from the Cul1 subunit of SCF. Spurred by this confluence of observations, we demonstrated that the Rpn11 subunit of the proteasome lid contains a putative JAMM metalloprotease active site analogous to that of Csn5, and this motif is essential for the deubiquitination of Sic1 *in vitro* and the degradation of multiple UPS substrates *in vivo*.

*Multiubiquitin chain receptors target substrate to the proteasome*: Although it has long been clear that a multiubiquitin chain targets an appended substrate to the proteasome for degradation, the mechanism of targeting has remained poorly understood. Genetic studies in yeast have suggested a potential role for multiubiquitin chain-binding proteins, including Rad23, Dsk2, Ddi1, and Rpn10. In contrast, biochemical studies in mammalian systems have emphasized a role for the proteasome ATPase Rpt5 as a multiubiquitin chain receptor, and have suggested that proteins such as Rad23 prevent premature metabolism of substrate-linked ubiquitin chains. We reasoned that our reconstituted system would enable us to address this fundamental question from a functional, mechanistic perspective. We first demonstrated using mutant proteasomes and add-back experiments that Rad23

and Rpn10 play a direct role in targeting ubiquitinated Sic1 to the proteasome for degradation (Verma *et al*., 2004a). We then went on to show that the multiubiquitin chain receptor activities of Rad23 and Rpn10 play a redundant role in sustaining turnover of Sic1 *in vivo*. Surprisingly, individual deletion of these and other receptor proteins led to the accumulation of different UPS substrates, suggesting that the receptors define a layer of specificity that resides downstream of the E3s and upstream of the proteasome. This hypothesis opens up a host of interesting questions about how specificity is achieved in the targeting step, and what its biological purpose is. We plan to address these key questions over the next several years using a combination of biochemical, molecular genetic, and proteomic approaches (see abstract by T. Gomez).

*Proteasome inhibitors*: Small molecules that inhibit protein turnover by the proteasome can selectively kill cancer cells. One inhibitor has already been approved by the FDA for the treatment of the blood cell cancer multiple myeloma, and others are currently in clinical development. We are interested to understand why these inhibitors kill some cancer cells (such as multiple myeloma and other hematological cancers) but are much less effective against solid tumors (see abstract by S. Radhakrishnan).

A couple of years ago, in collaboration with Dr. Randy King at Harvard, we discovered ubistatins, which are novel inhibitors of protein degradation by the proteasome. We demonstrated that ubistatin A binds to the ubiquitin chain in the same intersubunit cleft that is normally bound by the multiubiquitin chain receptors (Verma *et al*., 2004b). This binding prevents the ubiquitin chain from binding to receptors (Rpn10 and Rad23) that link it to the proteasome. A postdoctoral fellow who is just joining the lab (E. Miller) plans to study a second ubistatin (ubistatin B) that emerged from this screen. Ubistatin B shows promising activity in blocking protein degradation by the UPS in mammalian cells. We believe that ubistatins will be useful tools for studies on the UPS.

*Role of Cdc48 in targeting and degradation of ubiquitinated proteins*: Recently, we have become intrigued by a poorly understood protein, Cdc48, that, like the ubiquitin chain receptors, operates downstream of ubiquitin ligases to promote degradation of ubiquitinated proteins by the proteasome. The role of Cdc48 in protein turnover was originally thought to be confined to pulling malfolded secretory proteins through the endoplasmic reticulum membrane so that they can be degraded by the proteasome. However, several lines of evidence hint at a far broader role. Interestingly, there may be as many as seven distinct Cdc48 complexes in budding yeast, and our unpublished data points to the existence of ~15 distinct Cdc48 complexes in human cells (see abstract by G. Alexandru). Why all of this complexity? It is difficult to even begin to answer this question, because we know so little about Cdc48's function apart from its role in translocation across the ER membrane. Whatever Cdc48 is doing, it appears to be a fundamental component of the UPS, and thus, understanding how it works is important. We plan to attack this problem by first identifying substrates whose degradation depends on particular Cdc48 complexes. One strategy is to study candidate substrates such as Hsl1 or Cdc5 (see abstract by R. Verma). Another strategy is to employ the mass spec-based proteomics technology discussed below, as well as a novel *in vivo* substrate screen (see abstract by T. Chou). We will then reconstitute the degradation of these substrates using defined components. The objective will be to develop a reconstituted system in which turnover of the substrate is dependent upon Cdc48. We will then use this system to establish the mechanism-of-action of Cdc48. Armed with this information, we will be in a position to initiate investigations on how ubiquitin receptors and Cdc48 complexes collaborate to enable degradation of ubiquitinated substrates. Given the diversity of receptors that guide ubiquitinated proteins to the proteasome and the diversity of Cdc48 complexes that appear to act in concert with these receptors, there is clearly much about the targeting and degradation of ubiquitinated proteins that we do not understand, and thus, this topic may be fertile ground for making unexpected discoveries.

**Proteomics**

The Yates laboratory at Scripps has developed a method dubbed, "multidimensional protein identification technology" (MudPIT). In this method, a complex mixture of proteins is digested with protease and the resulting peptides are fractionated by two-dimensional chromatography on a column that is in-line with the electrospray interface of a mass spectrometer. Johannes Graumann and Thibault Mayor have applied MudPIT to the study of ubiquitination in yeast. In our first efforts we employed subtractive comparisons of samples from wild type and mutant cells to identify the ubiquitinated polypeptides that accumulate when the Rpn10 multiubiquitin chain receptor is absent (Mayor *et. al*., 2005). More recently, we have used differential labeling of mutant and wild-type cells with stable isotopes to obtain quantitative estimates of substrate accumulation in Rpn10-deficient cells (Mayor *et. al*., 2007). By identifying the set of substrates whose abundance is altered when a particular ubiquitin pathway component is mutated or blocked by the action of a drug, we hope to gain insight into enzyme-substrate relationships, which in turn may yield insights into the mechanisms that underlie specificity. Moreover, knowledge of the substrates affected can provide clues to the phenotypes that may occur upon inactivation of a particular component. Finally, the ability to quantify substrate accumulation may enable us to see subtle defects, such as those that occur when one member of a redundant pair of enzymes is mutated.

In a second project that we are just starting, we plan to use a combination of crosslinking and MudPIT to identify dynamic protein interactions that occur inside cells but do not survive the immunoprecipitation and washing steps that are normally employed in the affinity

purification of protein complexes for analysis by mass spectrometry (see abstract by E. Lee).

A third proteomics-related project is to screen an shRNA library to identify genes of the UPS that influence the differentiation of embryonic stem cells into cardiomyocytes (see abstract by N. Honarpour).

**Functions of the RENT complex in cell cycle control and nucleolar biogenesis**

Several years ago, a graduate student, Wenying Shou, discovered the RENT complex, and proposed that the mitotic exit network (MEN) specifies the exit from mitosis in budding yeast by promoting disassembly of RENT (Shou *et al*., 1999). RENT is comprised of the nucleolar anchor protein Net1, the cell cycle regulatory protein phosphatase Cdc14 and the chromatin silencing protein Sir2. Cdc14 is required for the exit from mitosis, which it promotes by dephosphorylating (and thereby activating) proteins that mediate the inactivation of cyclin/CDK activity at the end of mitosis. Throughout the cell cycle, Cdc14 is confined to the nucleolus through its interaction with Net1. At the end of mitosis, the successful completion of anaphase activates the MEN signaling pathway, which disengages Cdc14 from Net1. The emancipated Cdc14 goes on to inactivate cyclin/CDK and thereby trigger the exit from mitosis. This hypothesis for how the exit from mitosis is controlled in budding yeast was dubbed 'RENT control' by Shou *et al*., 1999. Over the past few years, it has become apparent that RENT is disassembled by a two-step mechanism. In early anaphase, Cdc14 is released from Net1 through a novel activity of separase. Separase is a protease that activates chromosome segregation in anaphase by cleaving the cohesin protein that holds sister chromatids together.

Throughout interphase and early mitosis, separase activity is repressed by a tightly bound inhibitor, securin. At the metaphase-anaphase boundary, securin is abruptly degraded, thereby liberating separase to cleave cohesin and initiate chromosome segregation. In addition to being a protease, separase has a second activity that promotes the phosphorylation of Net1 by cyclin B–Cdk. A graduate student in the lab, Ramzi Azzam, had identified this phosphorylation and demonstrated that it induces the dissociation of Cdc14 from Net1 (Azzam *et al*., 2004). Thus, the action of separase links initiation of the exit from mitosis with the initiation of chromosome segregation. In late anaphase the MEN serves to sustain Cdc14 release and enable the released Cdc14 to gain access to the cytoplasm, such that its substrates are dephosphorylated and the cell exits mitosis. Our current goal is to understand how the terminal signaling component of the MEN, the protein kinase Dbf2, mediates the sustained release of Cdc14 from the nucleolus and its translocation to the cytoplasm thereby triggering exit from mitosis (see abstract by D. Mohl).

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**37. p97 regulation via interaction with UBX domain-containing co-factors**

*Gabriela M. Alexandru*

p97/Cdc48 is a type II AAA (ATPase associated with a variety of activities) ATPase, highly conserved from archaebacteria to mammals. p97 plays a role in seemingly unrelated cellular activities, such as membrane fusion, endoplasmic reticulum-associated protein degradation (ERAD) and cell cycle regulation. All of these functions involve recognition of ubiquitinated protein-substrates and, at least in some cases, their subsequent degradation by the proteasome. In its active form, p97 forms homohexameric barrel structures in which the N-termini are free to bind substrate-recruiting co-factors. Thus, p97 in complex with p47 is thought to regulate membrane fusion, while p97/NPL4/UFD1 complexes are mainly required for ERAD. In an attempt to further understand the molecular basis for p97's diverse functions we have analyzed p97 immunoprecipitates from human tissue culture cells by MudPIT (Multidimensional Protein Identification Technology), searching for new p97 co-factors. This analysis revealed eight p97-binding partners, all having a UBX domain in their C-terminal region. Two of them have been linked to human diseases, such as atopic dermatitis and alveolar soft part sarcoma. However, the biological function for most of these proteins is largely unknown. Comparative analysis of Flag-UBX protein immunoprecipitates from human cells revealed that some of them bind ubiquitinated proteins and also interact with multiple E3 ubiquitin ligases, suggesting they might be involved in ubiquitin-dependent protein degradation. This analysis was initially aimed to identify substrates interacting specifically with each UBX-domain co-factor of p97. In particular, we found that UBXD7 mediated p97 interaction with the CUL2/VHL ubiquitin ligase and its substrate, the hypoxia-inducible factor 1a (HIF1a). Depletion of p97 by siRNA led to accumulation of endogenous HIF1a and increased expression of the HIF1a target carbonic anhydrase IX. Thereby our work revealed an unexpected role for p97 in functional regulation of HIF1a, which is the key governor of cellular and organismal responses to oxygen tension.

**38. Development of inhibitors for Cdc48/P97 AAA ATPase**

*Tsui-Fen Chou*

Cdc48/p97 is an important AAA ATPase not only due to its intriguing diverse cellular functions but also because it has been implicated in mediating turnover of many proteins involved in tumorgenesis**.** In an effort to develop small-molecule inhibitors for Cdc48/p97 based on its X-ray structure, we first searched for the scaffolds that are likely to bind to the ATP-binding pocket of the D2 domain of murine p97. Of particular interest to us are scaffolds that include an electrophile that can readily react with a natural active-site cysteine of p97. Inhibition is monitored by using an *in vitro* ATPase activity assay and the exact cysteine residue involved in the inactivation was confirmed by mass spectroscopy. The studies we have performed so far have established that it is feasible to identify specific and reasonably potent small molecule inhibitors of p97 that inhibit UPS activity in tissue culture cells. We have now initiated a much larger-scale HTS effort to identify the best possible p97 inhibitor for future cell culture and *in vivo* studies aimed at evaluating whether p97 is a good target in cancer. Our approach has been to establish a collaboration with the NIH screening center at Scripps that is directed by Dr. Hugh Rosen. This center has >250,000 screenable compounds at its disposal, including a large number that are based on a purine scaffold. Compounds identified from this study that show good inhibition potency toward the wild-type enzyme will be useful lead compounds for developing anticancer drugs that target the ubiquitin-proteasome system at a stage upstream of the proteasome. On the other hand, those compounds that inhibit engineered mutants but not the wild-type enzyme will provide a bio-orthogonal inhibitor/mutant pair that can serve as a valuable biological tool for study the physiological function and mechanism of Cdc48/p97.

**39. Regulation of SCF ubiquitin ligase activity**

*Ethan Emberley*

Ubiquitin-mediated protein degradation has emerged as a pivotal process in many areas of cell biology. The multi-subunit SCF (Skp1/Cul1/F-box protein) ubiquitin ligase enzymes transfer ubiquitin molecules onto target proteins destined for degradation by the 26S proteosome. Many cellular proteins, possibly hundreds, are targeted for degradation by SCF complexes. Inappropriate SCF activity can result in an enhancement in the degradation of a pool of proteins that may be required to maintain the cell in a normal state. As well, the SCF may no longer ubiquitinate a separate pool of substrates resulting in their intracellular accumulation and amplification of their function. Despite impressive advancements in our knowledge about the SCF protein complex, many fundamental aspects remain poorly understood, including its mechanism of action and regulation. We have begun to study the mechanism by which substrate binding to its respective F-box protein controls SCF activity. We propose that substrate binding either positively regulates the attachment of the ubiquitin-like protein Nedd8 to the SCF subunit Cul1 by Ubc12 (i.e., neddylation), or negatively regulates Cul1 deneddylation by the COP9 signalosome. The Nedd8 modification on Cul1 is necessary for SCF ubiquitin ligase activity and provides another layer of SCF regulation. The SCF and COP9 signalosome complexes have been previously shown to physically interact with each other and we aim to define the importance of this interaction further by characterizing the biochemical requirements that result in

the removal of Nedd8 from Cul1 and the eventual shutdown of SCF's ubiquitin ligase activity. By describing the specific effects of substrate binding on the neddylation status of SCF, we will better understand the steps needed for SCF activity, as well as described a mechanism by which the protein to be degraded is influencing its own ubiquitination. This new pathway controlling SCF activity could be the target of therapeutic intervention as deregulated SCF activity has been suggested to be involved in several human malignancies.

**40. Binding of 26S proteasome subunits to ubiquitin receptor proteins**

*Tara Gomez*

The mechanism by which ubiquitinated proteins are delivered to the 26S proteasome is poorly understood. The 26S proteasome is composed of a 20S catalytic core particle (CP) and a 19S regulatory particle (RP), which itself is composed of a base and a lid. In *Saccharomyces cerevisiae*, the base is composed of about eight proteins, one of which, *RPN1* is known to play an important role in binding to ubiquitin receptor proteins such as *RAD23*, *DSK2* and *UBP6*, all of which contain a ubiquitin-like domain (UBL) (1, 2). It is believed that *RAD23* and *DSK2* deliver ubiquitinated substrates to the proteasome to promote degradation, and may bind the same or overlapping sites of *RPN1* via their UBL domain (3,4,5). *UBP6*, a deubiquitinating enzyme, may preferentially deubiquinate substrates targeted by *RAD23,* but does not compete with *RAD23* for binding to *RPN1* (3). We plan to take a genetic approach to gain a better understanding of the mechanism and regulation of how UBL domain proteins are recruited to the proteasome.

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**41. Ubiquitination in stem cell differentiation and cardiovascular development**

*Narimon Honarpour*

Stem cell therapy is a developing technology with great potential to treat human disease. A major limitation, however, is that little is known about how stem cells differentiate. Thus, it is not currently possible to reliably generate tissue that could be used for cell-based therapy. Because the ubiquitin-proteosome system (UPS) plays a central role in regulating intracellular signaling, we hypothesize that key switches governing differentiation pathways are also controlled by the UPS. We propose to test this hypothesis by seeking UPS genes that influence embryonic stem cell differentiation into cardiovascular tissue. Our approach involves generating mouse embryonic stem cell lines that express lineage-specific reporter genes, transfecting these cells with a siRNA library, and screening for spontaneous or accelerated differentiation. After identifying siRNAs that promote differentiation, mechanisms by which silenced UPS genes affect differentiation will be further analyzed. As a reporter cell line for cardiac differentiation has been obtained, current efforts are directed towards characterizing the behavior of our screen for cardiac lineage commitment.

**42. Molecular mechanism of ubiquitin chain synthesis by Cdc34**

*Gary Kleiger*

Cdc34 is a ubiquitin-conjugating enzyme that is an essential component of the Ubiquitin Proteasome System (UPS). It functions by catalyzing the formation of polyubiquitin chains onto protein substrates destined for degradation by the 26S proteasome. Cdc34 works in concert with the ubiquitin ligase SCF, which is also an essential component of the UPS.

Cdc34 and SCF combine to form a potent ubiquitin ligase complex that processively labels its substrates. This processivity has important functional consequences for living cells, since it has been shown that a protein substrate must be labeled with at least four consecutive ubiquitins before it is recognized by the proteasome. In addition, living cells contain proteins that degrade ubiquitin chains. Therefore, ubiquitin ligases are working against the clock to put enough ubiquitins onto their substrates so that they will be degraded.

Our goal is to explain the molecular mechanism of Cdc34 and SCF processive enzyme activity. We are focused on the C-terminal tail domain of Cdc34, a conserved acidic stretch of 80 to 120 amino acids, found on all Cdc34 molecules from yeast to humans. We, and others, have demonstrated that deletion of the Cdc34 tail either drastically reduces or eliminates all SCF-dependent Cdc34 activity. We have expressed and purified the tail domain by itself in bacteria cells. The addition of free tail to SCF-dependent Cdc34 reactions competes with wild-type Cdc34, demonstrating that the function of the tail is at least partially retained in the absence of the catalytic domain of Cdc34. This result also hints that the function of the tail is to interact with SCF.

If the function of the Cdc34 tail domain is to mediate an interaction between Cdc34 with SCF, we hypothesized that the molecular fusion of the Cdc34 catalytic domain without its tail sequence to the SCF ligase should rescue the tail delete phenotype. Currently our data support this notion and future directions are focused on both the further biochemical characterization of the tail, as well as characterization of the SCF-Cdc34 fusion constructs.

**43. Identifying molecular components involved in Parkinson's disease**

*J. Eugene Lee*

Parkinson's disease is the most prevalent neurodegenerative movement disorder. Recent genetic findings have provided important insights into the pathogenetic mechanism of this diseases. In a large

number of familial Parkison's disease cases, *parkin* was found to contain deletion and missense mutations. *Parkin* encodes a 465-residue ubiquitin ligase. With the aid of ubiquitin-conjugating enzymes, ubiquitin ligases catalyze the synthesis of a polyubiquitin chain on the target substrate. Currently, it is unclear which proteins are modified by parkin in the cell. Moreover, the enzymatic function of parkin is controversial. Certain lines of evidence suggest that parkin mediates the typical K48-linked polyubiquitination for substrate degradation, while others implicate K63-linked polyubiquitination. K63-linked polyubiquitination signals cellular events that are distinct from substrate degradation. To elucidate how parkin functions, we will develop a sensitive protein identification method composed of *in vivo* cross-linking and quatitative mass spectrometry. Using this method, we will identify the substrates, and the ubiquitin-conjugating enzyme for parkin, which directs the conformation of the polyubiquitin chain synthesized. The successful completion of this proposal will present a new strategy for identifying dynamic protein–protein interactions, and could aid in the development of novel therapeutics for Parkinson's disease.

**44. Small molecule inhibitors of the proteasome's regulatory particle**

*Eric Miller*

Proteolysis via the ubiquitin proteasome system (UPS) involves the attachment of multiple ubiquitin (Ub) molecules to a target protein and degradation of that protein via the proteasome. The proteasome is a multicatalytic proteinase complex that consists of a cylindrical, proteolytic core particle (CP) capped on one or both ends by a regulatory particle (RP). The RP performs several key functions including recognition and binding of ubiquitinated substrates, unfolding of the substrate, enzymatic substrate deubiquitination, opening of the access pores in the CP and transfer of the unfolded protein into the CP where it is degraded.

The overall aim of this research is to completely characterize the biological activity of a small molecule (TLI-258) that blocks the RPs ability to recognize and bind ubiquitinated substrates. We will use this information to formulate more active derivatives of TLI-258 and determine how it could best be used to study the UPS. We propose to conduct multiple *in-vitro* and *in-vivo* experiments to completely understand the biological activity and specificity of TLI-258. We will utilize the information gleaned from these experiments to guide a collaborative medicinal chemistry effort geared toward formulating more cell-permeable, potent or selective derivatives of TLI-258. We will also use the information gleaned from the initial experiments to ascertain how TLI-258 could best be used as a research tool. Ultimately we plan to employ TLI-258 in conjunction with mass spectral-based proteomic analyses to more accurately determine the total number and identity of cellular proteins that are subject to ubiquitination.

Proteasome inhibition is a validated approach in anticancer chemotherapy. Unlike conventional targets, the proteasome represents a unique opportunity for the development of 2nd and 3rd generation inhibitors because it is a multi-enzyme, multi-functional complex. Inhibition of equally essential aspects of proteasome function that are distinct from CP activity such as Ub recognition and binding, should have the potential to exhibit considerable differences in therapeutic index and side-effect profile while maintaining efficacy.

**45. Regulation of *Saccharomyces cerevisiae* Cdc14**

*Dane Mohl*

Our work hopes to illuminate the mechanisms that allow the cell to sense the duplication of the DNA genome, and link completion of chromosome segregation to the initiation of cell division. In my research, I have used the model yeast system *Saccharomyces cerevisiae* to look more closely at the regulation of a key cell cycle phosphatase, Cdc14. Our work has demonstrated that a protein kinase complex called Dbf2/Mob1, that becomes active when each of the two new nuclei are segregated to opposite compartments of a pre-divisional cell, directly regulates Cdc14 phosphatase, thereby linking genome duplication to cytokinesis.

The aim of my project has been the identification of *cis*-acting sequences within Cdc14 and its inhibitor Net1p that are required for late mitotic regulation. We have also been searching for the transacting factors that act upon those epitopes. Live cell imaging and GFP fluorescence localization led us to conclude that a small portion of Cdc14-GFP visited the daughter bound spindle pole bodies of anaphase cells and could therefore, be a direct substrate of Dbf2/Mob1. Our research has, therefore, focused on identifying sequences within Cdc14 that could be sites of Dbf2/Mob1 phosphorylation. Mass spec peptide analysis of *in vitro*- and *in vivo*-phosphorylated Cdc14p has illuminated the importance of key C-terminal sequences within Cdc14. Genetic analysis and GFP fluorescence studies have strengthened our conclusion that Cdc14 is a direct substrate of Dbf2/Mob1 and that phosphorylation of Cdc14 within a tightly regulated nuclear localization signal may enable Cdc14 to escape nucleolar sequestration by Net1 thereby allowing it to reach targets within the cytoplasm.

Our most recent work hopes to find additional Dbf2/Mob1 substrates, as well as identify factors that may act as Cdc14 substrates or factors that partner with Cdc14 to facilitate its activity in both the cytoplasm and nucleus.

**46. Integrated dynamics of Sic1**

*Nathan Pierce*

Cell cycle progression in yeast is controlled by the activation and degradation of cyclins and cyclin-dependent kinases (Cdk). Our work focuses on the integrated dynamics of the B-type cyclin inhibitor Sic1. Sic1 is expressed at the end of mitosis, whereupon it binds and inhibits the protein kinase activity of Clb-Cdc28 Cdk complexes. Sic1 is multi-phosphorylated at the end of G1 phase by G1 cyclin (Cln)-Cdc28 Cdk complexes and is

subsequently ubiquitinated by the SCFCdc4 ubiquitin ligase.

Multi-ubiquitination of Sic1 targets it to the 26S proteasome for degradation. Declining levels of Sic1 unmask the kinase activity by Clb-Cdc28, thereby driving cells to enter S phase and replicate their DNA. The sequential phosphorylation, ubiquitination, and degradation of Sic1 define the G1/S phase transition. Our goal is to elucidate the kinetics of each event individually and together *in vitro* to better understand how they might occur *in vivo*.

**47. Proteasome inhibition as an anti-cancer strategy**

*Senthil K. Radhakrishnan*

Recent studies have indicated that proteasome inhibition could be an attractive strategy in anti-cancer therapy. However, one potential problem with inhibiting the proteasome is the existence of a feedback loop that results in proteasome resynthesis and recovery. We hypothesize that blocking this feedback loop by downregulating proteasome resynthesis could increase the efficacy of proteasome inhibitor therapy. We are currently investigating the covalent, irreversible proteasome inhibitor YU101 in combination with a transcriptional or a translational inhibitor (to block proteasome resynthesis) in cancer cell culture models. If promising, this approach could be extended to a mouse model harboring human xenograft tumors. As a part of this project we also intend to test if pulse treatment with YU101 (which mimics the clinical situation where proteasome inhibitors are rapidly cleared from the plasma) followed by inhibition of proteasome resynthesis can effectively sustain proteasome inhibition as a whole. Overall, our approach could lead to rational drug combinations and hence effective therapies against cancer.

**48. Targeting cancer-promoting proteins for ubiquitination and degradation**

*Agustin Rodriguez*

We are developing a new approach to cancer therapy that exploits the ubiquitin-dependent proteolytic system of eukaryotic cells. This approach employs cell permeable molecules that bind to the substrate-docking site of an ubiquitin ligase. Covalently linking such a molecule to compounds that bind to a desired target protein yields a heterobifunctional compound that we refer to as a ProTac (Proteolysis Targeting Chimeric Pharmaceuticals). ProTacs can be used, in theory, to trigger the destruction of any protein for which there exists a small, cell-permeable ligand. As "proof of principle," we designed a ProTac that contains a peptide that binds with high affinity to the substrate-docking domain of the ubiquitin ligase $SCF^{\delta\text{-}TRCP}$. We then chemically linked this peptide to the small molecule ovalicin, which binds covalently and specifically to the cellular enzyme methionine aminopeptidase-2 (MetAP-2). We demonstrated that the resulting peptide-ovalicin (ProTac) tethers MetAP-2 to $SCF^{\delta\text{-}TRCP}$, and targets MetAP-2 for ubiquitination and degradation[1]. To determine whether ProTacs could recruit different substrates to the $SCF^{\delta\text{-}TRCP}$ ubiquitin ligase for ubiquitination through non-covalent interactions, we generated ProTacs containing the same ligase-binding peptide linked to either estradiol or testosterone. Both the estrogen receptor (ER) and androgen receptor (AR) have been shown to enhance growth of breast and prostate cancer, respectively. We demonstrated that ProTacs containing estradiol or dihydroxytestosterone can trigger the degradation of ER and AR, respectively[2]. Making a cell-permeable ProTac was the next step to induce degradation of a target in living cells. The latest-generation ProTaCs are based on a hydroxyproline-containing peptide from HIF-1 that binds to the VHL ubiquitin ligase complex. These new ProTaCs-based on the HIF-1 peptide induced the degradation of AR and ER in tissue culture cells[3]. Induced degradation of ER arrested cell proliferation of estrogen-dependent breast cancer cells as a consequence of down regulation of ER signaling. On the other hand, breast cancer cell lines that do not express ER and are not dependent upon ER signaling for proliferation were not affected by treatment with ProTaCs. We are now focusing on developing ProTacs into effective drugs for cancer treatment *in vivo*.

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**49. Mechanism of ubiquitin chain assembly by SCF ligases**

*Anjanabha Saha*

Protein modification by ubiquitination is mediated by ubiquitin ligases (E3) that include the highly diverse and extensive superfamily of cullin-RING ubiquitin ligases (CRLs) that play a key role in regulating many cellular and organismal processes. We are utilizing the human SCF complex to investigate how the dynamics of substrate and E2 (ubiquitin conjugating enzyme) association with E3 regulate the specificity and the processivity of ubiquitination. Understanding the dynamics and the mechanism of E3 ligases is important in elucidating the chain linkages of various substrates ubiquitinated and correspondingly its cellular outcome. These studies will aid in establishing how SCF distinguishes substrates from non-substrates and provide insight into how SCF ligases achieve specificity *in vivo*. In addition, we are examining how reversible covalent modification of CRLs by neddylation stimulates substrate ubiquitination.

## 50. Receptor pathways of the Ubiquitin Proteasome System (UPS)

*Rati Verma, Robert Oania*

Labile substrates of the 26S proteasome are earmarked for proteolysis by the covalent attachment of a polyubiquitin (polyUb) chain on acceptor lysines. Our prior work has shown that although the Ub chain is a universal degradation signal, there is specificity in the receptor pathway that is preferentially deployed to target the ubiquitinated protein to the proteasome (Verma *et al*., 2004). Currently, there are about ten different polyUb-binding receptors known in budding yeast. Although all known receptors have a polyUb-binding domain (UBD), only a subset of them have a proteasome-binding domain (PBD). The prototype of the latter is Rpn10, which is an intrinsic subunit of the 26S proteasome. Other receptors such as Rad23 and Dsk2 are present in substoichiometric amounts in the 26S proteasome preparations, leading to the "shuttle hypothesis" of delivery of ubiquitinated substrates (reviewed in Elsasser and Finley, 2005). Rpn10 is believed to be the obligate terminal step in this hypothesis. For substrates such as the ubiquitinated Cdk inhibitor Sic1, this may indeed be the case. However, Rpn10 is dispensable for viability. Moreover, Rpn10 is not required for the degradation of substrates such as misfolded CPY* which is degraded at the endoplasmic reticulum (ER). Instead, Cdc48 and its adaptors are needed for degradation of CPY*. The questions that follow from these, and other, observations are: 1) Are there other examples of ubiquitinated substrates that are preferentially recognized by Cdc48 and its adaptors? 2) If yes, are they all localized at the ER, or are there also cytoplasmic and nuclear substrates? 3) How are these ubiquitinated proteins delivered to the 26S proteasome, given that Cdc48 and its adaptors have no *bona fide* PBD? 4) How is Rpn10, an intrinsic subunit of the 26S proteasome, bypassed in such pathways?

These are some of the questions that we are attempting to answer in studies on the different receptor pathways of the UPS.

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**Support**: The work described in the following research reports has been supported by:
Ellison Medical Research Foundation
Gordon Ross Fellowship
National Institutes of Health, USPHS

**Summary**: In eukaryotic cells, the cyclin-dependent kinases (Cdks) control the progression of the cell cycle by regulating the accurate replication of the genome during S-phase and the faithful segregation of the chromosomes at mitosis (M-phase). The entry into these phases of the cell cycle is controlled by Cdks called S-phase promoting factor (SPF) and M-phase promoting factor (MPF). The action of these Cdks must be controlled both temporally and spatially in a very stringent manner. This strict regulation is imparted by a number of checkpoint mechanisms. For example, cells containing unreplicated DNA cannot enter mitosis due to the mobilization of the replication checkpoint. The Dunphy laboratory is engaged in the elucidation of the molecular mechanisms underlying the regulation of SPF and MPF during the cell cycle. Most of these experiments are conducted with *Xenopus* egg extracts, a system in which the entire cell cycle can be reconstituted *in vitro*.

The first member of the cyclin-dependent protein kinase family described is M-phase promoting factor (MPF), which contains the Cdc2 protein kinase and a regulatory subunit known as cyclin B. Since the identification of the molecular components of MPF, there has been rapid and extensive progress in unraveling the biochemistry of mitotic initiation. It is now well established that MPF acts by phosphorylating a myriad of structural and regulatory proteins that are involved directly in mitotic processes such as nuclear membrane disintegration, chromosome condensation, and mitotic spindle assembly. An ongoing challenge to the cell cycle field is the elucidation of how these phosphorylation reactions regulate the structural and functional properties of the various targets of MPF.

We have been most interested in how the cyclin-dependent protein kinases are regulated during the cell cycle. The principal focus of our laboratory has been on the regulatory mechanisms that govern the activation of MPF at the G2/M transition. Some immediate and long-term issues that we are tackling include:

1. What controls the timing of MPF activation so that it occurs at a defined interval following the completion of DNA replication?

2. How do various checkpoint or feedback controls influence the Cdc2/cyclin B complex?

3. What are the molecular differences between the simple biphasic cell cycle found in early embryonic cells and the more complex cell cycles that arise later in development?

More recently, we have been able to study at the molecular level some of the key events leading to the initiation of DNA replication at the G1/S transition. These events involve a cooperative interaction between the Origin Recognition Complex (ORC), the Cdc6 protein, and members of the Mcm family. These studies may ultimately help us understand how S-phase and M-phase are integrated with one another.

In principle, the regulation of cyclin-dependent kinases such as MPF could occur at any of several levels, including synthesis of the cyclin protein, association between the Cdc2 and cyclin proteins, or posttranslational modification of the Cdc2/cyclin complex. The posttranslational regulation of the Cdc2/cyclin complex is particularly important, even in early embryonic cells that manifest the simplest cell cycle programs. In recent years, many of the elaborate details of this Cdc2 modification process have been defined. For example, the binding of cyclin results in three phosphorylations of Cdc2: one at threonine 161 that is required for Cdc2 activity, and two dominantly inhibitory phosphorylations at threonine 14 and tyrosine 15. A variety of genetic and biochemical experiments have established that the inhibitory tyrosine phosphorylation of Cdc2 is an especially important mechanism of cell cycle regulation. As described in greater detail below, there is now strong evidence that the decision to enter mitosis involves considerably more than the tyrosine dephosphorylation of Cdc2. However, a thorough understanding of the kinase/phosphatase network that controls the phosphotyrosine content of Cdc2 will provide a firm foundation for understanding other facets of mitotic regulation.

Our laboratory has made substantial contributions to understanding the molecular mechanisms controlling the activation of the Cdc2 protein. For our studies, we utilize cell-free extracts from *Xenopus* eggs. Due to pioneering work in a number of the laboratories, it is now possible to re-create essentially all of the events of the cell cycle in these extracts. Consequently, it is feasible to study the molecular mechanisms of Cdc2 regulation in intricate detail with this experimental system. To facilitate these studies, we make extensive use of recombinant DNA technology to overproduce cell cycle proteins in either bacteria or baculovirus-infected insect cells. Moreover, in conjunction with our biochemical studies, we are taking advantage of the fission yeast system to exploit genetic approaches to identify novel *Xenopus* regulators of the cell cycle.

**51. Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1**

*Akiko Kumagai, Soo-Mi Kim, William G. Dunphy*

Claspin is necessary for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. ATR possesses a regulatory partner called ATRIP. We have studied the respective roles of ATR-ATRIP and Claspin in the activation of Chk1. ATR-ATRIP binds well to various DNA templates in *Xenopus* egg extracts. ATR-ATRIP bound to a single-stranded DNA template is weakly active. By contrast, the ATR-ATRIP complex on a DNA template containing both single-stranded and double-stranded regions displays a large increase in kinase activity. This observation suggests that ATR-ATRIP normally undergoes activation upon association with specific nucleic acid structures at DNA replication forks. Without Claspin, activated ATR-ATRIP phosphorylates Chk1 weakly in a cell-free reaction. Addition of Claspin to this reaction strongly stimulates the phosphorylation of Chk1 by ATR-ATRIP. Claspin also induces significant autophosphorylation of Chk1 in the absence of ATR-ATRIP. Taken together, these results indicate that the checkpoint-dependent phosphorylation of Chk1 is a multi-step process involving activation of the ATR-ATRIP complex at replication forks and presentation of Chk1 to this complex by Claspin.

**52. Roles of replication fork interacting and Chk1-activating domains from Claspin in a DNA replication checkpoint response**

*Joon Lee, Daniel A. Gold, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy*

Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. Claspin associates with replication forks upon origin unwinding. We show that Claspin contains a replication fork-interacting domain (RFID, residues 265-605) that associates with Cdc45, DNA polymerase epsilon, RPA, and two RFC complexes on chromatin. The RFID contains two basic patches (BP1 and BP2) at amino acids 265-331 and 470-600, respectively. Deletion of either BP1 or BP2 compromises optimal binding of Claspin to chromatin. Absence of BP1 has no effect on the ability of Claspin to mediate activation of Chk1. By contrast, removal of BP2 causes a large reduction in the Chk1-activating potency of Claspin. We also find that Claspin contains a small Chk1-activating domain (CKAD, residues 776-905) that does not bind stably to chromatin, but is fully effective at high concentrations for mediating activation of Chk1. These results indicate that stable retention of Claspin on chromatin is not necessary for activation of Chk1. Instead, our findings suggest that only transient interaction of Claspin with replication forks potentiates its Chk1-activating function. Another implication of this work is that stable binding of Claspin to chromatin may play a role besides the activation of Chk1.

**53. Phosphorylation of Chk1 by ATR in *Xenopus* egg extracts requires binding of ATRIP to ATR but not the stable DNA-binding or coiled-coil domains of ATRIP**

*Soo-Mi Kim, Akiko Kumagai, Joon Lee, William G. Dunphy*

ATR, a critical regulator of DNA replication and damage checkpoint responses, possesses a binding partner called ATRIP. We have studied the functional properties of *Xenopus* ATR and ATRIP in incubations with purified components and in frog egg extracts. In purified systems, ATRIP associates with DNA in both RPA-dependent and RPA-independent manners, depending on the composition of the template. However, in egg extracts, only the RPA-dependent mode of binding to DNA can be detected. ATRIP adopts an oligomeric state in egg extracts that depends upon binding to ATR. In addition, ATR and ATRIP are mutually dependent on one another for stable binding to DNA in egg extracts. The ATR-dependent oligomerization of ATRIP does not require an intact coiled-coil domain in ATRIP and does not change in the presence of checkpoint-inducing DNA templates. Egg extracts containing a mutant of ATRIP that cannot bind to ATR are defective in the phosphorylation of Chk1. However, extracts containing mutants of ATRIP lacking stable DNA-binding and coiled-coil domains show no reduction in the phosphorylation of Chk1 in response to defined DNA templates. Furthermore, activation of Chk1 does not depend upon RPA under these conditions. These results suggest that ATRIP must associate with ATR in order for ATR to carry out the phosphorylation of Chk1 effectively. However, this function of ATRIP does not involve its ability to mediate the stable binding of ATR to defined checkpoint-inducing DNA templates in egg extracts, does not require an intact coiled-coil domain, and does not depend on RPA.

**54. TopBP1 activates the ATR-ATRIP complex**

*Akiko Kumagai, Joon Lee, Hae Yong Yoo, William G. Dunphy*

ATR is a key regulator of checkpoint responses to incompletely replicated and damaged DNA, but the mechanisms underlying control of its kinase activity are unknown. TopBP1, the vertebrate homolog of yeast Cut5/Dpb11, has dual roles in initiation of DNA replication and regulation of checkpoint responses. We show that recombinant TopBP1 induces a large increase in the kinase activity of both *Xenopus* and human ATR. The ATR-activating domain resides in a conserved segment of TopBP1 that is distinct from its numerous BRCT repeats. The isolated ATR-activating domain from TopBP1 induces ectopic activation of ATR-dependent signaling in both *Xenopus* egg extracts and human cells. Furthermore, *Xenopus* egg extracts containing a version of TopBP1 with an inactivating point mutation in the ATR-activating domain are defective in checkpoint regulation. These studies establish that activation of ATR by TopBP1 is a crucial step in the initiation of ATR-dependent signaling processes.

**55. Site-specific phosphorylation of a checkpoint mediator protein controls its responses to different DNA structures**

*Hae Yong Yoo, Seong-Yun Jeong, William G. Dunphy*

The checkpoint mediator protein Claspin is indispensable for the ATR-dependent phosphorylation of Chk1 in response to stalled DNA replication forks in *Xenopus* egg extracts. We show that Claspin also participates in the detection of chromosomal double-stranded DNA breaks (DSBs) in this system. Significantly, removal of Claspin from egg extracts only partially abrogates the activation of Chk1 in response to chromatin with DSBs, whereas depletion of both Claspin and BRCA1 completely abolishes this activation. The function of Claspin in this DSB-triggered pathway depends upon phosphorylation of T817 and S819 by ATR. Conversely, neither phosphorylation of Claspin on these sites nor the presence of BRCA1 is necessary for activation of Chk1 in response to stalled replication forks. Thus, site-specific phosphorylation of a checkpoint mediator protein is a crucial determinant in the discrimination between various checkpoint-inducing structures. Furthermore, checkpoint mediator proteins exhibit functional overlap that varies depending on the nature of the checkpoint-triggering DNA signal.

**56. Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM**

*Hae Yong Yoo, Akiko Kumagai, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy*

ATM is necessary for activation of Chk1 by ATR in response to double-stranded DNA breaks (DSBs) but not to DNA replication stress. TopBP1 has been identified as a direct activator of ATR. We show that ATM regulates *Xenopus* TopBP1 by phosphorylating S1131 and thereby strongly enhancing association of TopBP1 with ATR. *Xenopus* egg extracts containing a mutant of TopBP1 that cannot be phosphorylated on S1131 are defective in the ATR-dependent phosphorylation of Chk1 in response to DSBs but not to DNA replication stress. Thus, TopBP1 is critical for the ATM-dependent activation of ATR following production of DSBs in the genome.

**57. The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR**

*Joon Lee, Akiko Kumagai, William G. Dunphy*

TopBP1 serves as an activator of the ATR-ATRIP complex in response to the presence of incompletely replicated or damaged DNA. This process involves binding of ATR to the ATR-activating domain of TopBP1, which is located between BRCT domains VI and VII. TopBP1 displays increased binding to ATR-ATRIP in *Xenopus* egg extracts containing checkpoint-inducing DNA templates. We show that an N-terminal region of TopBP1 containing BRCT repeats I-II is essential for this checkpoint-stimulated binding of TopBP1 to ATR-ATRIP. The BRCT I-II region of TopBP1 also binds specifically to the Rad9-Hus1-Rad1 (9-1-1) complex in *Xenopus* egg extracts. This binding occurs via the C-terminal domain of Rad9 and depends upon phosphorylation of its Ser-373 residue. Egg extracts containing either a mutant of TopBP1 lacking the BRCT I-II repeats or a mutant of Rad9 with an alanine substitution at Ser-373 are defective in checkpoint regulation. Furthermore, an isolated C-terminal fragment from Rad9 is an effective inhibitor of checkpoint signaling in egg extracts. These findings suggest that interaction of the 9-1-1 complex with the BRCT I-II region of TopBP1 is necessary for binding of ATR-ATRIP to the ATR-activating domain of TopBP1 and the ensuing activation of ATR.

**Publications**

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**Support:** The work described in the following reports has been supported by:
Agouron
Beckman Institute
DOE
Moore
NIH
Searle Foundations

**Summary:** If we could simply look inside a cell and see its molecular components in all their complexes and conformations, cell biology would be all but finished. While this is of course still just a dream, we are developing electron-cryomicroscopy–based technologies to do this for at least the largest structures, hoping to show both how individual proteins work together as large "machines" and how those machines are organized into "assembly lines" within living cells.

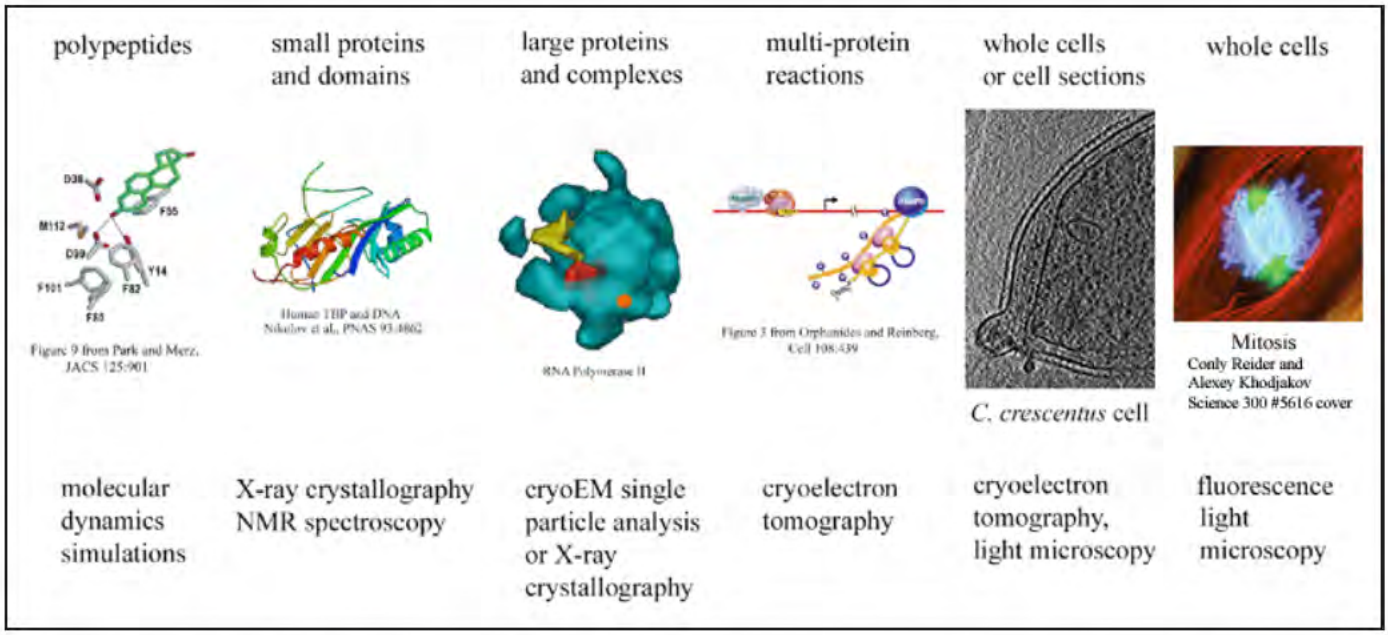
The principle technique we're developing is electron cryotomography (ECT). Briefly, purified proteins, viruses, or even cell cultures are spread into thin films across EM grids and plunge-frozen in liquid ethane. Quick-freezing causes the water to form vitreous ice around the proteins and other macromolecules, preserving their native structure but solidifying the sample so it can withstand the high vacuum within an electron microscope. Projection images are then recorded through the sample as the sample is tilted incrementally along one or two axes. The microscope we use is one of only a few like it in the world: a 300 kV, helium-cooled, energy-filtered, dual-axis tilting, FEG cryo-TEM with a lens-coupled 4k x 4k CCD. Three-dimensional reconstructions, or "tomograms," are then calculated from the images. In this way we can produce 3-D structures of heterogeneous proteins, viruses, and even whole bacterial cells in near-native states to "molecular" (~4-7 nm) resolution.

The first cells we've begun imaging are small bacteria. Now that nearly a thousand bacterial genomes have been sequenced, a variety of "omic" technologies are being used to document which genes are transcribed and when, which macromolecules are synthesized and how many of each type are present in the cell, and how they interact in pathways to mediate metabolism and regulate gene expression. Despite this encouraging progress, however, our persistent ignorance about many of the fundamental physical and mechanical processes that occur in a bacterial life cycle is sobering. We still don't know, for instance, how bacteria generate and maintain their characteristic shapes, establish polarity, organize their genomes, segregate their chromosomes, divide, and in some cases move. Thus, in some sense the "omics" technologies are giving us lists of parts and reactions, but bacterial cells are not merely bags of enzymes. Structural and mechanical details are also needed. This is where ECT is poised to make an important contribution.

In recent years we have used ECT to show by direct visualization that bacteria do indeed have an elaborate cytoskeleton. We have documented structural details of different cell motility mechanisms, chemoreception apparati, flagellar motors, and metabolic microcompartments. We continue to work on all these subjects and hope also to begin shedding light on the structure and management of the nucleoid and cell wall. One of the projects below describes the application of ECT to the smallest known eukaryote, *Ostreococcus tauri*.

We have also worked to apply the power of ECT to the structure and maturation of the human immunodeficiency virus type 1 (HIV-1). HIV-1 is an interesting structural story: following its discovery in the mid-1980's, thousands (!) of different structures of its 15 different proteins and pieces of its RNA genome have been solved. Nevertheless, we still don't know just how these proteins fit together to form intact, infectious virions, or how their organization changes during assembly, maturation, and infection. The main technical obstacle is that like people, while all HIV-1 virions have the same basic features, each virion is unique in its details. Techniques like X-ray crystallography or NMR spectroscopy, which require a large number of identical objects, have not therefore been able to reveal "supramolecular" details. So far we have imaged HIV-1 in its immature and mature states, and are now analyzing these at higher resolution and endeavoring to image HIV-1 structures in living host cells, as well as host factors involved in the HIV-1 life cycle.

Technologically, we are working on optimizing sample preservation, recording better images through improved instrumentation, obtaining more images through automation, and extracting as much biological insight as possible from the images through more sophisticated image processing. For more information, see http://www.jensenlab.caltech.edu




**58. 3-D Ultrastructure of *O. tauri*: Electron cryotomography of an entire eukaryotic cell**

*Gregory P. Henderson, Lu Gan, Grant J. Jensen*

The hallmark of eukaryotic cells is their segregation of key biological functions into discrete, membrane-bound organelles. Creating accurate models of their ultrastructural complexity has been difficult in part because of the limited resolution of light microscopy and the artifact-prone nature of conventional electron microscopy. In this study we explored the potential of the emerging technology electron cryotomography to produce three-dimensional images of an entire eukaryotic cell in a near-native state. *Ostreococcustauri* was chosen as the specimen because as a unicellular picoplankton with just one copy of each organelle, it is the smallest known eukaryote and was therefore likely to yield the highest resolution images. Whole cells were imaged at various stages of the cell cycle, yielding 3-D reconstructions of complete chloroplasts, mitochondria, endoplasmic reticula, Golgi bodies, peroxisomes, microtubules, and putative ribosome distributions *in situ*. Surprisingly, the nucleus was seen to open long before mitosis, and while one microtubule (or two in some predivisional cells) was consistently present, no mitotic spindle was ever observed, prompting speculation that a single microtubule might be sufficient to segregate multiple chromosomes.

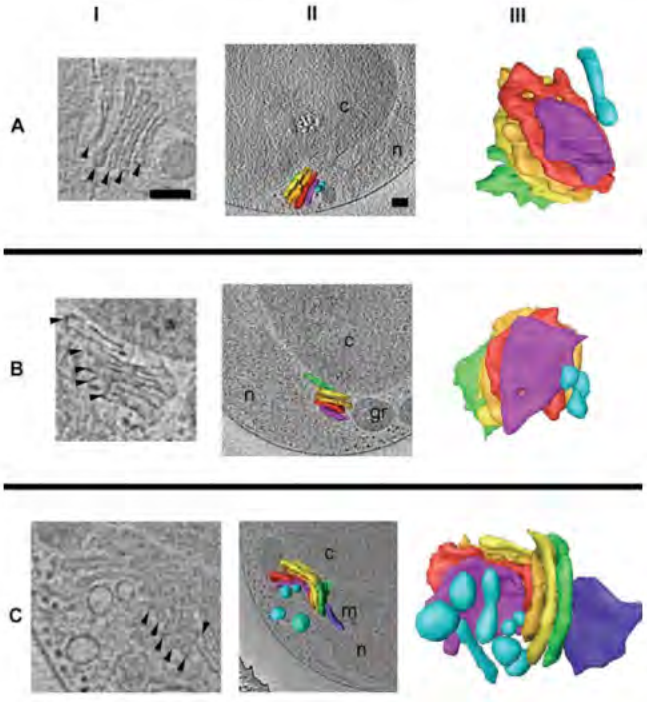



Figure 2. Golgi body. The Golgi bodies from three different cells are shown (rows A–C).

**Reference**

Henderson, G.P., Gan, L. and Jensen, G.J. (2007) 3-D *PLoS One* **2**(8):e749, doi:10.1371/journal.pone.0000749.

**59. *Haloquadratum walsbyi* gen. nov., sp. nov., the square haloarchaeon of Walsby, isolated from saltern crystallizers in Australia and Spain**

*David G. Burns,[1] Peter H. Janssen,[1] Takashi Itoh,[2] Masahiro Kamekura,[3] Zhuo Li,[4] Grant Jensen,[4] Francisco Rodríguez-Valera,[5] Henk Bolhuis,[6] Mike L. Dyall-Smith[1]*

Strains C23T and HBSQ001 were isolated from solar salterns and are novel square-shaped, aerobic, extremely halophilic members of the domain Archaea and family *Halobacteriaceae*. Cells stained Gram-negative and grew optimally in media containing 18 % salts at around neutral pH. Mg2+ is not required. The DNA G+C content of both isolates was 46.9 mol% and DNA-DNA cross-hybridization showed a relatedness of 80%. Their 16S rRNA gene sequences showed only two nucleotide differences (99.9 % identity) and phylogenetic tree reconstructions with other recognized members of the *Halobacteriaceae* indicated that they formed a distinct clade, with the closest relative being *Halogeometricum borinquense* PR 3T (91.2 % sequence identity). The major polar glycolipid of both isolates was the sulfated diglycosyl diether lipid S-DGD-1. Electron cryomicrosopy of whole cells revealed similar internal structures, such as gas vesicles and polyhydroxyalkanoate granules, but the cell wall of isolate HBSQ001 displayed a more complex S-layer compared with that of isolate C23T. The phenotypic characterization and phylogenetic data support the placement of isolates C23T and HBSQ001 in a novel species in a new genus within the Halobacteriaceae, for which we propose the name *Haloquadratum walsbyi* gen. nov., sp. nov. The type strain of *Haloquadratum walsbyi* is C23T (=JCM 12705T=DSM 16854T).

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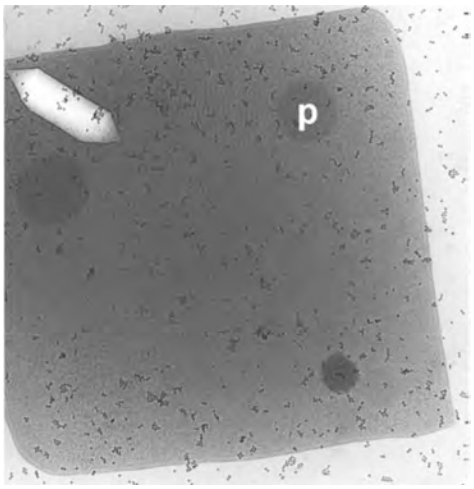


Figure 3. Cell of strain C23T showing a gas vesicle and PHA granules (one is labelled p).

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Burns, D.G, Janssen, P.H., Itoh, T., Kamekura, M., Li, Z., Jensen, G.J., Rodriguez-Valera, F., Bolhuis, H. and Dyall-Smith, M.L. (2007) *Int. J. Syst. Evol. Microbiol.* **57**:387-392.

**60. Electron cryotomography of immature HIV-1 virions reveals the structure of the CA and SP1 Gag shells**

*Elizabeth R. Wright, Jordan B. Schooler, H. Jane Ding, Collin Kieffer*, Christopher Fillmore*, Wesley I. Sundquist*, Grant J. Jensen*

The major structural elements of retroviruses are contained in a single polyprotein, Gag, which in human immunodeficiency virus type 1 (HIV-1) comprises the MA, CA, spacer peptide 1 (SP1), NC, SP2, and p6 polypeptides. In the immature HIV-1 virion, the domains of Gag are arranged radially with the N-terminal MA domain at the membrane and C-terminal NC-SP2-p6 region nearest to the center. In this project we saw the three-dimensional structures of individual immature HIV-1 virions, as obtained by electron cryotomography. The concentric shells of the Gag polyprotein were clearly visible, and radial projections of the different Gag layers revealed patches of hexagonal order within the CA and SP1 shells. Averaging well-ordered unit cells lead to a model in which each CA hexamer is stabilized by a bundle of six SP1 helices. This model suggested why the SP1 spacer is essential for assembly of the Gag lattice and how cleavage between SP1 and CA acts as a structural switch controlling maturation.

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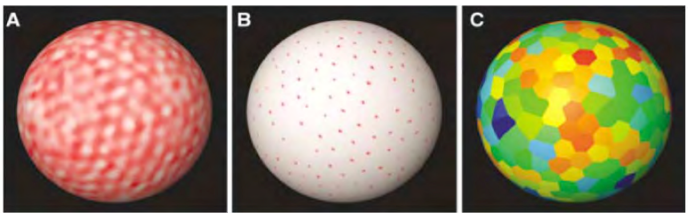


**Figure 4.** Identification and ranking of unit cells according to local order. (A) Surface projection of the CA NTD layer of Gag in one virion, where red indicates high density. (B) Result of a peak search algorithm, with peaks marked by red dots. (C) Putative unit cells color coded according to the degree of local hexagonal order (red: greatest order, blue: least order).

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**61. The structure of isolated *Synechococcus* strain WH8102 carboxysomes as revealed by electron cryotomography**

*Cristina V. Iancu, H. Jane Ding, Dylan M. Morris, D. Prabha Dias, Arlene D. Gonzales*[*]*, Anthony Martino*[*]*, Grant J. Jensen*

Carboxysomes are organelle-like polyhedral bodies found in cyanobacteria and many chemoautotrophic bacteria that are thought to facilitate carbon fixation. Carboxysomes are bounded by a proteinaceous outer shell and filled with ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the first enzyme in the CO2 fixation pathway, but exactly how they enhance carbon fixation is unclear. We can show the three-dimensional structure of purified carboxysomes from *Synechococcus* species strain WH8102 as revealed by electron cryotomography. We found that while the sizes of individual carboxysomes in this organism varied from 114 nm to137 nm, surprisingly, all were approximately icosahedral. There were on average ~250RuBisCOspercarboxysome, organized into three to four concentric layers. Some models of carboxysome function depend on specific contacts between individual RuBisCOs and the shell, but no evidence of such contacts was found: no systematic patterns of connecting densities or RuBisCO positions against the shell's presumed hexagonal lattice could be discerned, and simulations showed that packing forces alone could account for the layered organization of RuBisCOs.

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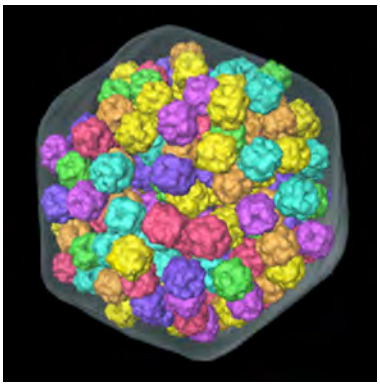

**Figure 5.** 3-D model of carboxysomes filled with RuBisCO.

**Reference**

Iancu, C.V., Ding, H.J., Morris, D.M., Martino, A. and Jensen, G.J. (2007) *J. Mol. Biol*. **372**:764-773.

**62. The structure of FtsZ filaments *in vivo* suggests a force-generating role in cell division**

*Zhuo Li, Michael J. Trimble*[*]*, Yves V. Brun*[*]*, Grant J. Jensen*

In prokaryotes, FtsZ (the filamentous temperature sensitive protein Z) is a nearly ubiquitous GTPase that localizes in a ring at the leading edge of constricting plasma membranes during cell division. We found electron cryotomographic reconstructions of dividing *Caulobacter crescentus* cells wherein individual arc-like filaments were resolved just underneath the inner membrane at constriction sites. The filaments' position, orientation, time of appearance, and resistance to A22 all suggested that they were FtsZ. Predictable changes in the number, length, and distribution of filaments in cells where the expression levels and stability of FtsZ were altered supported that conclusion. In contrast to the thick, closed-ring-like structure suggested by fluorescence light microscopy, throughout the constriction process the Z-ring was seen here to consist of just a few short (approximately 100 nm) filaments spaced erratically near the division site. Additional densities connecting filaments to the cell wall, occasional straight segments, and abrupt kinks were also seen. An 'iterative pinching' model is proposed wherein FtsZ itself generates the force that constricts the membrane in a GTP-hydrolysis-driven cycle of polymerization, membrane attachment, conformational change, depolymerization, and nucleotide exchange.

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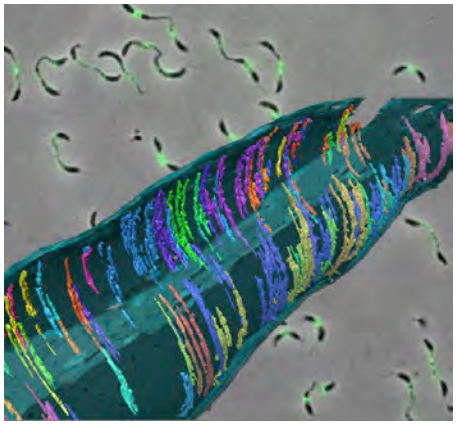

**Figure 6**. 3-D segmentation of a *Caulobacter crescentus* constriction site full of FtsZ filaments against a fluorescence light micrograph of similar cells at much smaller scale.

**Reference**

Li, Z., Trimble, M.J., Brun, Y.V. and Jensen, G.J. (2007) *EMBO J.* **26**:4694-4708.

**63. Novel ultrastructures of *Treponema primitia* and their implications for motility**

*Gavin E. Murphy, Eric G. Matson[1], Jared R. Leadbetter[1], Howard C. Berg[2], Grant J. Jensen*

Members of the bacterial phylum *Spirochaetes* are generally helical cells propelled by periplasmic flagella. The spirochete *Treponema primitia* is interesting because of its mutualistic role in the termite gut, where it is believed to cooperate with protozoa that break down cellulose and produce H(2) as a by-product. In this study we showed the ultrastructure of *T. primitia* as obtained by electron cryotomography of intact, frozen-hydrated cells. Several previously unrecognized external structures were revealed, including bowl-like objects decorating the outer membrane, arcades of hook-shaped proteins winding along the exterior and tufts of fibrils extending from the cell tips. Inside the periplasm, cone-like structures were found at each pole. Instead of the single peptidoglycan layer typical of other Gram-negative bacteria, two distinct periplasmic layers were observed. These layers formed a central open space that contained two flagella situated adjacent to each other. In some areas, the inner membrane formed flattened invaginations that protruded into the cytoplasm. High-speed light microscopic images of swimming *T. primitia* cells showed that cell bodies remained rigid and moved in a helical rather than planar motion. Together, these findings support the 'rolling cylinder' model for *T. primitia* motility that posits rotation of the protoplasmic cylinder within the outer sheath.

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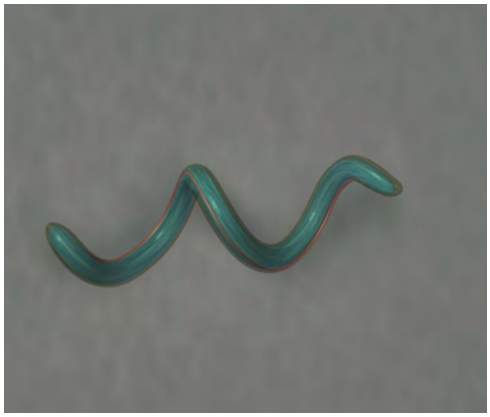

**Figure 7.** Computer animation model of a *Treponema primitia* cell (see movie on Jensen lab website).

**Reference**

Murphy, G.E., Matson, E.G., Leadbetter, J.R., Berg, H.C. and Jensen, G.J. (2008) *Mol. Microbiol.* **67**(6):1184-1195.

**64. Cryo-EM structure of dodecameric Vps4p and its 2:1 complex with Vta1p**

*Zhiheng Yu, Malgorzata D. Gonciarz[*], Wesley I. Sundquist[*], Christopher P. Hill[*], Grant J. Jensen*

The type I AAA (ATPase associated with a variety of cellular activities) ATPase Vps4 and its co-factor Vta1p/LIP5 function in membrane remodeling events which accompany cytokinesis, multivesicular body biogenesis, and retrovirus budding, apparently by driving disassembly and recycling of membrane-associated ESCRT (endosomal sorting complex required for transport)-III complexes. In this project we presented electron cryomicroscopy reconstructions of dodecameric yeast Vps4p complexes with and without their microtubule interacting and transport (MIT) N-terminal domains and Vta1p co-factors. The ATPase domains of Vps4p form a bowl-like structure composed of stacked hexameric rings. The two rings adopt dramatically different conformations, with the "upper" ring forming an open assembly that defines the sides of the bowl and the lower ring forming a closed assembly that forms the bottom of the bowl. The N-terminal MIT domains of the upper ring localize on the symmetry axis above the cavity of the bowl, and the binding of six extended Vta1p monomers causes additional density to appear both above and below the bowl. The structures suggest models in which Vps4p MIT and Vta1p domains engage ESCRT-III substrates above the bowl and help transfer them into the bowl to be pumped through the center of the dodecameric assembly.

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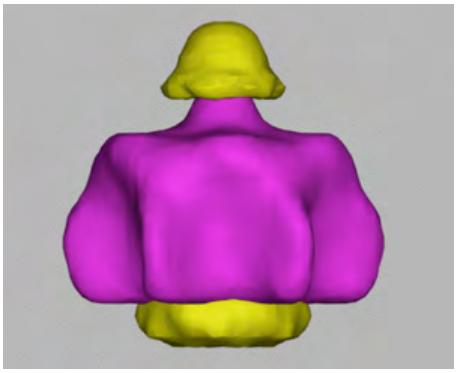

**Figure 8.** 3-D single-particle reconstruction of Vps4, a AAA-ATPase involved in HIV budding.

**Reference**

Yu, Z., Gonciarz, M.D., Sundquist, W.I., Hill, C.P. and Jensen, G.J. (2008) *J. Mol. Biol.* **377**:364-377.

**65. Location and architecture of the *Caulobacter crescentus* chemoreceptor array**

*Ariane Briegel, H. Jane Ding, Zhuo Li, John Werner[1], Zemer Gitai[1], D. Prabha Dias, Rasmus B. Jensen[2], Grant J. Jensen*

A new method for recording both fluorescence and cryo-EM images of small bacterial cells was developed and used to identify chemoreceptor arrays in cryotomograms of intact *Caulobacter crescentus* cells. We showed that in wild-type cells preserved in a near-native state, the chemoreceptors are hexagonally packed with a lattice spacing of 12 nm, just a few tens of nanometers away from the flagellar motor that they control. The arrays were always found on the concave side of the cell, further demonstrating that *Caulobacter* cells maintain dorsal/ventral as well as anterior/posterior asymmetry. Placing the known crystal structure of a trimer of receptor dimers at each vertex of the lattice accounts well for the density and agrees with other constraints. Based on this model for the arrangement of receptors, there are between one and two thousand receptors per array.

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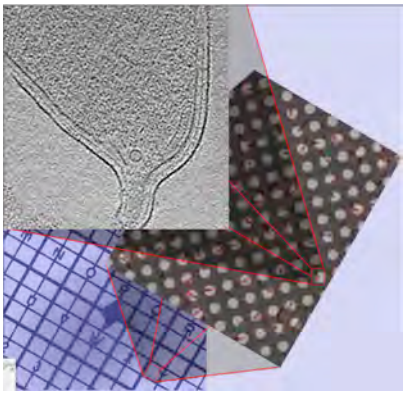

**Figure 9.** Image montage illustrating how correlated light and electron microscopy allows us to study supramolecular structures like the chemoreceptor array at multiple scales.

**Reference**

Briegel, A.B., Ding, H.J., Li, Z., Gitai, Z., Dias, P.D. and Jensen, G.J. (2008) Location and architecture of the *Caulobacter crescentus* chemoreceptor array by electron cryotomography. *Mol. Micro.* Available online.

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Briegel, A.B., Ding, H.J., Li, Z., Gitai, Z., Dias, P.D. and Jensen, G.J. (2008) Location and architecture of the *Caulobacter crescentus* chemoreceptor array by electron cryotomography. *Mol. Micro.* Available online.

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Li, Z., Trimble, M.J., Brun, Y.V. and Jensen, G.J. (2007) The structure of FtsZ filaments *in vivo* suggests a force-generating role in cell division. *EMBO J.* **26**:4694-4708.

Murphy, G.E., Matson, E.G., Leadbetter, J.R., Berg, H.C. and Jensen, G.J. (2008) Novel ultrastructures of *Treponema primitia* and their implications for motility. *Mol. Microbiol.* **67**(6):1184-1195.

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Yu, Z., Gonciarz, M.D., Sundquist, W.I., Hill, C.P. and Jensen, G.J. (2008) Cryo-EM structure of dodecameric Vps4 and its 2:1 complex with Vta1. *J. Mol. Biol.* **377**:364-377.

**Professor of Biology and Chemistry**: Stephen L. Mayo
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**Graduate Students**: Benjamin D. Allen, Mohsen Chitsaz, Jennifer R. Keeffe, Matthew M. Moore, Yun Mou, Alex Nisthal, Heidi K. Privett, Christina Vizcarra
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**Computational Research Specialist**: Barry D. Olafson

**Support**: The work described in the following research report has been supported by:
Colvin Fund for Research Initiatives in Biomedical Science
DARPA
Donald Bren Foundation
Howard Hughes Medical Institute
National Institutes of Health
National Science Foundation
Ralph M. Parson's Foundation
Weston Haven's Foundation

**Summary:** The focus of the lab has been the coupling of theoretical, computational, and experimental approaches for the study of structural biology. In particular, we have placed a major emphasis on developing quantitative methods for protein design with the goal of developing a fully systematic design strategy that we call "protein design automation." Our design approach has been captured in a suite of software programs called ORBIT (Optimization of Rotamers By Iterative Techniques) and has been applied to a variety of problems ranging from protein fold stabilization to enzyme design.

### 66. Efficient optimization with structural flexibility in computational protein design

*Benjamin D. Allen*[*]*, Barry D. Olafson, Stephen L. Mayo*

Current computational procedures for protein design rely on the use of fixed backbone coordinates and libraries of discrete, rigid side-chain conformations. This static model has been favored because it leads to discrete and tractable combinatorial optimization problems, given reasonable design goals. Unfortunately, this methodology fails to treat the conformational flexibility of protein backbones and side chains, resulting in many false negatives during sequence selection. Protein designers typically use large rotamer libraries to simulate continuous side-chain conformational space. However, the use of very large rotamer libraries can negate much of the efficiency gains allowed by the static structural model without necessarily improving predictive power.

As available computational power has increased, protein design methods that allow explicit structural flexibility have become viable. They have also become more attractive from an efficiency perspective, since processing power has increased more rapidly than physical memory, and structurally flexible design methods do not rely on the precomputation of pairwise energies. However, the on-the-fly calculation of energies used in flexible design models still requires significantly more compute time than previous-generation static-model designs.

Our previous work has shown the FASTER combinatorial optimization procedure consistently outperforms more commonly used methods based on Monte Carlo with simulated annealing. FASTER consists of an iterative batch relaxation phase (iBR) followed by additional relaxations in the context of perturbations (sPR). Modifying iBR so that relaxed rotamers at each position are chosen according to a power-law probability distribution resulted in dramatically improved optimization efficiency. The use of power iBR (piBR) with energy minimization and a small rotamer library was able to match the conformational sampling ability of standard methods with a very large rotamer library, showing that flexible design can be viable. We plan to test power FASTER on a variety of challenging protein design problems, such as multistate design, enzyme design, and polar interface design.

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### 67. Redesign of arylmalonate decarboxylase to synthesize enantiopure amino acids

*Swathi Amere, Stephen L. Mayo*

Arylmalonate decarboxylase catalyzes the enantioselective decarboxylation of simple α-arylmalonic acids to produce optically pure α-arylpropionic acid. This enzyme is of special interest because it does not require cofactors or coenzymes, and consequently could be used as an efficient biocatalyst. The crystal structure of this enzyme has been reported very recently [1]. The high optical yields obtained in this decarboxylation reaction prompted us to redesign this enzyme to act on more structurally and functionally diverse substrates in order to synthesize enantiopure natural and non-natural amino acids. Enantio-discrimination of the present transformation arises from the selective addition of a proton to one of the enantiotopic faces of the intermediate enolate. Using in-house protein design software, we have been targeting specific residues in the binding pocket of the enzyme for mutations that will stabilize the transition state structure of α-amino-α-arylmalonic acid decarboxylation. Computationally designed sequences will be tested experimentally to determine whether they are able to produce the desired chemical transformations.

**Reference**

[1] Kuettner, E.B., Keim, A., Kircher, M., Rosmus, S. and Sträter, N. (2008) *J. Mol. Biol.* **377**:386-394.

### 68. Modifying the spectral properties of a red fluorescent protein

*Roberto A. Chica, Benjamin D. Allen*[*]*, Stephen L. Mayo*

Fluorescent proteins have found widespread application in cell biology as reporters for the cellular localization of various proteins. Those that emit in the red portion of the visible spectrum are of particular interest because longer wavelengths are less damaging to cells and because cells are more transparent to red light. One such

red fluorescent protein, mCherry, has an emission maximum of 610 nm. Our goal is to generate mCherry mutants with improved spectral properties such as a red-shifted emission maximum, higher quantum yield, and increased brightness. We have been using a computational design method to generate focused combinatorial libraries of mCherry. The protein sequences in these libraries have a high probability of folding properly since our method helps to eliminate mutations that would destabilize the protein fold. A total of 13 residues surrounding the chromophore were designed, yielding four libraries of 240-540 mutants each, a size that can easily be screened for the desired properties using a 96-well plate-based assay. We have thus far identified 20 mutants displaying fluorescence emission red-shifts of up to 16 nm. The quantum yields and extinction coefficients of these mutants are currently being determined. In addition, exhaustive sequencing of all the fluorescent mutants is being done, which will provide information on structure/function relationships in mCherry.

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**69. Evolutionary search algorithms for rotamer optimization**

*Mohsen Chitsaz[*], Stephen L. Mayo*

Search strategies are a key feature of computational protein design algorithms. Most protein design algorithms are based on searching astronomically large spaces of possible rotamer configurations to find the global minimum in the energy landscape. This type of global optimization problem is reducible to a traveling salesman problem (TSP), which is nondeterministic polynomial-time hard (NP-hard). Consequently, employing efficient global search algorithms is crucial in this field. Several approaches can be employed in this context. These include the use of evolutionary stochastic search algorithms, which typically involve applying evolutionary strategies to stochastically improve a random initial result into obtaining the global optimum. Some of these algorithms have proven to be effective in some contexts, but no uniquely effective solution has been found for all problems. We intend to investigate the utility of several evolutionary search algorithms for rotamer optimization, including genetic algorithms, linkage learning algorithms, and linkage learning genetic algorithms. The results obtained with these algorithms will be compared with the best optimization methods currently available, such as FASTER and Monte Carlo with simulated annealing.

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**70. Designing cyanovirin-N variants to enhance HIV neutralization**

*Jennifer R. Keeffe[*], Priyanthi Peiris, Stephen L. Mayo*

Cyanovirin-N (CV-N), a protein originally isolated from the cyanobacterium *Nostoc ellipsosporum*, has been shown to bind specifically to glycosylated gp120 on HIV particles, preventing viral fusion [1]. We have successfully created dimeric and trimeric variants of CV-N to investigate the effect of avidity on CV-N-mediated HIV neutralization. The proteins were expressed and purified to homogeneity then assessed for their ability to prevent HIV infection in a cell culture-based neutralization assay [2]. We found that covalently linking two CV-N monomers ($CVN_2$) through a flexible polypeptide linker decreased the $IC_{50}$ of neutralization ten-fold compared to wild-type CV-N. The addition of a third CV-N monomer ($CVN_3$), however, showed minimal further neutralization enhancement. Our data also suggest that neutralization activity depends on linker length, although variability in the assays currently masks a definitive trend. We have successfully created a CV-N variant that is ten-fold more effective in preventing HIV infection in a cell-based system. Structural studies are currently underway to investigate any differences between the structures of the engineered variants and wild type, which may help elucidate a mechanism for the increased efficacy. In addition to our work creating CV-N oligomers, we are also investigating a chimeric CV-N-Fc fusion protein (CVN-Fc). Initial experiments indicate that CVN-Fc expressed in and purified from HEK-293 cells is able to neutralize HIV with an $IC_{50}$ similar to wild type. Future studies are aimed at optimizing expression, performing structural studies, and continuing the functional assays.

[*]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

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**71. LTP-based biosensor development for computational design of a new phospholipase**

*Matthew M. Moore[*], Stephen L. Mayo*

Lipid transfer proteins (LTPs) are a ubiquitous class of small soluble proteins found in plants. These proteins are characterized by their primarily α-helical structures and distinct topological pattern of four disulphide bonds. A recombinant expression system for an LTP isolated from *Zea mays* (maize) was developed in *Escherichia coli*, and one is currently being developed in *Pichia pastoris* for potentially higher levels of protein expression using a more straightforward purification scheme.

Previously, a computational design strategy for stabilizing a mutant of this LTP by individual removal of each of its disulphide bonds led to an LTP-based biosensor device that could be tagged with thioreactive fluorescent probes to more easily detect lipid-binding events [1]. Broader experimental testing of various thioreactive probes and additional application of rational design strategies aimed at enhancing the stability of the sensor

could improve its functionality. Eventually, such a biosensor could be used as a foundation for an assay to detect lipid-binding events in computationally designed LTPs with prospective phospholipase activity.

Reliable rational design of catalytic activity in protein scaffolds is at the forefront of computational biology. Recently, crystal structures of phospholipases like patatin have shown that a Ser-Asp catalytic dyad is sufficient for phospholipase-A activity in lipid-binding proteins. Given that the orientation of lipid binding for maize LTP is known from atomic resolution crystallographic data, installation of a catalytic dyad could be guided by computational protein design strategies. Coupled with the development of a biosensor device described above, future sequence designs could be easily probed for ligand binding capability and ultimately catalytic activity.

[*]*Division of Chemistry and Chemical Engineering, Caltech*

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**72. Library designs to expand calmodulin-binding diversity**

*Y. Mou[*], Stephen L. Mayo*

Biologically active proteins often carry out their functions by interacting with other proteins. Many essential processes in living organisms, such as the activation of signaling pathways, the initiation of the immune response, and the formation of functionally active oligomeric states, are made possible via protein-protein interactions. Designing a protein with high affinity to a novel target would not only improve our understanding of protein-protein interactions, but also aid in the generation of new therapeutics, diagnostics, and research tools.

Calmodulin (CaM) is a small $Ca^{2+}$-binding protein that binds to and regulates a number of different protein targets. Its ability to tightly bind to a diverse set of small peptides makes it an ideal system for searching for new binding targets. Hundreds of sequences are already known to bind CaM, leading to suggestions regarding the basic elements required for binding [1]. Several high-resolution CaM-peptide complexes have also been solved by X-ray crystallography. Further, the ORBIT computational design software was successfully used to generate a CaM variant with increased specificity toward a particular target [2]. Using this abundant knowledge of CaM binding affinity and specificity, we intend to design libraries of CaM variants to bind novel peptides with high affinity. To be able to experimentally validate the libraries, we first need to develop a high-throughput assay to determine CaM-peptide binding. Förster resonance energy transfer (FRET) is a state-of-the-art method to characterize interactions between molecules. Color variants of green fluorescent protein (GFP) can be attached to a host protein (CaM), and FRET can be used to determine protein-protein interactions *in vivo*, thus allowing identification of CaM-binding peptides. Using a 96-well plate-based assay, we expect high-number libraries can be tested experimentally and novel targets can be found efficiently.

[*]*Division of Chemistry and Chemical Engineering, Caltech*

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**73. Improving the throughput of protein stability determination**

*Alex Nisthal[1], Benjamin D. Allen[2], Stephen L. Mayo*

Chemical denaturation by circular dichroism is a commonly used method for determining protein stability. However, because of the time consuming nature of the procedure, it's not ideal for screening libraries of mutants. A medium-throughput screen using chemical denaturation was developed as a microplate-based assay monitoring tryptophan fluorescence. After gene assembly of the designed library, constructs are transformed, sequenced, and expressed, and the hex-histidine tagged proteins are purified in a 96-well format by Ni-NTA filter plates. Mutant proteins are then denatured using guanidinium chloride on 96-well microplates. Chemical denaturation midpoints are calculated from the linear extrapolation method. Accuracy and precision of the assay proved to be in good agreement with results from circular dichroism and a cuvette-based fluorimeter. A computationally designed combinatorial library attempted to optimize the β-sheet surface of the β1 domain of streptococcal protein G. Experimental characterization by the plated-based stability assay showed the library was composed of soluble but less stable mutants. Libraries containing up to 96 members have been successfully screened by this procedure, providing a wealth of protein stability data not usually available to computational protein designers. This methodology will allow for the comparison of library quality in response to changes in protein design parameters and energy functions.

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**74. Crystallographic confirmation of an inactive computationally designed enzyme active site**

*Heidi K. Privett[*], Leonard Thomas, Stephen L. Mayo*

A general method for the design of highly efficient protein catalysts for arbitrary chemical transformations has been an attractive but elusive goal since the advent of computational protein design. In cases where designed enzymes were shown to be inactive, few studies have been performed to determine the cause of this inactivity. We have determined the X-ray crystal structure of a 7-point mutant of *T. aurantiacus xylanase* (1.2.1), which was designed using ORBIT to catalyze the general base catalyzed Kemp elimination of 5-nitrobenzsoxazole.

This protein was shown to provide no significant rate enhancement over the background reaction; however, crystallographic analysis confirmed that the conformations of most of the active site residues, including the general base, were very similar to the conformations predicted by ORBIT. One exception was the aromatic stacking residue (W275), which is rotated out of the active site, preventing predicted binding interactions with the substrate and reducing the predicted pKa of the general base by exposing the active site to solvent. Based on this crystallographic evidence, modifications have been made to the design to correct the positioning of the tryptophan. The crystal structure of this variant (1.2.3) showed that a single mutation results in conformations of both the tryptophan and an additional contacting residue that are more like the predicted conformations; however, this positioning may be affected by domain swapping of the dimer in the asymmetric unit. We are currently pursuing a crystal structure of 1.2.3 that is monomeric in order to confirm the effects of our mutations on the active site structure.

[*]*Division of Chemistry and Chemical Engineering, Caltech*

**75. The plasticity of surface residues on engrailed homeodomain**

*Christina Vizcarra*[*]*, Stephen L. Mayo*

I have created a hyper-thermostable variant of *Drosophila melanogaster* engrailed homeodomain by mutating its solvent-exposed residues. This variant, HT_ENH, was designed using structure-based computational optimization of the amino acid sequence, with sequence constraints for N-capping and helix dipole rules. HT_ENH has a thermal denaturation temperature approximately 50°C higher than the wild-type sequence. This result is yet another example of protein stabilization via electrostatic optimization of surface residues. In order to reconcile HT_ENH's high stability with recent reports of well-folded proteins with poor surface electrostatic interactions and large formal charges, I also created a negatively "super-charged" variant of engrailed homeodomain with a formal charge of −13. The super-charged variant has a circular dichroism spectrum similar to that of the wild-type sequence. The thermostability is also close to that of the wild type. As would be predicted for electrostatic repulsion or optimization, the super-charged variant is stabilized, while HT_ENH is destabilized, by the addition of monovalent salt. These data indicate that for the engrailed homeodomain fold, the plasticity of surface residues can be exploited to either impart highly unfavorable electrostatic interactions without disrupting the fold or to increase the protein's thermostability by optimizing electrostatic interactions.

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**76. Establishing the entatic in folding metallated azurin**

*Corey J. Wilson, Chenghang Zong*[*]*, Peter G. Wolynes*[*]*, Stephen L. Mayo*

Understanding how the folding of proteins establishes their functional characteristics at the molecular level challenges both theorists and experimentalists. The simplest testbeds for confronting this issue are provided by electron transfer proteins. The environment provided by the folded protein to the cofactor tunes the metal's electron transport capabilities as envisioned in the entatic hypothesis. According to the entatic hypothesis, the so-called "entatic state" occurs in proteins when a group, metal or nonmetal, is forced into an unusual, energetically strained geometric or electronic state (or rack-induced state). The rigidity of the protein scaffold speeds charge transfer by minimizing the nuclear reorganization energy. To see how the entatic state is achieved, one must study how the folding landscape affects and in turn is affected by the metal. In this study, we have developed a coarse-grained Hamiltonian (i.e., a minimalist metalloprotein representation) to explicitly model how the coordination of the metal modifies the folding of *Pseudomonas aeruginosa* azurin, a blue copper protein [1]. Our free energy functional based approach directly yields the proper non-linear extra-thermodynamic free energy relationships for the kinetics of folding the wild type as well as several point-mutated variants of the metallated protein. The results agree quite well with corresponding laboratory experiments. Moreover, our modified free energy functional provides a sufficient level of detail to explicitly model how the geometric entatic state of the metal modifies the dynamic folding nucleus of azurin.

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**Support:** The work described in the following research reports has been supported by:
California Institute of Regenerative Medicine
Ellison Medical Foundation
Howard and Gwen Laurie Smits Professorship in Cell Biology
National Institutes of Health, USPHS
Sandler Program in Asthma Research

**Summary**: Our main subject is the ubiquitin system. The field of ubiquitin and regulated protein degradation was created in the 1980s, largely through the complementary discoveries by the laboratory of A. Hershko (Technion, Israel) and by my laboratory, then at MIT. These discoveries revealed three sets of previously unknown facts:

1. ATP-dependent protein degradation involves a new protein modification, ubiquitin conjugation, which is mediated by specific enzymes, termed E1, E2 and E3.

2. The selectivity of ubiquitin conjugation is determined by specific multipartite degradation signals (degrons) in short-lived proteins, including degrons that give rise to the N-end rule.

3. Ubiquitin-dependent processes play a strikingly broad, previously unsuspected part in cellular physiology, primarily by controlling the *in vivo* levels of specific proteins. Ubiquitin conjugation was shown by us to be required for protein degradation *in vivo*, for cell viability, and also, specifically, for the cell cycle, DNA repair, protein synthesis, transcriptional regulation, and stress responses. We also cloned the first ubiquitin genes (discovering their divergent functions), the first specific E3 ubiquitin ligase (UBR1), the first deubiquitylating enzymes (DUBs), and identified the first physiological substrate of the ubiquitin system, the MATalpha2 transcriptional repressor. In addition, we discovered that ubiquitin-dependent proteolysis involves an essential, substrate-linked polyubiquitin chain of unique topology. The ubiquitin system was also discovered to possess the critical property of *subunit selectivity*, i.e., the ability to destroy a specific subunit of a multisubunit protein, leaving the rest of the protein intact and thereby making possible *protein remodeling*. This fundamental process underlies the cell cycle (the replacement of cyclin subunits in cell-cycle kinases), the activation of transcription factors such as, for example, NF-kappaB, and a multitude of other biological pathways.

The Hershko laboratory produced the first of these fundamental advances (item 1), and my laboratory produced the other two (items 2 and 3). Our function-based studies in the 1980s yielded the overall discovery of the *physiological regulation by intracellular protein degradation*. The complementary "chemical" and "biological" insights by Hershko's and my laboratories caused a massive expansion of the ubiquitin field in the 1990s. It became one of the largest arenas in biomedical science, the point of convergence of many disparate disciplines. Because perturbations of the cell cycle, DNA repair and stress response pathways are hallmarks of malignant transformation, the 1987-88 discoveries by my lab with the CDC34, RAD6 and UBI4 proteins opened up ubiquitin studies in cancer research as well. For accounts of the early history of the ubiquitin field, see Hershko *et al*. (2000); Varshavsky (2006, 2008).

Our biological discoveries in the 1980s yielded the modern paradigm of cellular physiology, in which regulated proteolysis is of central importance. These advances, together with later studies by many groups, revealed that the control through regulated protein degradation rivals, and often surpasses in significance the classical regulation through transcription and translation. This altered understanding of the design of biological circuits is of major importance for medicine, given the astounding functional range of the ubiquitin system and the multitude of ways in which ubiquitin-dependent processes can malfunction in disease or in the course of aging, from cancer and neurodegenerative syndromes to perturbations of immunity and many other illnesses, including birth defects. Our work at Caltech continues to focus on ubiquitin-dependent processes.

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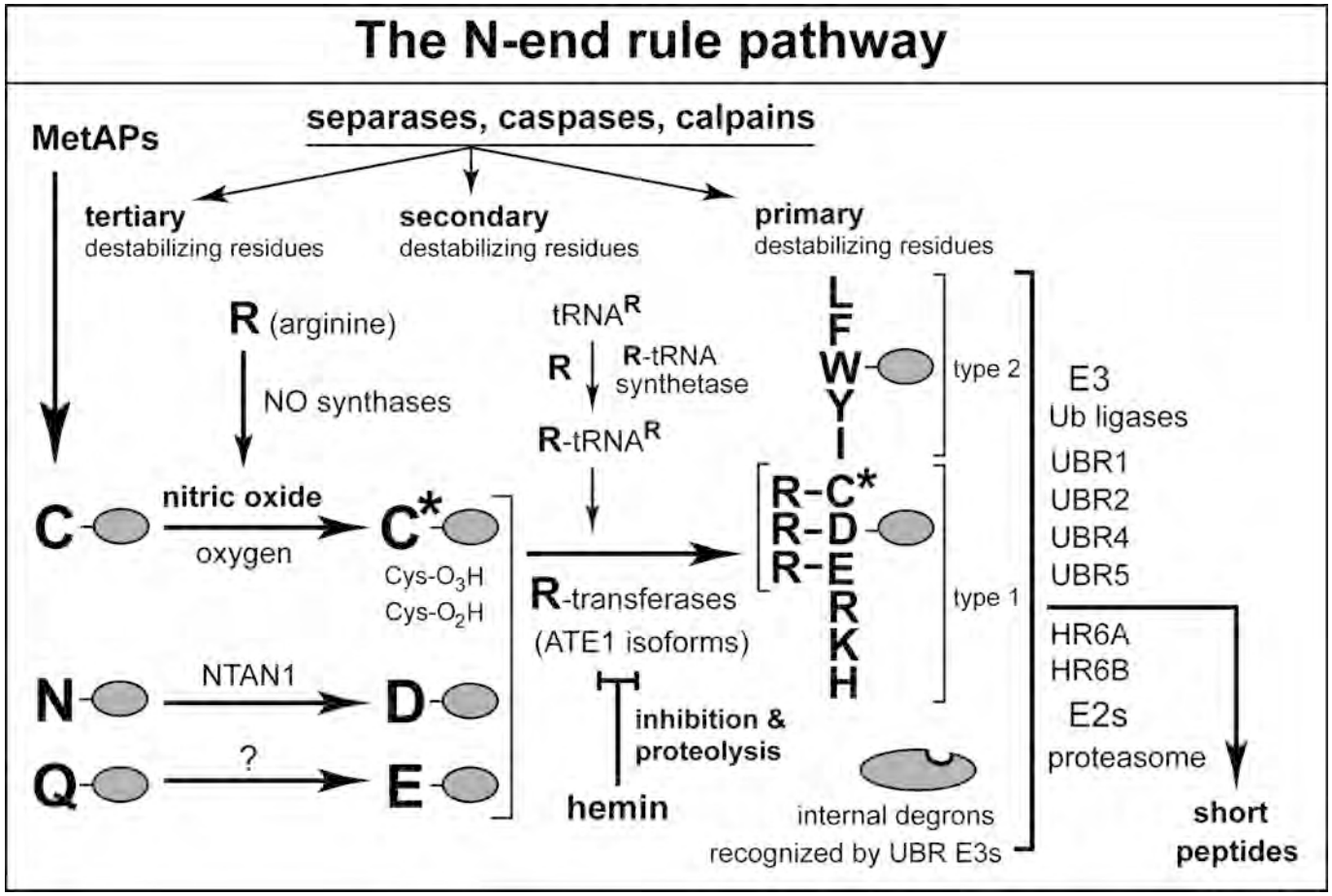


**Fig. 1.** The mammalian N-end rule pathway. N-terminal residues are indicated by single-letter abbreviations for amino acids. Yellow ovals denote the rest of a protein substrate. A sign, above *"hemin"* in the middle of diagram, is a modified "downregulation" sign that denotes a downregulation mediated, at least in part, by target's degradation. MetAPs, methionine aminopeptidases. C* denotes oxidized Cys, either Cys-sulfinate or Cys-sulfonate, produced in reactions mediated by nitric oxide (NO), oxygen ($O_2$) and their derivatives. Oxidized N-terminal Cys is arginylated by *ATE1*-encoded isoforms of R-transferase. Type-1 and type-2 primary destabilizing N-terminal residues are recognized by the pathway's E3 Ub ligases, called N-recognins. Through their other substrate-binding sites, these E3s also recognize internal (non-N-terminal) degrons in other substrates of the N-end rule pathway, denoted by a larger oval. As shown in the diagram, hemin ($Fe^{3+}$-heme) interacts not only with Ate1 (R-transferase) but with UBR-family Ub ligases as well, and downregulates at least some E3s of this family.

Ubiquitin (Ub)-dependent proteolysis involves the "marking" of a substrate through covalent conjugation of Ub to a substrate's internal Lys residue. Ub conjugation is mediated by the E1-E2-E3 enzymatic cascade. E1, the Ub-activating enzyme, forms a thioester bond between the C-terminal Gly of Ub and a Cys residue of E1. In the second step, activated Ub is transesterified to a Cys residue of a Ub-conjugating (E2) enzyme. Thereafter a complex of E2 and another enzyme, E3, conjugates Ub to a Lys residue of a substrate. Proteolytic pathways of the Ub system have in common their dependence on Ub conjugation and the 26S proteasome (which processively degrades Ub-protein conjugates), and differ largely through their utilization of distinct E2-E3 complexes. Specific E3s recognize (bind to) specific degradation signals (degrons) of their substrates. Ub has nonproteolytic functions as well.

One pathway of the Ub system is the N-end rule pathway (Fig. 1). The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue. The N-end rule pathway recognizes several kinds of degradation signals, including a set called N-degrons (Fig. 1). Although prokaryotes lack the Ub system, they still contain the N-end rule pathway, albeit Ub-independent versions of it. In eukaryotes, an N-degron consists of three determinants: a destabilizing N-terminal residue of a protein substrate, one (or more) of its internal Lys residues (the site of formation of a poly-Ub chain), and a nearby disordered region. The N-end rule has a hierarchic structure (Fig. 1). In eukaryotes, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their enzymatic deamidation, to yield the secondary destabilizing N-terminal residues Asp and Glu. The destabilizing activity of N-terminal Asp and Glu

requires their conjugation to Arg, one of the primary destabilizing residues, by Arg-tRNA-protein transferase (R-transferase). In mammals and other eukaryotes that produce nitric oxide (NO), the set of arginylated residues contains not only Asp and Glu but also N-terminal Cys, which is arginylated after its oxidation to Cys-sulfinate or Cys-sulfonate. The *in vivo* oxidation of N-terminal Cys requires NO, as well as oxygen ($O_2$) or its derivatives (Fig. 1). The N-end rule pathway is thus, a sensor of NO, through the ability of this pathway to destroy proteins with N-terminal Cys, at rates controlled by NO, $O_2$ and their derivatives.

E3 Ub ligases of the N-end rule pathway, called N-recognins, bind to primary destabilizing N-terminal residues, including Arg (Fig. 1). (The term "Ub ligase" denotes either an E2-E3 holoenzyme or its E3 component.) At least four N-recognins, including Ubr1, mediate the N-end rule pathway in mammals. The known N-recognins share a ~70-residue motif called the UBR box. Mouse Ubr1 and Ubr2 are sequelogous (similar in sequence) 200-kD RING-type E3 Ub ligases that are 47% identical. Several other UBR-containing N-recognins, either confirmed or putative ones, are HECT-type or SCF-type E3 Ub ligases that share the UBR motif with the RING-type Ubr1/Ubr2 but are largely nonsequelogous to them otherwise. (*A note on terminology*: "sequelog" and "spalog" denote, respectively, a sequence that is similar, to a specified extent, to another sequence, and a 3D (spatial) structure that is similar, to a specified extent, to another 3D structure (Varshavsky, 2004). Besides their usefulness as separate terms for sequence and spatial similarities, the rigor-conferring advantage of "sequelog" and "spalog" is their *evolutionary neutrality*, in contrast to interpretation-laden terms such as "homolog," "ortholog" and "paralog." The latter terms are compatible with the sequelog/spalog terminology and can be used to convey understanding about functions and common descent, if this (additional) information is available.)

The N-end rule pathway of *S. cerevisiae* is mediated by a single N-recognin, Ubr1, a 225-kD sequelog of mammalian Ubr1 and Ubr2 (Fig. 1). *S. cerevisiae* Ubr1 contains at least three substrate-binding sites. The type-1 site is specific for basic N-terminal residues of protein substrates (Arg, Lys, His), while the type-2 site is specific for bulky hydrophobic N-terminal residues (Trp, Phe, Tyr, Leu, Ile). The third binding site of UBR1 targets proteins through their internal (non-N-terminal) degrons, and is allosterically "activated" through a conformational change that is caused by the binding of short peptides to the Ubr1's other two binding sites, type-1 and type-2. The known substrate of the third binding site of Ubr1 is Cup9, a transcriptional repressor whose regulon includes *PTR2*, a gene-encoding transporter of di- and tripeptides. The reversal of Ubr1 autoinhibition by imported peptides accelerates the Ubr1-dependent ubiquitylation of Cup9, leads to its faster degradation, and thereby causes a derepression of *PTR2*. The resulting positive-feedback circuit allows *S. cerevisiae* to detect the presence of extracellular peptides and to react by increasing their uptake.

The functions of the N-end rule pathway were discovered largely over the last decade, in part through our studies. These functions include (i) the sensing of heme, owing to inhibition of the pathway's ATE1 R-transferase, in both yeast and mammals, by hemin ($Fe^{3+}$-heme), which also inhibits N-recognins, the latter at least in yeast; (ii) the sensing of NO and oxygen, and the resulting control of signaling by transmembrane receptors, through the conditional, $NO/O_2$-mediated degradation of G-protein regulators RGS4, RGS5 and RGS16; (iii) regulation of import of short peptides, through the degradation, modulated by peptides, of Cup9, the import's repressor; (iv) fidelity of chromosome segregation, through degradation of a separase-produced cohesin fragment; (v) regulation of apoptosis, through degradation of a caspase-processed inhibitor of apoptosis; (vi) a multitude of processes mediated by the transcription factor c-FOS, a conditional substrate of the N-end rule pathway; (vii) regulation of the human immunodeficiency virus (HIV) replication cycle, through degradation of HIV integrase; (viii) regulation of meiosis, spermatogenesis, neurogenesis and cardiovascular development in mammals, and leaf senescence in plants. Mutations in human Ubr1 (Fig. 1) are the cause of Johansson-Blizzard Syndrome (JBS), which comprises mental retardation, physical malformations, and severe pancreatitis.

Functional and mechanistic studies of the N-end rule pathway in yeast and mammals are a major theme of our current work.

## 77. Mechanistic and functional studies of N-terminal arginylation

*Christopher Brower, Rong-Gui Hu, Alexander Varshavsky*

This Abstract mentions some of our ongoing projects that aim to further advance the understanding of the arginylation branch of the N-end rule pathway, beyond the understanding described above.

(**i**) Construction and functional analyses of mouse strains (and cells derived from them) in which the expression of *Ate1*-encoded Arg-tRNA-protein transferases (R-transferases) is selectively and conditionally abolished in specific cell lineages during embryogenesis, or postnatally. This set of projects should make possible, among other things, a functional dissection of N-terminal arginylation in specific organ systems and cell types of adult mice. (A nonconditional *Ate1*$^{-/-}$ genotype is embryonic lethal [Kwon *et al.*, 2002]).

(**ii**) Construction and functional analysis of knock-in mouse strains that contain a doxycycline-inducible allele of *Ate1*, and thus, can overproduce R-transferases, in a controllable manner, in specific cell types during embryogenesis, or postnatally.

**(iii)** Analysis of chromosome stability and regulation of apoptosis in mouse *Ate1*$^{-/-}$ cells. These projects stem from the discovery of the function of the *S. cerevisiae* N-end rule pathway in the maintenance of chromosome stability (Rao *et al.*, 2001), and from the conjecture that an analogous function in mammalian cells involves the (*Ate1*-dependent) arginylation branch of the N-end rule pathway. Recent work indicated that

N-terminal arginylation is essential for the *in vivo* degradation of the separase-produced fragment of Scc1/Rad21, a subunit of mouse cohesin. Moreover, *Ate1(-/-)* fibroblasts were also found exhibit a strong chromosome instability (CIN), presumably because, at least in part, of their inability to destroy the separase-produced cohesin's fragment.

**(iv)** Identification of *ATE1*-dependent circuits (i.e., circuits that involve N-terminal arginylation) through the identification of mouse genes whose expression is significantly altered during embryonic development in *Ate1-/-* embryos, using microarray techniques, differential display and analogous methods with *Ate1(-/-)* and congenic +/+ embryos or EF cells.

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**78. Biochemical and genetic studies of UBR3, a ubiquitin ligase with a function in olfactory and other sensory systems**

*Takafumi Tasaki*[*]*, Yong Tae Kwon*[*]*, Alexander Varshavsky*

Our previous work identified E3 ubiquitin ligases, termed Ubr1-Ubr7, that contain the ~70-residue UBR box, a motif important for the targeting of N-end rule substrates. In this pathway, specific N-terminal residues of substrates are recognized as degradation signals by UBR box-containing E3s that include Ubr1, Ubr2, Ubr4 and Ubr5. The other E3s of this set, Ubr3, Ubr6 and Ubr7, remained uncharacterized. In the present work, we cloned and analyzed mouse Ubr3. The similarities of Ubr3 to the Ubr1 and Ubr2 E3s of the N-end rule pathway include the RING and UBR domains. We found that Hr6A and Hr6B, the E2 enzymes that bind to Ubr1 and Ubr2, also interact with Ubr3. However, in contrast to Ubr1 and Ubr2, Ubr3 does not recognize N-end rule substrates. We also constructed Ubr3-lacking mouse strains. In the 129SvImJ background, *Ubr3*$^{-/-}$ mice died during embryogenesis, whereas the C57BL/6-background *Ubr3*$^{-/-}$ mice exhibited neonatal lethality and suckling impairment that could be partially rescued by litter size reduction. The adult *Ubr3*$^{-/-}$ mice had female-specific behavioral anosmia. Cells of the olfactory pathway were found to express beta-galactosidase (LacZ) that marked the deletion/disruption *Ubr3*$^{-}$ allele. The *Ubr3*-specific LacZ expression was also prominent in cells of the touch, vision, hearing and taste systems, suggesting a regulatory role of Ubr3 in sensory pathways, including olfaction. By analogy with functions of the UBR domain in the N-end rule pathway, we propose that the UBR box of Ubr3 may recognize small compounds that modulate the targeting, by this E3, of its currently unknown substrates.

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**79. Regulation of peptide import through phosphorylation of Ubr1, the ubiquitin ligase of the N-end rule pathway**

*Cheol-Sang Hwang, Alexander Varshavsky*

Ubr1 is the N-recognin of the yeast *S. cerevisiae* (see Introduction). Extracellular amino acids or short peptides upregulate the peptide transporter gene *PTR2*, thereby increasing the capacity of a cell to import peptides. Cup9 is a transcriptional repressor that downregulates *PTR2*. The induction of *PTR2* by peptides or amino acids involves accelerated degradation of Cup9 by the N-end rule pathway. We found that the Ubr1 N-recognin, which conditionally targets Cup9 for degradation, is phosphorylated *in vivo* at multiple sites, including Ser300 and Tyr277 (Hwang *et al*., 2008). We also found that the type-I casein kinases Yck1 and Yck2 phosphorylate Ubr1 on Ser300, and thereby make possible ("prime") the subsequent (presumably sequential) phosphorylations of Ubr1 on Ser296, Ser292, Thr288 and Tyr277 by Mck1, a kinase of the glycogen synthase kinase 3 (Gsk3) family. Phosphorylation of Ubr1 on Tyr277 by Mck1 is the first example of a cascade-based tyrosine phosphorylation by a Gsk3-type kinase outside of autophosphorylation. We show that the Yck1/Yck2-mediated phosphorylation of Ubr1 on Ser300 plays a major role in the control of peptide import by the N-end rule pathway. In contrast to phosphorylation on Ser300, the subsequent (primed) phosphorylations, including the one on Tyr277, have at most minor effects on the known properties of Ubr1, including regulation of peptide import. Thus, a biological role of the rest of Ubr1 phosphorylation
cascade remains to be identified.

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**80. The N-end rule pathway as a sensor of heme**

*Rong-Gui Hu, Haiqing Wang, Alexander Varshavsky*

Heme is an iron-containing protoporphyrin IX. Two major species of heme are ferrous ($Fe^{2+}$) heme and its ferric ($Fe^{3+}$) counterpart, called hemin. Intracellular proteins whose functions depend on their binding to heme include hemoglobins, cytochrome oxidases, NO synthases, cGMP cyclases, and catalases, as well as specific kinases, transcription factors, ion channels, and regulators of iron

metabolism. A major aspect of heme is its ability to interact with physiologically relevant gases such as $O_2$, NO and carbon monoxide (CO). Over the last decade, it became clear that reactive oxygen species (ROS), if they are present at "signaling" (i.e., sufficiently low) levels, can act as regulators of circuits that underlie not only stress responses but other functions as well, including the cell cycle, transcription and differentiation. The proximal sensors of $H_2O_2$ and analogous compounds are cysteine-containing proteins, some of which contain heme as well. ROS-mediated formation of disulfides or other Cys derivatives changes the activity of sensor proteins, which transduce their altered states into the outputs of circuits they control. In some hemoproteins of these circuits, it is the heme moiety, rather than Cys residues, that functions as a redox sensor.

We discovered (Hu *et al*., 2008) that the arginyl-transferase Ate1, which mediates the arginylation branch of the N-end rule pathway (see Fig. 1 and Introduction), is inhibited by hemin ($Fe^{3+}$-heme), via a specific redox mechanism that involves the formation of disulfide between cysteine-71 and cysteine-72 of Ate1. Remarkably, hemin also induces degradation of R-transferase *in vivo* thus, acting as both a "stoichiometric" and "catalytic" down-regulator of the N-end rule pathway. This proteolytic circuit, a known sensor of short peptides, nitric oxide and oxygen, is now a sensor of heme as well. One function of the N-end rule pathway may be to coordinate the activities of small effectors, both reacting to and controlling the redox dynamics of heme, oxygen, nitric oxide and thiols, in part through conditional degradation of specific transcription factors and G-protein regulators.

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Hu, R.-G., Wang, H., Xia, Z. and Varshavsky, A. (2008) *Proc. Natl. Acad. Sci. USA* **105**:76-81.

**81. Enzymatic N-terminal addition of noncanonical amino acids to peptides and proteins**

*Connor, R.E., Piatkov, K.P., Varshavsky, A., Tirrell, D.A.*

The production of well-defined protein conjugates is essential for many therapeutic and biochemical technologies. To achieve site-specific modification of proteins, the N-terminus has been targeted by a variety of chemical and enzymatic methods. We developed a fully enzymatic *in vitro* method for the N-terminal addition of non-canonical amino acids to peptides and proteins. The *Escherichia coli* leucyl, phenylalanyl-transferase (Aat), encoded by the *aat* gene, catalyzes the conjugation of leucine (Leu), phenylalanine (Phe) or methionine (Met) from an aminoacylated tRNA to any protein that bears N-terminal arginine or lysine (Connor *et al*., 2008). The transfer of Leu or Phe to the N-terminus of a protein in wild-type *E. coli* cells results in a decrease of the protein's *in vivo* half-life through degradation by ClpAP, an ATP-dependent protease. Aat, ClpAP, and the adapter protein, ClpS, comprise the *E. coli* N-end rule pathway. Aat is tolerant of structural variation in its amino acid substrates and is known to accept Phe analogs through chemically aminoacylated-tRNAs. We used a simple chromatographic assay to demonstrate Aat-mediated transfer of non-canonical amino acids to the acceptor peptide lysylalanyl-7-amino-4-methylcoumarin. Bio-orthogonal reactive functional groups, such as alkenes, alkynes, azides and ketones, can be transferred and used to prepare bio-conjugates in high yield. The method introduced here allows new approaches to the engineering of therapeutic proteins through pegylation; to the study of protein interactions through crosslinking; and to the immobilization of proteins for use in sensors, microarrays, and catalytic systems. Furthermore, the HPLC assay introduced here for monitoring Aat activity can be applied to any non-canonical amino acid.

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Connor, R.E., Piatkov, K.P., Varshavsky, A. and Tirrell, D.A. (2008) *ChemBiochem.* **9**:366-369.

**82. Listeriolysin O secreted by *Listeria monocytogenes* into the host cell cytosol is degraded by the N-end rule pathway**

*Schnupf, P., Zhou, J., Varshavsky, A., Portnoy, D.A.*

The intracellular pathogen *Listeria monocytogenes* escapes from a phagosomal compartment into the cytosol by secreting the pore-forming cytolysin listeriolysin O (LLO). During proliferation of *L. monocytogenes* in the mammalian cell cytosol, the secreted LLO is targeted for degradation by the ubiquitin system. We show here that LLO is a substrate of the ubiquitin-dependent N-end rule pathway, which recognizes LLO through its N-terminal Lys residue (Schnupf *et al*., 2008). Specifically, we demonstrated, by reverse-genetic and pharmacological methods, that LLO was targeted for degradation by the N-end rule pathway in reticulocyte extracts, in mouse 3T3 cells, and after its secretion, by intracellular bacteria, into the mouse cell cytosol. Replacing the N-terminal Lys of LLO with a stabilizing residue such as Val increased the *in vivo* half-life of LLO but did not strongly affect the intracellular growth or virulence of *L. monocytogenes*. Nevertheless, this replacement decreased the virulence of *L. monocytogenes* by nearly 2-fold, suggesting that a destabilizing N-terminal residue of LLO may stem from positive selection during the evolution of this and related bacteria. A double-mutant *L. monocytogenes*, in which an upregulated secretion of LLO was combined with a stabilizing N-terminal residue, was severely cytotoxic to infected mammalian cells, resulting in reduced intracellular growth of bacteria and a ~100-fold lower virulence. In summary, we showed that LLO is degraded by the N-end rule pathway and that degradation of LLO can reduce its toxicity during infection, a property of LLO that may have been selected for its positive effects on fitness during the evolution of *L. monocytogenes*.

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Schnupf, P., Zhou, J., Varshavsky, A. and Portnoy, D.A. (2007) *Infection & Immunity* **75**:5135-5147.

**83. Substrate-binding sites of Ubr1, the ubiquitin ligase of the N-end rule pathway**

*Xia, Z., Webster, A., Du, F., Piatkov, K., Ghislain, M., Varshavsky, A.*

Substrates of a ubiquitin-dependent proteolytic system called the N-end rule pathway include proteins with destabilizing N-terminal residues. N-recognins, the pathway's ubiquitin ligases, contain three substrate-binding sites. The type-1 site is specific for basic N-terminal residues (Arg, Lys, His). The type-2 site is specific for bulky hydrophobic N-terminal residues (Trp, Phe, Tyr, Leu, Ile). We show here that the type-1/2 sites of Ubr1, the sole N-recognin of the yeast *Saccharomyces cerevisiae*, are located in the first ~700 residues of the 1,950-residue Ubr1. These sites are distinct in that they can be selectively inactivated by mutations, identified through a genetic screen. Mutations inactivating the type-1 site are in the previously delineated ~70-residue UBR motif characteristic of N-recognins. Fluorescence polarization and surface plasmon resonance were used to determine that Ubr1 binds, with $K_d$ of ~1 microM, to either type-1 or type-2 destabilizing N-terminal residues of reporter peptides, but does not bind to a stabilizing N-terminal residue such as Gly (Xia *et al*., 2008). A third substrate-binding site of Ubr1 targets an internal degron of Cup9, a transcriptional repressor of peptide import. We show that the previously demonstrated *in vivo* dependence of CUP9 ubiquitylation on the binding of cognate dipeptides to the type-1/2 sites of Ubr1 can be reconstituted in a completely defined *in vitro* system. We also found that purified Ubr1 and Cup9 interact nonspecifically, and that specific binding (which involves, in particular, the binding by cognate dipeptides to the Ubr1's type-1/2 sites) can be restored either by a chaperone such as Ef1A or through macromolecular crowding.

**Reference**

Xia, Z., Webster, A., Du, F., Piatkov, K., Ghislain, M. and Varshavsky, A. (2008) *J. Biol. Chem.* **283**:24011-24028.

**84. Amino acids induce peptide uptake via accelerated degradation of Cup9, the transcriptional repressor of the Ptr2 peptide transporter**

*Xia, Z., Turner, G.C., Hwang, C.-S., Byrd, C. Varshavsky, A.*

Multiple pathways link expression of Ptr2, the transporter of di- and tripeptides in the yeast *S. cerevisiae*, to the availability and quality of nitrogen sources. Previous work has shown that induction of *PTR2* by extracellular amino acids requires, in particular, *SSY1* and *PTR3*. Ssy1 is structurally similar to amino acid transporters, but functions as a sensor of amino acids. Ptr3 acts downstream of Ssy1. Expression of the Ptr2 peptide transporter is induced not only by amino acids but also by dipeptides with destabilizing N-terminal residues. These dipeptides bind to Ubr1, the ubiquitin ligase of the N-end rule pathway, and allosterically accelerate the Ubr1-dependent degradation of Cup9, a transcriptional repressor of *PTR2*. Ubr1 targets Cup9 through its internal degron. In the present study, we found that the repression of *PTR2* by Cup9 requires Tup1 and Ssn6, the corepressor proteins that form a complex with Cup9 (Xia *et al*., 2008). We also show that the induction of *PTR2* by amino acids is mediated by the Ubr1-dependent acceleration of Cup9 degradation that requires both Ssy1 and Ptr3. The acceleration of Cup9 degradation is shown to be attained without increasing the activity of the N-end rule pathway toward substrates with destabilizing N-terminal residues. We also found that Gap1, a general amino acid transporter, strongly contributes to the induction of *PTR2* by Trp. While several aspects of this complex circuit remain to be understood, our findings establish new functional links between the amino acids-sensing SPS system, the Cup9-Tup1-Ssn6 repressor complex, the Ptr2 peptide transporter and the UBR1-dependent N-end rule pathway.

**Reference**

Xia, Z., Turner, G.C., Hwang, C.-S., Byrd, C. and Varshavsky, A. (2008) *J. Biol. Chem.* **283**:28958-28968.

**85. Targeting the absence: homozygous deletions as immutable signposts for cancer therapy**

*Alexander Varshavsky*

A major obstacle to drug-based therapies of human diseases that are both efficacious and substantially free of side effects is the massive interconnectedness and redundancy of molecular circuits in living cells. In the case of cancer, the problem is exacerbated by genomic instability of many, possibly most, cancers. This property increases heterogeneity of malignant cells in the course of tumor progression or anticancer treatment, and is one reason for the failure of most drug-based cancer therapies. A few relatively rare cancers, such as testicular carcinoma, Wilm's kidney tumor, and some leukemias in children, can often be cured through chemotherapy, but require cytotoxic treatments of a kind that cause severe side effects and are themselves carcinogenic. Several recent advances, including the use of antiangiogenic compounds and inhibitors of specific kinases, hold the promise of efficacious, curative therapies. Nevertheless, major human cancers are still incurable once they have metastasized.

I recently suggested a new approach to cancer therapy. It involves homozygous deletions (HDs). Previous studies have demonstrated that many human cancers, including major ones, contain a significant number of scattered homozygous deletions. A salient property of an HD that involves DNA sequences not present elsewhere in the genome is that HD cannot revert. Employing homozygous deletions – *not their effects on tumor suppression and RNA/protein circuits but deletions themselves* – as a target of therapy is a new idea, to my knowledge. The difficulty here is that HD is an "absence," and therefore it cannot be a conventional molecular target. Nevertheless, an HD-specific anticancer regimen is feasible, through an approach termed *deletion-specific targeting* (DST).

The DST strategy (Varshavsky, 2007) is implemented by molecular circuits that combine, in a novel way, both existing and new methodologies. One of them is the ubiquitin (Ub) fusion technique (Varshavsky, 2005). In addition, an essential part of DST strategy is

based on "helper-dependent" split-protein devices, introduced by Johnsson and Varshavsky (1994) with the split-Ub assay and thereafter extended to other split-protein constructs, such as dihydrofolate reductase (DHFR), green fluorescent protein (GFP), and beta-lactamase. Split-protein domains coupled to DNA-recognizing proteins (Stains *et al*., 2005) are also a component of DST strategy. Yet another part of DST is a feedback mechanism that receives input from a circuit operating as a Boolean OR gate and involves the activation of split nucleases, which destroy DST vector in normal (non-target) cells. The DST strategy is independent, to a striking extent, of considerations that underlie other approaches to cancer therapy. For example, DST does not involve a function of deleted DNA, or its levels of expression in normal cells, or tumorigenic alterations of RNA/protein circuits in cancer cells, or cell-surface differences between them and normal cells. The logic of DST makes possible an incremental and essentially unlimited increase in the selectivity of therapy. If DST strategy can be implemented in a clinical setting, it may prove to be curative and substantially free of side effects.

At this stage, the nearest aim is not a clinically realistic DST design but rather the circuit's ability to function as a DST device. Work to verify the feasibility and medical relevance of DST is under way.

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Xia, Z., Turner, G.C., Hwang, C.-S., Byrd, C. and Varshavsky, A. (2008b) Amino acids induce peptide uptake via accelerated degradation of Cup9, the transcriptional repressor of the Ptr2 peptide transporter. *J. Biol. Chem.* **283**:28958-28968.

# Molecular, Cellular and Integrative Neuroscience

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**Senior Research Fellow**: Lynn Paul
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**Lab website**: http://www.emotion.caltech.edu

**Support**: The work described in the following research reports has been supported by:
Cure Autism Now Foundation
Gimble Neuroscience Fund
Gordon and Betty Moore Foundation
Hanson Fund
James S. McDonnell Foundation
Japan Society for the Promotion of Science
National Institute of Mental Health
National Institute of Neurological Disorders and Stroke
Pfeiffer Foundation
Simons Foundation

**Summary**: Our laboratory investigates the psychological and neural bases of social cognition, using a number of different approaches. Some studies focus on the psychological level, using behavioral data from healthy people to make inferences about how emotion modulates memory, attention, or conscious awareness. A second approach uses neuroimaging to investigate the neural mechanisms behind emotional and social processing. A third approach studies the performances, and the brains, of special populations. At Caltech, we have been recruiting people with agenesis of the corpus callosum to investigate the functional consequences of disruption in long-range connectivity. Dr. Lynn Paul spearheads this work. In collaboration with Joe Piven at the University of North Carolina, we have also been studying people with autism, as well as their first-degree relatives (the parents). At the University of Iowa, we have ongoing collaborations that involve neurological populations with focal brain lesions, and that involve neurosurgical populations in whom we can record intracranially.

A major focus has been on two brain structures: the amygdala, and the prefrontal cortex, both structures known to participate in emotion and social cognition. We are interested in how these structures modulate memory for emotional events, how they modulate allocation of attention to emotional stimuli, and how they guide decisions about options whose outcomes have emotional value. One particularly active area of development is to better understand how amygdala and prefrontal cortex interact, and how they interface with other brain structures that also participate in emotional processing. Another active area of research investigates brain connectivity. We hope that a better understanding of the neural basis of social cognition will help with the diagnosis and treatment of people diagnosed with autism, agenesis of the corpus callosum, or mood disorders. Equally important, we hope that such a better understanding can educate everybody about people with these disorders and their consequences for social behavior.

### 86. Decoding facial information from direct intracranial recordings of the human brain

*Naotsugu Tsuchiya, Hiroto Kawasaki, Hiroyuki Oya, Matthew A. Howard, III, Ralph Adolphs*

Functional neuroimaging has revealed a distributed network of cortical regions that participate in processing faces, but the points in time at which they come into play, and the electrophysiological signatures of their activation, remain poorly understood. Yet such information is essential to construct a mechanistic model of how distinct facial attributes are extracted and used by the brain. In collaborations with neurosurgeons at the University of Iowa, we recorded wide-band activity from over 100 contacts on the ventral and lateral temporal cortex of nine neurosurgical patients undergoing epilepsy monitoring while they viewed static and dynamic facial expressions. Applying a novel decoding analysis to optimally combine information from simultaneously recorded channels and/or different frequency bands, we found sites in ventral middle temporal cortex that discriminated faces from non-face objects within 100 msec. The critical information for discriminating faces and emotions was carried by power modulation above 50Hz, extending into 150 Hz, considerably higher than the classic gamma band. Task-relevant attention improved decoding accuracy, and its effects were widely distributed across frequencies. Though a dominant theory of face perception postulates independent representations of invariant (identity) and changeable (emotional expression) aspects of faces, our results challenge such a model: information about both was better decoded from common anatomical locations, especially from the middle fusiform gyri.

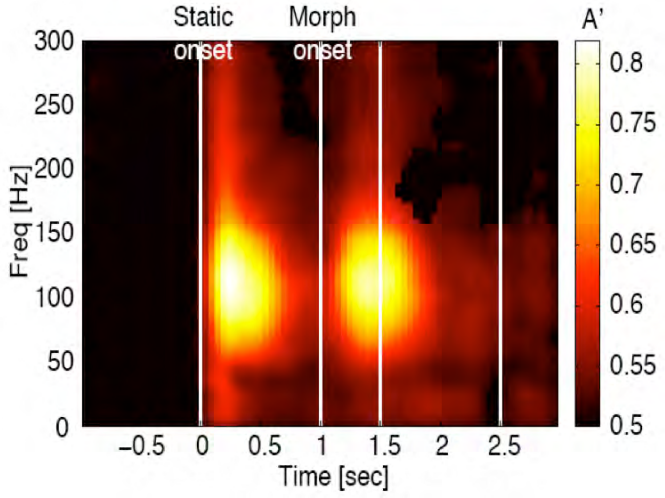


**Figure 1**. High frequency (50-150Hz) power from the intracranial EEG recorded in the ventral temporal cortex can be used to decode whether subjects are viewing faces

or not with an accuracy of 80% (chance is 50% correct). The colors encode the decoding accuracy as a function of frequency (y-axis) and time relative to the stimulus (x-axis), which consisted of the presenting of a static face (at t=0) followed by a dynamic morph (at t=1).

**87. Intact rapid detection of fear in the absence of the amygdala**

*Naotsugu Tsuchiya, Farshad Moradi, Csilla Felsen, Ralph Adolphs*

A prevalent view of the amygdala's contribution to processing facial expressions of fear is that it comes into play early and automatically, possibly through a subcortical route that mediates rapid detection of fear. We discovered that a patient with complete bilateral amygdala lesions, who is impaired in recognizing fearful faces, nonetheless showed entirely normal rapid detection of fear. The findings argue against an essential early-processing role for the amygdala, and instead favor a contribution that incorporates contextual and cognitive modulation.

Subjects were shown two faces (one emotional, one neutral) or two scenes (one threatening, one neutral) side-by-side and asked, as rapidly as possible, to push a button to indicate the side of the emotional stimulus. The amygdala patient was as fast and as accurate as controls were (**Figure 2**), despite the fact that her recognition and ratings of emotional stimuli are impaired.

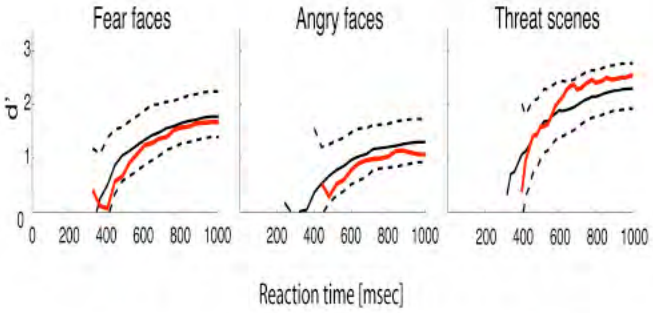


**Figure 2.** Amygdala patient (red) can detect fearful and angry faces as well as threat scenes as rapidly and accurately as age-matched controls (black, 95% confidence interval is dotted). The y-axis plots d', a measure of accuracy.

**88. Bimanual motor coordination in patients with callosal agenesis**

*J. Michael Tyszka, Lynn K. Paul, Matt Leonard, Ralph Adolphs*

The human motor system is frequently studied to understand the role that callosal and subcortical pathways play in the generation of coordinated motion. Differences in symmetric (mirrored) and asymmetric bimanual tasks have been used with EEG and fMRI. The stability of mirror movement is largely attributed to the role of dominant motor cortex in ipsilateral movements [1]. Facilitation and inhibition of bilateral coordinated motion by callosal fibers is supported by strong evidence from callosotomy patients [2] and paired transcranial magnetic stimulation (TMS) [3]. Little is known about the functional network involved in coordinated bimanual motor tasks in congenitally acallosal subjects (AgCC). Callosal transfer normally aids in bimanually coordinated motor activity [4, 5]. Numerous studies have shown that individuals with AgCC are considerably more capable of interhemispheric integration than "split-brain" subjects [6]. In this study, the network of motor areas associated with bimanual coordination, namely primary motor (M1), dorsal premotor (dPM), supplementary motor area (SMA), and dorsal cerebellar motor areas (Cb) was mapped using BOLD fMRI. A simple, self-paced mirrored finger tapping blocked task revealed the preservation of the SMA location in AgCC despite marked structural white and gray matter reorganization due to the absence of the corpus callosum. Of equal interest were differences between unimanual and bimanual tasks when compared to control subjects (**Figure 3**). The contrast between the combination of unimanual tapping (Left + Right) and the bimanual tapping equivalent revealed almost no significant activation difference between the two conditions in acallosal subjects. Whether unimanual and bimanual tapping are equally easy or equally difficult for acallosal subjects is an open question and will be studied using behavioral measures of performance in coordinated bimanual tasks.

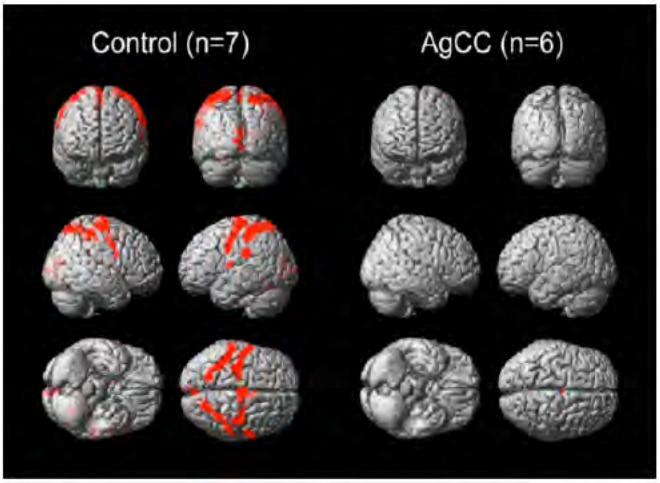


**Figure 3.** Functional MRI of unimanual and bimanual tasks in AgCC. Comparison of group BOLD activity for unimanual and bimanual coordinated finger tapping in control and AgCC subjects. The contrast shown is (Right + Left) > Both and reveals increased activity in parietal, primary and premotor areas of control subjects for the unimanual tasks. The null contrast in AgCC subjects may arise either from equal difficulty or equal facility of bimanual and unimanual tasks in these subjects.

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**89. Virtual fiber tracking in an acallosal mouse model of autism**

*J. Michael Tyszka, Ralph Adolphs*

Mouse models of autism generally focus on a small set of behaviors held in common with human autism spectrum disorders. Examples include deficits in reciprocal social interactions, repetitive behaviors, and communication deficits [1, 2]. The BTBR mouse strain is perhaps the premier mouse model of autism and, intriguingly, is also congenitally acallosal [3]. BTBR therefore offers a unique opportunity to isolate the role of interhemispheric communication in autistic behavior with obvious comparisons to similar behavior observed in acallosal human subjects [4, 5]. In order to better understand the complex relationship between structure and behavior in the BTBR mouse strain, we are undertaking a comprehensive imaging study, initially in fixed brain samples, using state-of-the art methods including virtual fiber tracking. Magnetic resonance imaging is uniquely sensitive to the microscopic motion of water molecules [6]. Directional variations in diffusion measured by MRI lead to estimates of fiber orientation, and ultimately to models of long-range structural connectivity within the brain. For this study, high angular resolution diffusion imaging (HARDI) is being used to generate three-dimensional maps of water diffusion anisotropy in perfusion fixed brains from BTBR and control C57BL/6 mice. All data is acquired using the 11.7 Tesla magnetic resonance microscope supported by the Biological Imaging Center of the Beckman Institute at Caltech. Initial results are encouraging, and allow the territories associated with the Probst bundles and residual commissural fibers to be mapped in a probabilistic sense across many mouse brains. *This work is in collaboration with Elliott Sherr (UCSF) and Jackie Crawley (NIH/NIMH).*

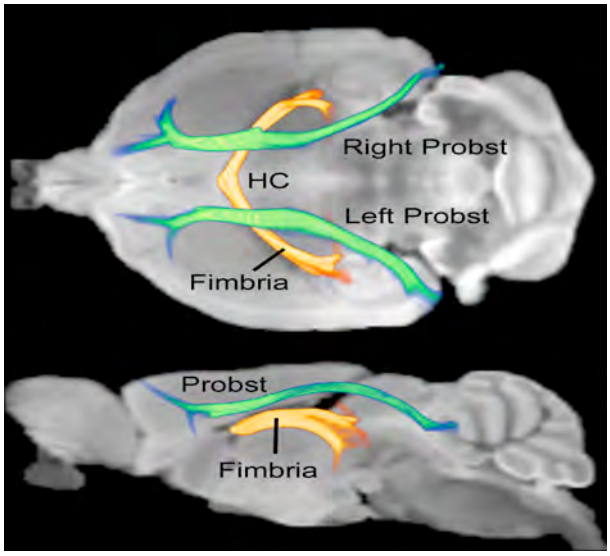


**Figure 4**. Probabilistic fiber tracking of the Probst bundle and the hippocampal commissure (HC) in a BTBR mouse brain using high angular resolution diffusion MR microscopy and a multi-fiber model of diffusion anisotropy. The Probst bundles are comprised of callosal fibers that fail to cross midline in this strain, and are also observed in congenitally acallosal humans. The functional role played by the Probst bundles currently is not understood. The remaining interhemispheric commissures (hippocampal, anterior and posterior) are typically intact in this strain, but can be reduced in size. Virtual fiber density is rendered in orange (HC and fimbria) and green (Probst bundles).

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**90. Connectivity of the macaque brain from diffusion imaging**

*Dirk Neumann, J. Parvizi, J. Michael Tyszka, Ralph Adolphs*

Long-range corticocortical connections have been studied using *in vivo* tracers in the macaque brain, but this has been examined only for a relatively small subset of regions, and is impossible in humans. Diffusion-weighted imaging and fiber tracking algorithms offer an alternative that can examine the entire brain, and that can be applied also to humans. While effective, this method is limited by its relatively low spatial resolution, making it difficult to resolve small fibers as well as crossing fibers. As well, what precisely constitutes a traceable fiber and how many of the corticocortical connections can potentially be studied with diffusion-weighted imaging is currently unknown. Here, we scanned two macaque brains in a 7T and 9.4T Bruker MR system and identified connections between 72 cortical areas with a probabilistic algorithm. For every pairing between the cortical areas, we estimated the diffusion-based evidence that a fiber connects the pair. If the evidence is strong, then it is very likely that the connection is indeed there and can be identified with a chemical tracing technique. By increasing the cut-off for the diffusion-estimate strength, we can raise the reliability of our algorithm. By decreasing the cut-off we can increase its sensitivity, but this comes at the expense of increasing the number of incorrectly identified connections. We compared our fiber tracking results against cortical connections known from tracer studies. We found A' values of an ROC analysis of around 0.72, indicating that a fairly good sensitivity in finding correct connections can be obtained with a relatively low false positive rate (**Figure 5**).

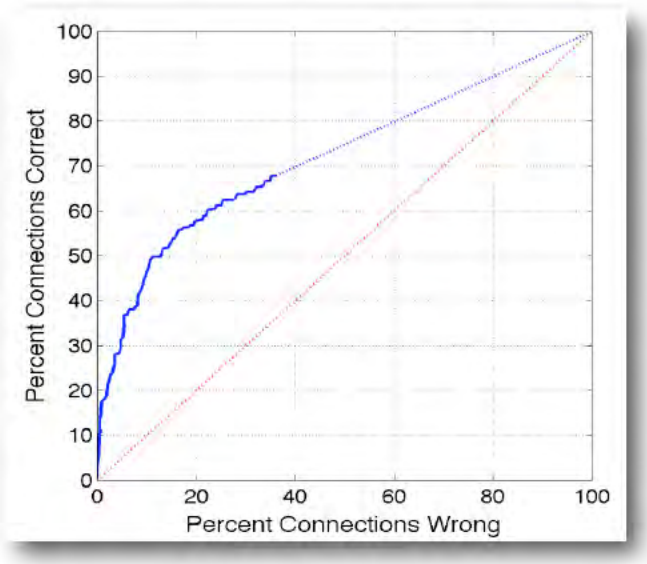


**Figure 5:** ROC curve showing the sensitivity (y-axis) and specificity (x-axis) of our tractography (blue line) compared against chance (red line).

**91. Unusual face processing in first-degree relatives of people with autism**

*Ralph Adolphs, Michael Spezio, Morgan Parlier, Joseph Piven*

In his original description of autism, Kanner [1] noted that the parents of autistic children often exhibited unusual social behavior themselves, consistent with what we now know about the high heritability of autism [2]. We investigated this so-called "Broad Autism Phenotype" in the parents of children with autism, who themselves did not have a diagnosis of any psychiatric illness. Building on recent quantifications of social cognition in autism [3], we investigated face processing using the "Bubbles" method [4] to measure how viewers make use of information from specific facial features in order to judge emotions. Parents of autistic children who were assessed as socially aloof (N=15), a key component of the phenotype [5], showed a remarkable reduction in processing the eye region in faces, compared to a control group of parents of neurotypical children (N=20), as well as to non-aloof parents of autistic children (N=27, whose pattern of face processing was intermediate) (**Figure 6**). The pattern of face processing seen in the Broad Autism Phenotype showed striking similarities to that previously reported to occur in autism [3], and for the first time provides a window into the endophenotype that may result from a subset of the genes that contribute to social cognition.

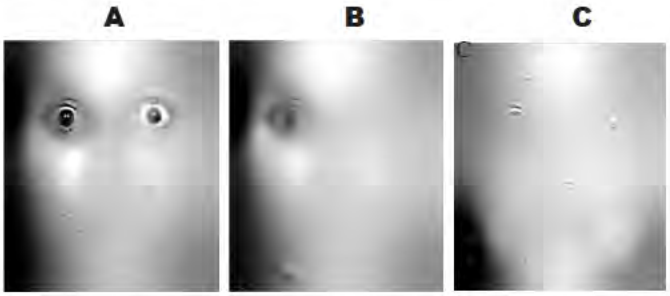


**Figure 6:** Classification images showing the use of facial information. **A**: Controls (parents of a child without autism). **B**: Difference between Controls and aloof parents who had a child with autism (the image shows the region of the face used more by controls). **C**: Difference between Controls and parents who had a child with autism but who were not socially aloof. The regions of the face shown in these images show which parts of the face were used statistically significantly more in an emotion discrimination task.

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**92. Temporal isolation of neural processes underlying face preference decisions**

*H. Kim, R. Adolphs, J.P. O'Doherty, S. Shimojo*

Decisions about whether we like someone are often made so rapidly from first impressions that it is difficult to examine the engagement of neural structures at specific points in time. Here we used a novel temporally extended decision-making paradigm to examine brain activation with fMRI at sequential stages of the decision-making process. Activity in reward-related brain structures, the nucleus accumbens and orbitofrontal cortex, was found to occur at temporally dissociable phases while subjects decided which of two unfamiliar faces they preferred. Increases in activation in the orbitofrontal cortex occurred late in the trial, consistent with a role for this area in computing the decision of which face to choose. Signal increases in the nucleus accumbens occurred early in the trial, consistent with a role for this area in initial preference formation. Moreover, early signal increases in the nucleus accumbens also occurred while subjects performed a control task (judging roundness of the faces), when the data were analyzed on the basis of which of those faces were later chosen as preferred. The findings support a model in which rapid, automatic engagement of the nucleus accumbens conveys a preference signal to the representation of choice in orbitofrontal cortex.

**Reference**
Kim *et al*., 2007.

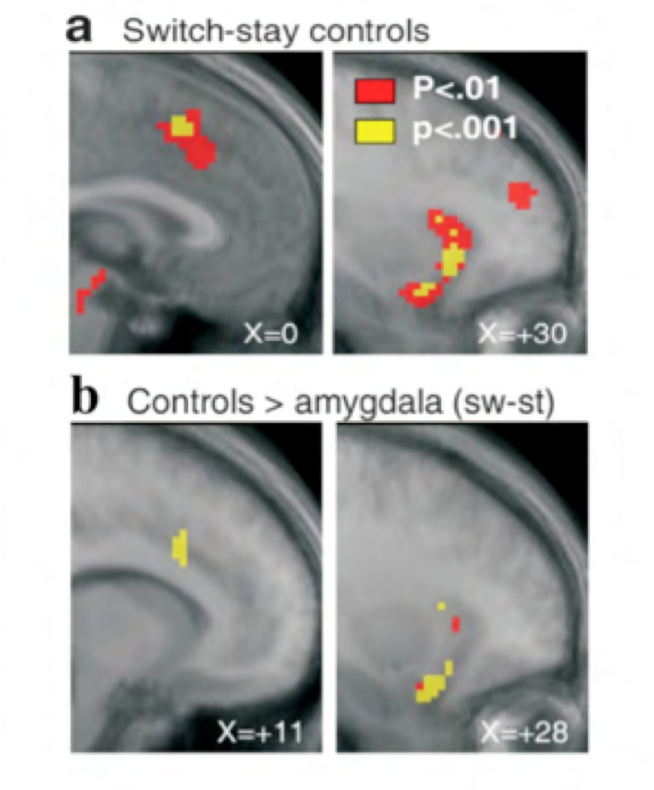


**Figure 7:** Regions of the prefrontal cortex inferred to receive expected reward signals from the amygdala. (a) Regions in the prefrontal cortex of normal individuals that show greater activation when subjects switch on a reversal learning task than when they continue to choose an unrewarded option. These regions thus track changes in the reward expected for a given choice. (b) Within the regions normally activated in (a), there were sub-regions showing significantly greater reward modulation in normal individuals than in the two individuals with amygdala regions. We interpret this finding as showing that these regions track expected reward in part via signals that are normally conveyed from the amygdala.

**93. The amygdala's influence on reward signals in the prefrontal cortex**

*A.N. Hampton, R. Adolphs, J.M. Tyszka, J.P. O'Doherty*

The prefrontal cortex receives substantial anatomical input from the amygdala, and these two structures have long been implicated in associative learning between stimuli and outcomes, as well as between actions and their consequences. Yet little is known about how the two structures interact, especially in humans. We investigated the contribution of the amygdala to reward-related signals in prefrontal cortex in two rare subjects with focal bilateral amygdala lesions. We measured blood oxygenation-level dependent (BOLD) responses using fMRI, while the subjects performed a reversal learning task in which they first had to learn which of two choices was the more rewarding, and then flexibly switch their choice when contingencies changed. Compared to healthy controls, both subjects showed a profound change in BOLD responses in ventromedial prefrontal cortex associated with reward expectation and behavioral choice (**Figure 7**). These findings support a critical role for the human amygdala in establishing expected reward representations in prefrontal cortex, which in turn may be used to guide behavioral choice.

**Reference**
Hampton *et al*., 2007.

**94. Does damage to the prefrontal cortex result in moral judgments that are more utilitarian?**

*M. Koenigs, L. Young, R. Adolphs, D. Tranel, F. Cushman, M. Hauser, A. Damasio*

In a previous study [1] we had tested the hypothesis that patients with lesions to the ventromedial prefrontal cortex make abnormal judgments about moral dilemmas. This hypothesis was based on the abnormal emotional responses seen in such patients, together with a prevalent theoretical view that emotional responses causally contribute to moral judgments that can be counter to the greatest good for the greatest number of people (the "utilitarian" option). For instance, when faced with a dilemma in which one must choose between smothering one's baby to silence its cries, or be found by enemy soldiers in wartime who will kill a larger number of people including the baby if it continues to cry, people are typically torn and sometimes choose not to kill the baby. We had found [1] that lesions to the ventromedial prefrontal cortex resulted in a greater proportion of utilitarian choices on such dilemmas (i.e., they would more frequently endorse killing the baby in order to save everybody else). It had been countered [2] that these findings did not demonstrate a bias specifically for "utilitarian" choices, since those could be defined in different ways. In a rebuttal [3], we re-analyzed our data according to alternate schemes for defining "utilitarian" (as choosing duty versus consequences based on the content of the dilemma) and still obtained results. We concluded that damage to the ventromedial prefrontal cortex, and the reduced emotional response it produces, results in an abnormal propensity to make utilitarian moral judgments, regardless of exactly how "utilitarian" is defined. This work was done in collaboration with the University of Iowa, where the lesion patients were tested.

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**Support**: The work described in the following research reports has been supported by:
The Brown University/NIH
The James S. McDonnell Foundation
Simons Foundation Autism Research Initiative

**Summary**: We have continued our exploration of the neurobiology and neuropathology of the Von Economo Neurons and the connections of fronto-insular (FI) and anterior cingulate cortex (ACC), which contain them. We have also begun a collaboration with the Wold lab in which we are using a new technology, RNA-Seq, to determine the quantitative expression of virtually the complete set of genes in FI, ACC and visual cortex in autistic and control brains.

## 95. Selective reduction of Von Economo neuron number in agenesis of the corpus callosum

*Jason A. Kaufman, Lynn K. Paul[1,2], Kebreten F. Manaye[3], Andrea E. Granstedt[4], Patrick R. Hof[5], Atiya Y. Hakeem, John M. Allman*

Von Economo neurons (VENs) are large spindle-shaped neurons localized to anterior cingulate cortex (ACC) and fronto-insular cortex (FI). VENs appear late in development in humans, are a recent phylogenetic specialization, and are selectively destroyed in frontal temporal dementia, a disease which profoundly disrupts social functioning and self-awareness. Agenesis of the corpus callosum (AgCC) is a congenital disorder that can have significant effects on social and emotional behaviors, including alexithymia, difficulty intuiting the emotional states of others, and deficits in self- and social-awareness that can impair humor, comprehension of non-literal or affective language, and social judgment. To test the hypothesis that VEN number is selectively reduced in AgCC, we used stereology to obtain unbiased estimates of total neuron number and VEN number in postmortem brain specimens of four normal adult controls, two adults with isolated *callosal dysgenesis*, and one adult whose corpus callosum and ACC were severely atrophied due to a non-fatal cerebral arterial infarction. The partial agenesis case had approximately half as many VENs as did the four normal controls, both in ACC and FI. In the complete agenesis case, the VENs were almost entirely absent. The percentage of total neurons in VEN-containing regions that are VENs was reduced in callosal agenesis, while this percentage was normal in FI the stroke patient. These results indicate that the VEN population is selectively reduced in AgCC, but that the VENs do not depend on having an intact corpus callosum. We conclude that in agenesis of the corpus callosum, the reduction in the number of VENs is not the direct result of the failure of this structure to develop, but may instead be another consequence of the genetic disruption that caused the agenesis. The reduction of the VEN population could help to explain some of the social and emotional deficits that are seen in this disorder.

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## 96. DISC1 (disrupted in schizophrenia) is preferentially expressed in VENs

*Nicole Tetreault, John M. Allman*

The product of the gene DISC1 (disrupted in schizophrenia) labels the soma and dendrites of the VENs. Figure 1 illustrates the strong immunocytochemical staining by an antibody to Disc1 of the VEN somas and dendrites in area FI. We found that 90% of the VENs were Disc1-positive but only 37% of the other layer five neurons were positive in subject #1, a 51 year-old male. 94% of the VENs were Disc1-positive, whereas only 34% of the other neurons were positive in subject #2, a 39 year-old male. Both subjects with neurologically normal and died of myocardial infarctions. Thus, Disc1 is selectively expressed in the VENs versus other layer 5 neurons in area FI. Most of the other layer 5 DISC1 positive numbers are compass and mace cells, which appear to be closely related to the VENs. The selective expression of DISC1 on the VEN soma and dendrites has several interesting implications. Duan *et al.* (2007) blocked the expression of Disc1 in postnatally generated neurons in the dentate gyrus of the mouse hippocampus through the use of selective interference of Disc1 RNA. When DISC1 expression is blocked in these neurons, there is considerably more branching of the secondary and tertiary dendrites than when Disc1 expression is normal. Thus, DISC1 reduces secondary and tertiary dendritic branching in these neurons, and DISC1 could be part of the genetic circuitry responsible for the relative absence of higher order branching in the VENs. Crespi *et al.* (2007) showed that several parts of the DISC1 gene have undergone substantial positive selective changes since hominoids diverged from other anthropoids and since humans diverged other hominoids. The site of the largest evolutionary divergence maps to exons 1 and 2 in the part of the DISC1 gene that binds to MAP1a (microtubule associated protein 1a), which is involved in the control of activity dependent dendritic branching (Szebenyi *et al.*, 2005). A single nucleotide polymorphism in another part of the Disc1 gene is linked to increased risks of major depression and schizophrenia, and this polymorphism is also linked to reduced volume of ACC in a large MRI study (Hashimoto *et al.*, 2006). Variations in the DISC1 have been frequently associated with changes in cognitive

functioning, particularly with respect to tasks that involve maintaining and shifting attention (see Chubb *et al*., 2008 for review). Thus, Disc1 is a gene that has undergone considerable recent evolution and may be related to enhanced cognition in humans. DISC1 is expressed selectively on the soma and dendrites of the VENs and may be related to their distinctive dendritic architecture. The recent evolutionary changes associated with the VENs and DISC1 may carry increased vulnerabilities to dysfunction which are manifest in autism, frontotemporal dementia, agenesis of the corpus callosum, depression and schizophrenia (Allman *et al*., 2005; Crespi *et al*., 2007; Seeley *et al*., 2006; Hashimoto *et al*., 2006; Kaufman *et al*., in press. Further investigation of gene expression in the VENs could provide insights into the dysfunctions of neuropsychiatric illnesses and into the recent VEN evolution.

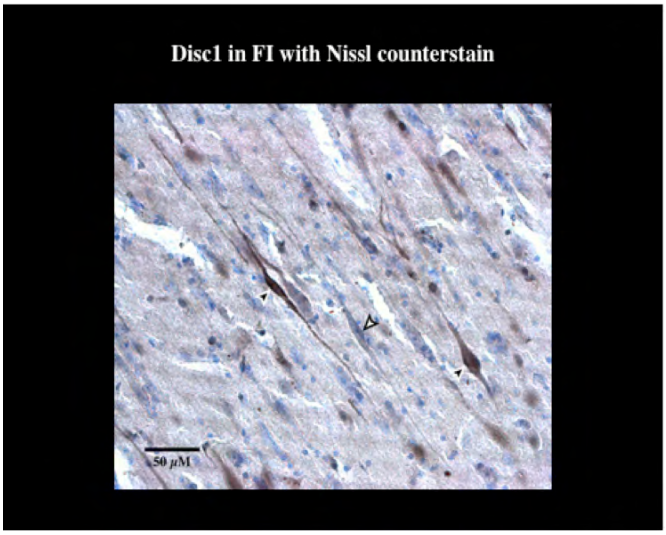


**Figure 1** - The DISC1 immunocytochemically labeled cells are dark brown. Two DISC1 positive VENs are indicated with the solid arrowheads. The section was also counterstained with cresyl violet so that that the blue staining indicates cells which are DISC1 negative. The outline arrowhead indicates a DISC1 negative VEN.

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**97. Von Economo neurons in the elephant brain**

*Atiya Hakeem*

Von Economo neurons (VENs), previously found in humans, all of the great ape species, and four cetacean species, are also present in African and Indian elephants. The VENs in the elephant are primarily found in similar locations to those in the other species. They are most abundant in the fronto-insular cortex (area FI) and are also present at lower density in the anterior cingulate cortex. Additionally, they are found in a dorso-lateral prefrontal area and less abundantly in the region of the frontal pole. The VEN morphology appears to have arisen independently in hominids, cetaceans, and elephants, and may reflect a specialization for the rapid transmission of crucial social information in very large brains.

**98. Mapping the connections of FI and ACC in apes with diffusion tensor imaging**

*Jason Kaufman, Mike Tyszka, John M. Allman*

We mapped the connections of the VEN containing areas, FI and ACC with probabilistic tractography based on the movement of water molecules in fiber tracts assayed with diffusion tensor imaging. The brains of apes who had died of natural causes and provided by Zoo veterinarians were imaged at 7 and 9 Tesla at the Broad Imaging Center using conventional structural imaging and high angular resolution diffusion imaging (HARDI).

The first finding is a connection between ACC and FI. By seeding in the VEN-containing regions of ACC, we are able to reconstruct a pathway that projects ventrally and contacts FI, as shown in the figure X.

The connection between ACC and FI is a pathway we had previously hypothesized (given their commonality of VEN populations), but the diffusion MRI results are the first empirical evidence we have for this pathway.

A second finding is that FI connects to the temporal poles and the inferior temporal cortex, as illustrated in the Figure 2.

A third finding is that both FI and ACC project to the frontal poles. Inferior temporal cortex (IT) is known to function in the recognition and interpretation of faces and facial expressions – functions that are integral to appropriate social behavior.

Finally, both ACC and FI project to the frontal pole (BA10). The frontal pole is known to function in mentalizing (imagination), decision-making, multitasking, and episodic memory retrieval. Moreover, BA10 is thought to have undergone expansion during evolution of apes and humans. Evidence of connections between the frontal pole and the VEN-containing regions of ACC and FI is therefore significant from both a functional and an evolutionary perspective.

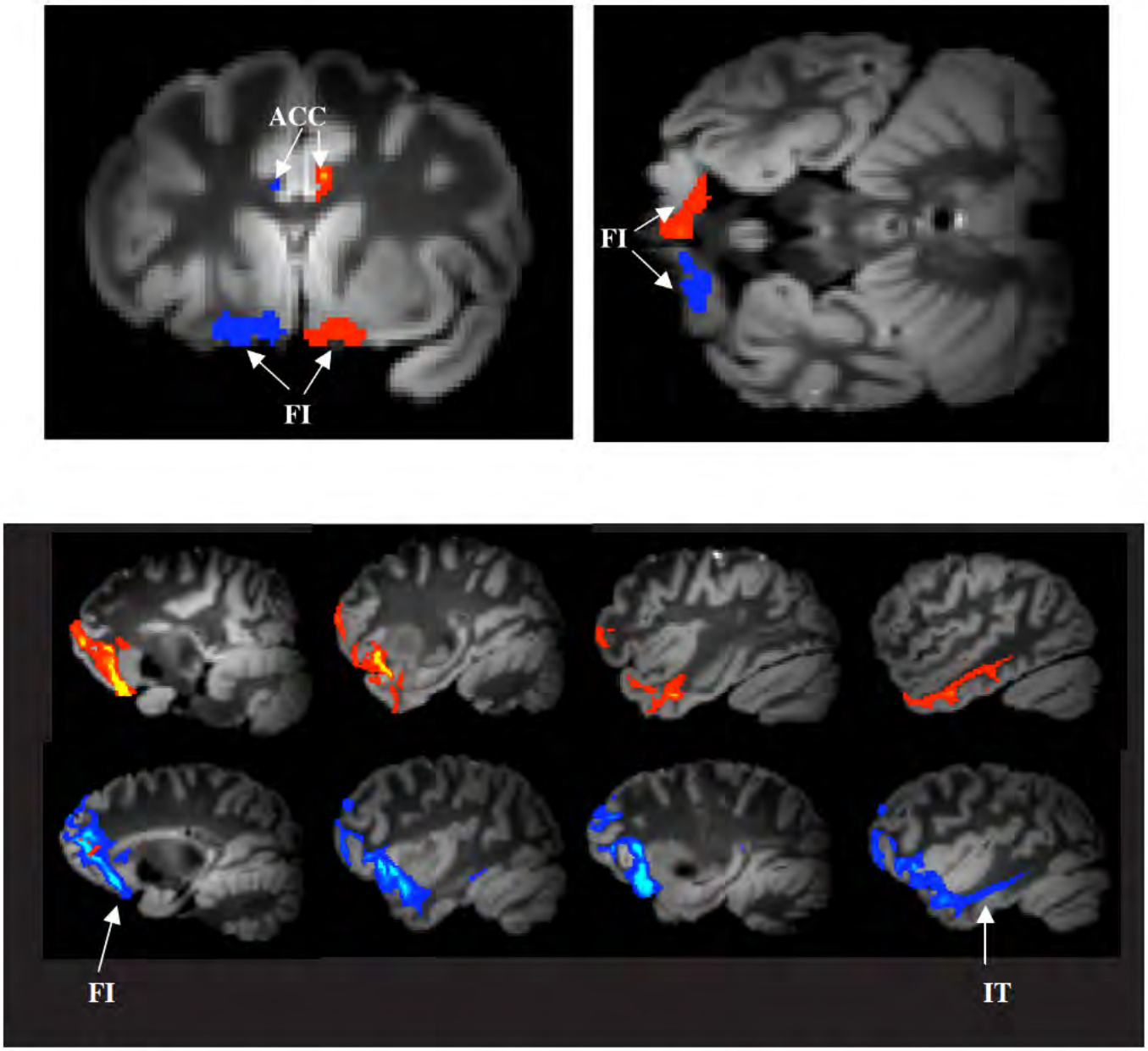


**Figure 2**. The fiber pathway connections of fronto-insular cortex (FI) in the left (blue) and right (red-yellow) hemispheres of a gorilla based on diffusion tenor imaging (DTI). The upper left panel represents a coronal section, the upper right panel a horizontal section, and the lower panel a series of parasagittal sections proceeding from medial (right side) to lateral (left side).

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**Support:** The work described in the following research reports has been supported by:
James G. Boswell Professor of Neuroscience
Defense Advance Research Project Agency (DARPA)
ERATO
McKnight Endowment Fund for Neuroscience
Moore Foundation
National Institutes of Health (USPHS)
Sandia National Laboratories
Sloan Foundation
Swartz Foundation

**Summary:**

*Neural mechanisms for visual-motor integration, spatial perception and motion perception.*

While the concept of artificial intelligence has received a great deal of attention in the popular press, the actual determination of the neural basis of intelligence and behavior has proven to be a very difficult problem for neuroscientists. Our behaviors are dictated by our intentions, but we have only recently begun to understand how the brain forms intentions to act. The posterior parietal cortex is situated between the sensory and the movement regions of the cerebral cortex and serves as a bridge from sensation to action. We have found that an anatomical map of intentions exists within this area, with one part devoted to planning eye movements and another part to planning arm movements. The action plans in the arm movement area exist in a cognitive form, specifying the goal of the intended movement rather than particular signals to various muscle groups.

*Neuroprosthetics.* One project in the lab is to develop a cognitive-based neural prosthesis for paralyzed patients. This prosthetic system is designed to record the electrical activity of nerve cells in the posterior parietal cortex of paralyzed patients, interpret the patients' intentions from these neural signals using computer algorithms, and convert the "decoded" intentions into electrical control signals to operate external devices such as a robot arm, autonomous vehicle or a computer.

Recent attempts to develop neural prosthetics by other labs have focused on decoding intended hand trajectories from motor cortical neurons. We have concentrated on higher-level signals related to the goals of movements. Using healthy monkeys with implanted arrays of electrodes we recorded neural activity related to the intended goals of the animals and used this signal to position cursors on a computer screen without the animals emitting any behaviors. Their performance in this task improved over a period of weeks. Expected value signals related to fluid preference, or the expected magnitude or probability of reward were also decoded simultaneously with the intended goal. For neural prosthetic applications, the goal signals can be used to operate computers, robots and vehicles, while the expected value signals can be used to continuously monitor a paralyzed patient's preferences and motivation.

*Movable probes.* In collaboration with Joel Burdick's laboratory at Caltech, we have developed a system that can autonomously position recording electrodes to isolate and maintain optimal quality extracellular recordings. It consists of a novel motorized microdrive and control algorithm. The microdrive uses very small piezoelectric actuators that can position electrodes with micron accuracy over a 5 mm operating range. The autonomous positioning algorithm is designed to detect, align, and cluster action potentials, and then command the microdrive to move the electrodes to optimize the recorded signals. This system has been shown to autonomously isolate single unit activity in monkey cortex. In collaboration with Yu-Chong Tai's lab and the Burdick lab we are now developing an even more compact system that uses electrolysis-based actuators designed to independently move a large number of electrodes in a chronically implanted array of electrodes.

*Coordinate frames.* Our laboratory also examines the coordinate frames of spatial maps in cortical areas of the parietal cortex coding movement intentions. Recently, we have discovered that plans to reach are coded in the coordinates of the eye. This is particularly interesting finding because it means the reach plan at this stage is still rather primitive, coding the plan in a visual coordinate frame rather than the fine details of torques and forces for making the movement. We have also discovered that when the animal plans a limb movement to a sound, this movement is still coded in the coordinates of the eye. This finding indicates that vision predominates in terms of spatial programming of movements in primates.

We have also been examining the coordinate frame for coordinated movements of the hand and eyes. In the dorsal premotor cortex we find a novel, "relative" coordinate frame is used for hand-eye coordination. Neurons in this cortical area encode the position of the eye to the target, the position of the hand to the target, and the

relative position of the hand to the eye. A similar relative coding may be used for other tasks that involve the movements of multiple body parts such as bimanual movements.

*Motion perception.* Another major effort of our lab is to examine the neural basis of motion perception. One series of experiments is determining how optic flow signals and efference copy signals regarding eye movements are combined in order to perceive the direction of heading during self-motion. These experiments are helping us understand how we navigate as we move through the world. A second line of investigation asks how motion information is used to construct the three-dimensional shape of objects. We asked monkeys to tell us which way they perceived an ambiguous object rotating. We found an area of the brain where the neural activity changed according to what the monkey perceived, even though he was always seeing the same stimulus. In other experiments we have been examining how we rotate mental images of objects in our minds, so-called mental rotation. In the posterior parietal cortex we find that these rotations are made in a retinal coordinate frame, and not an object based coordinate frame, and the mental image of the object rotates through this retinotopic map.

*Local field potentials.* The cortical local field potential (LFP) is a summation signal of excitatory and inhibitory dendritic potentials that has recently become of increasing interest. We report that LFP signals in the saccade and reach regions provide information about the direction of planned movements as well as the state of the animal; e.g., baseline, planning a saccade, planning a reach, executing a saccade, or executing a reach. This new evidence provides further support for a role of the parietal cortex in movement planning. It also shows that LFPs can be used for neural prosthetics application. Since LFP recordings from implanted arrays of electrodes are more robust and do not degrade as much with time compared to single cell recordings, this application is of enormous practical importance.

*fMRI in monkeys.* We have successfully performed functional magnetic resonance imaging (fMRI) experiments in awake, behaving monkeys. This development is important since this type of experiment is done routinely in humans and monitors the changes in blood flow during different cognitive and motor tasks. However, a direct correlation of brain activity with blood flow cannot be achieved in humans, but can in monkeys. Thus, the correlation of cellular recording and functional MRI activation in monkeys will provide us with a better understanding of the many experiments currently being performed in humans. A 4.7 Tesla vertical magnet for monkey imaging has recently been installed in the new imaging center in the Broad building. We are using this magnet, combined with neural recordings, to examine the correlation between neural activity and fMRI signals.

**99. Changing your mind - contributions of primate OFC to self-initiated free-choice behavior**

*Campos, M., Koppitch, K., Andersen, R.A., Shimojo, S.*

The orbitofrontal cortex (OFC) encodes the subjective value of behavioral options, but evidence that OFC encodes behavioral choices is lacking. In this study we investigate how monkey (*macaca mulatta*) OFC neurons support choice behavior in a self-initiated free-choice paradigm. Monkeys were free to press one of two buttons in alternating liquid-reward or video-watching periods. In the liquid-reward period the monkey could choose between apple juice and water. In the video-watching period the monkey could choose to watch the subsequent five second clip of the current video channel or to watch a clip from the next channel. In the video-watching period no appetitive reward was delivered. Each period lasted 20 minutes regardless of the monkey's behavior. Reward contingencies and amounts were stable for a given period, leaving only the changing subjective valuation of the outcomes to drive the monkey to make different choices. We found that while OFC neurons rarely encoded the specific choice that the monkey made, many neurons encoded a behavioral switch. First, we found neurons that predicted and/or detected a switch between runs of a given choice to the other available choice. These neurons would increase in firing rate before and/or after a switch, suggesting that the neuron's activity reflected the decision to switch. However, firing rates did not discriminate between the direction of the switch, such as apple juice to water or water to apple juice, and therefore did not encode the specific behavioral choice. Second, we found neurons that encoded a change in the current behavioral context (video-watching to juice-reward, or vice versa). A third class of neurons was modulated over the course of a task period, possibly reflecting satiety. We speculate that these three types of neurons could flexibly guide behavior based on changing subjective preferences, if the satiety-sensitive neurons were to drive one or both classes of the switch-sensitive neurons. For example, if the monkey were to become bored with the current video channel, the neural firing that encodes subjective valuation would diminish, which would in turn drive the switch-sensitive neurons to fire, ultimately driving the monkey to change the channel. A similar mechanism might account for the monkey choosing to switch from drinking liquid to watching television, though in our current paradigm the experimenter makes the choice to switch from one behavioral context to the next. Overall, our results suggest that in a behavioral paradigm with free, self-initiated choices between well-known outcomes, OFC neurons contribute to decision-making by driving the monkey to switch from one behavioral option to another.

**100. Distinct neural correlates of decision making and motor preparation in the posterior parietal cortex**

*Cui, H., Andersen, R.A.*

Recent neurophysiological studies suggest that potential plans for movements to multiple alternative targets are simultaneously represented in a collection of parietal and frontal areas, indicating that plan selection and movement preparation involve the same brain regions and are performed in an integrated manner, as opposed the traditional psychological theory in which decision making has been considered as a separated neural process occurring before action planning (Cisek, *J. Neurosci.*, 2006). However, this has only been tested for spatial target selection, which involves spatial attention, which in turn engages numerous cortical areas. It still remains unclear whether plan selection and action preparation are represented in segregated areas for other kinds of decision making. The lateral intraparietal area (LIP) and parietal reach region (PRR) of rhesus monkeys has been found to prospectively encode the movement plan while monkeys chose to acquire a target either by saccading or reaching in the absence of direct sensory instructions specifying the effectors (Cui and Andersen, *Neuron*, 2007). In the present study, we extended the non-spatial effector choice paradigm to examine single-cell activity in dorsal area 5 (area 5d) and compared it to activity in the LIP and PRR. Both LIP and PPR showed vigorous responses when effector information was ambiguous, and activity was maintained elevated only if their own preferred motor plans (saccade and reach, respectively) were chosen after the monkey selected a particular movement. In contrast, area 5d did not show any response to the stimulus and its activity was not elevated until the effector was unambiguously specified as the arm, so area 5d does not appear to encode potential action plans. Apparently, LIP and PRR neurons encode both alternative plans and the decision outcome, whereas cells in area 5d only reflected reach plan selected by upstream brain areas. Furthermore, the analysis of the time courses demonstrated that the activity in the area 5d became significantly elevated about 100 ms after the PRR population showed significant differential activity for saccade and reach chosen trials. Nevertheless, the same population of area 5d neurons began to fire right after target onset during the classical delayed reach trials in which the stimulus was indicated as the reach target at the beginning. The absence of activity related to potential plans in area 5d suggest a distinct neural correlate of motor preparation downstream to plan selection in favor of the serial hypothesis that decision making occurs before action planning. This result suggests there are different neural dynamics for non-spatial effector choice and spatial target selection.

**101. Decoding instantaneous hand position from posterior parietal cortex in a natural task**

*Hauschild, M., Mulliken, G.H., Loeb, G.E., Andersen R.A.*

Posterior parietal cortex (PPC) is known to be involved in the sensorimotor transformations required during planning and execution of reaching movements. During the delay period of a memory guided reach task, the parietal reach region (PRR) encodes reach targets in a gaze-centered reference frame. Recently it has been shown that during the execution of reaching movements in a 2D center-out-task, PRR contains information about the instantaneous hand position. We explored whether information about the intended hand trajectory is similarly represented in the activity of PRR and area 5 neurons in a more realistic task under unconstrained gaze conditions. Monkeys (*Macaca mulatta*) were instructed to acquire randomly placed reach targets in a 3D cubic virtual reality workspace. First, PRR and area 5 spiking activity was examined during the execution of movements, and later the results were used to obtain an online decode model for closed loop feedback brain control tasks where the animal was required to guide a cursor using the neural activity only, without being allowed to move his limb. Despite free gaze conditions, we were able to decode the current state of the hand in 3D, using linear regression and Kalman filter algorithms in a screen-centered reference frame. Throughout the brain-control phase of the experiment, we observed a gradual improvement of the monkey's ability to acquire targets successfully over time. This increase in behavioral performance was accompanied by a change of the neural ensemble's tuning properties. Furthermore, we observed similar learning effects when the decode was intentionally perturbed by imposing artificial dynamics (a robotic arm) on the decode-driven cursor. Despite poor initial behavioral performance, the monkey was able to learn the imposed robotic arm. Our results indicate that the decoding of instantaneous hand position in 3D workspace with a rich set of non-center-out targets is feasible under unconstrained gaze conditions. Neural plasticity resulted in gradual but substantial performance improvement, even in the case of artificially imposed dynamics. The fact that the targeted brain regions are known to rely on gaze centered reference frames (PRR) or a combination of gaze and hand centered coordinates (area 5) to encode reach-targets during the delay period of a memory guided reach, raises the question whether for the encoding of the instantaneous hand trajectory, the neural ensemble under investigation relies on gaze centered reference frames, or an alternate coordinate system such as body-centered.

**102. Integrated parylene-cabled silicon probes for neural prosthetics**

*Huang, R., Pang, C., Tai, Y.C., Emken, J., Ustun, C., Andersen, R.A., Burdick, J.*

Recent advances in the field of neural prosthetics have demonstrated the thought control of a computer cursor. This capability relies primarily on electrode array surgically implanted into the brain as an acquisition source of neural activity. Various technologies have been developed for signal extraction; however most suffer from either fragile electrode shanks and bulky cables or inefficient use of surgical site areas. We have designed and tested high electrode density, silicon-based array system with an integrated parylene cable. The greatly reduced flexible rigidity of the parylene cable is believed to relief possible mechanical damages due to relative motion between a brain and its skull.

**103. Brain-control of movement execution time using LFP in PRR**

*Hwang, E.J., Andersen R.A.*

One important question in cortical motor prosthetics is when to execute a decoded movement plan. A recent study from the parietal reach region (PRR) suggested that the local field potentials (LFPs) in this area might be useful in decoding the execution time information because of the striking difference in the LFP spectrum between the plan and execution states. More specifically, the LFP power in the 0-10 Hz band sharply rises while the power in the 20-40 Hz sharply falls as the state transition from plan to execution. However, in that study, the movement onset was time-locked to the extinction of a visual stimulus, raising a possibility that the spectrum reversal might be driven by the visual event instead of the movement onset per se. To rule out this possibility, we examined the LFP signal in PRR while the primate subjects were performing self-paced delayed reaches in the absence of visual stimulus change. The LFP spectrum reversal was still time-locked to the movement onset in the self-paced reaches, rejecting the visual response hypothesis. Furthermore, we successfully trained the subjects to utilize the LFP spectrum reversal as a "go" signal in a closed-loop, brain-control task in which the limb did not move. The execution time was signaled by the change in the LFP spectrum while the spike firing rate was used to control the position of the cursor. The results corroborate that the LFP spectrum reversal in PRR is a robust indicator for the movement onset and can be trained for control the execution time in a cortical motor prosthesis.

**104. The parietal reach region represents the spatial goal in symbolically instructed reaches**

*Hwang, E.J., Andersen, R.A.*

The parietal reach region (PRR) located in the dorsal visual pathway is thought to form an abstract reaching goal using the spatial information of a target object. Then, one intriguing question is whether PRR still represents the reaching goal when the goal must be computed using non-spatial information, e.g., shape or color. To this end, we examined PRR spiking and LFP activity during a symbolically instructed reaching task wherein the subject reached to the peripheral location at which a centrally-located arrow pointed in the absence of any visual stimulus at the target location. To compare, we also performed a spatially instructed reaching task in which the subject reached to a visual target. In both tasks, the majority of neurons and LFPs were spatially tuned to the upcoming reaching target location with the similar tuning strength and preference. However, the spatial tuning arose earlier by approximately 100 ms in the spatially instructed reaching task, implying that extra computational steps were involved in the symbolically instructed reaching task. These findings suggest that PRR encodes the spatial goal of reach regardless of whether the goal is instructed by an explicit spatial cue or an arbitrarily-associated, non-spatial cue.

Furthermore, we successfully trained the subject to modulate both the spiking and LFP activity based on the symbol instruction in a closed-loop brain-control task in which a cursor is positioned by decoding neural activity in the absence of arm movements. The subject could sustain performance accuracy at 85% over 400 trials. In sum, we report that PRR invariably represents the spatial goal of the upcoming reach and serves as an ideal high-level brain area from which the spatial goal of movement can be robustly decoded for motor prosthetic applications.

**105. Neural signatures of choice-overload and choice set-value in human posterior parietal cortex**

*Lindner, A., Reutskaja, E., Nagel, R.C., Andersen, R.A., Camerer, C.F.*

Whereas classical economics argues that more choice is always beneficial, recent studies indicate that large choice sets can be demotivating and lead to "choice paralysis." We investigated the neural bases of these phenomena by providing twenty subjects with increasing numbers of choice alternatives (N=6, 12 or 24 items) while measuring time-resolved brain activity using event-related fMRI. Subjects faced different-sized choice sets of landscape photographs from which they had to choose their most preferred one. One of these choices was then used to produce a consumer product with an imprint of the respective photograph (e.g., a mug, a T-shirt, etc.). Subjects generally rated the smaller sets as having too few items and the larger sets as having too many, i.e., subjective value of the choice set was an inverted U-shaped function of the number of choice alternatives. All said choosing from the larger sets was more difficult. Preparatory fMRI activity (i.e., activity which preceded the actual choice) increased with such perceived choice difficulty in the anterior cingulate and dorsal premotor cortex. Areas exhibiting fMRI activity that was rather correlated with the subjective value of the choice set were mapped within posterior parietal cortex, namely SPL. Yet,

large choice sets can also be pleasing when they include a highly-preferred item. This was implied by increased levels of fMRI activity within the striatum, in comparison to trials where all the available alternatives were similarly preferred. This pattern of fMRI activity provides the first insight into how the brain combines the quality of choices from a set with the difficulty of making these choices into a signal that can be interpreted as the value of a choice set. Specifically, posterior parietal cortex seems to represent such subjective set-value and thus might play a major role in decision processes.

**106. Encoding and decoding information by the phase of action potentials**

*Nadasdy, Z., Andersen R.A.*

Experimental evidence, such as task-dependent coherency between single-unit activity and local field potentials (LFPs), together with the dependency of action potential (AP) initiation on the subthreshold membrane oscillation (SMO) suggest that: *i*) the probability of action potentials is controlled by a common oscillatory mechanism; *ii*) the SMOs across individual neurons are not independent but rather form a coherent field of oscillations; and iii) nearly-synchronized SMOs may propagate through neuronal connections, creating a constant-phase gradient of SMO between neighbor neurons. Based on these assumptions, we formulated a model in which neurons encode information by the phase of APs relative to the SMO. The model consists of four stages: encoding with phase, gamma alignment, information transfer, and reconstruction. We demonstrated by means of simulations that information encoded by the phase of APs can reliably be transferred and reconstructed at distant target areas. Moreover, since the phase code is a compressed representation of the spatio-temporal features of the stimulus, it enables the transfer of information in parallel pathways without distortion from conductance differences. We illustrate by examples how phase coding may account for a number of unresolved physiological observations related to sparse coding, motion processing, phase precession, and invariance detection, as well as anatomical principles, such as the columnar organization and grid cell architecture. Furthermore, we show empirical evidence for stimulus-dependent phase coding in V1 from simultaneous single-unit and LFP recordings.

**107. Spike field coherence and cortico-cortical communication**

*Stetson, C., Nelson, M., Cui, H., Andersen, R.A.*

How do different parts of the brain communicate with each other? Perhaps spikes synchronize with large-scale oscillatory signals in order to communicate more effectively over long cortical distances. Recent data from our laboratory have shown that when a monkey freely chooses a reach target among several alternatives, cells in the dorsal premotor cortex (PMd) become coherent with local field potentials (LFPs) in the parietal reach region (PRR) at low frequencies (10-15 Hz), and vice versa. Does this increased spike-field coherence represent selective communication between spikes in one lobe of the brain, and LFPs in another? One way to look at this question is to determine whether larger-scale signals, such as LFPs, between lobes also show increased coherence. Partial coherence analysis has previously shown that this spike-field coherence between areas exceeds what would be expected by the within area spike-field and between-area field-field coherence. In fact, we show here that the average magnitude of the field-field coherence between PMd and PRR actually decreases almost at the same time that the spike-field coherence increases. We find a similar relationship in a related decision-making task, where the monkey chooses whether to reach or saccade toward a target (an effector-choice task). These data suggest rich cortical dynamics during decision-making, whereby a neuron in one cortical area can selectively synchronize with a distant cortical area, even as the fields become desynchronized between the same two cortical areas.

**108. Circuit structure of the Parietal Reach Region is linked to temporal dynamics learning**

*Torres, E.B., Buneo, C.A., Cui, H., Andersen, R.A.*

It has been known that primates decouple the spatial and the temporal components of the movement trajectories during natural learning but how neurons in the arm system learn the timing of a new reach is unknown. Movement dynamics and motor learning have thus far been exclusively studied in relation to motor regions. This work instead investigated a more sensory-motor area, the posterior parietal reach region (PRR). We assessed the role of this area in the learning of new temporal dynamics as a new motor memory was being acquired by rhesus monkeys. They were required to navigate in the dark along new arm motion paths towards remembered targets while avoiding the remembered locations of obstacles. Comparisons were made between over-trained straight reaches without obstacles and the new, curved reaches with obstacles. These experiments also dissociated between activities related to arm postural changes prior to the curved movements and activity related to the learning of the timing of these new movements. Prior to movement, in the planning period that lasted about 1 second, the responses of the neurons segregated into two complementary response types during the learning phase: those that showed a reduction in firing rate and those that showed an increase. These two response types directly correlated with their spike-widths. Broad-spike neurons had reduced firing rates during learning and narrow-spike neurons had increased firing rates. Upon learning completion the PRR cells usually returned to the same firing rates for both the curved and straight trajectories. Since broad and narrow spiking cortical neurons have been suggested to correspond to output neurons and inter-neurons, this work suggests a circuit structure within the PRR that is directly linked to temporal dynamics learning.

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**Graduate Students**: Anne C. Hergarden, Christian Hochstim, Liming Wang, Suzuko Yorozu
**Joint appointment with Professor Pietro Perona, Engineering and Applied Science, Caltech*

**Support:** The work described in the following research reports has been supported by:
Beckman Institute
California Institute of Regenerative Medicine (CIRM)
Helen Hay Whitney
Howard Hughes Medical Institute
Human Frontier Science Program
Jane Coffin Childs Memorial Research Fund
Merck
NARSAD
National Institutes of Mental Health
National Institutes of Health
National Science Foundation
Pritzker Neurogenesis Research Consortium

**Summary**: There are currently three areas of investigation in this laboratory: the development of neural stem cells; the functional neuroanatomy of emotional behaviors in mice; and the functional neuroanatomy of innate behaviors in *Drosophila*.

**Neural stem cell biology**
Stem cells are multipotent, self-renewing progenitor cells. In the central nervous system (CNS), neurons, astrocytes and oligodendrocytes-the three major cell classes of the CNS-are thought to be derived from self-renewing stem cells. We have recently shown that astrocytes are much more heterogeneous than previously appreciated and exhibit positional heterogeneity in the spinal cord, similar to interneurons (Hochstim *et al.*, 2008). Current work is aimed at identifying genes that control the apparently irreversible switch from neurogenesis to gliogenesis. An understanding of this mechanism might eventually permit controlled reversion of adult glial cells to a stem cell-like state, which could be useful for therapeutic purposes.

**Neural circuitry of behavior**
We are developing and applying molecular biological tools to map and manipulate the neural circuitry underlying emotional behaviors, in mice. These studies focus on two distinct but related states: pain and fear. Our studies of pain are based on our discovery of a novel family of G protein-coupled receptors (GPCRs) for neuropeptides, called Mrgs, which are specifically expressed in subsets of primary nociceptive sensory neurons. Using homologous recombination in embryonic stem cells, we have marked the neurons that express different Mrgs with genetically-encoded axonal tracers. Remarkably, different Mrg-expressing neurons project to different, and highly specific, peripheral target tissues (Zylka *et al*., 2005; Liu *et al*., 2007). We are now engaged in genetic inactivation or killing of these neurons, as well as genetic activation, to understand their function. We are also developing new methods to trace the higher-order projections of these neurons into the brain, to determine the point at which these novel and distinct sensory circuits converge.

In a separate project, we have identified genes that are expressed in subpopulations of neurons in the central nucleus of the amygdala, which is thought to be a major output structure that coordinates different aspects of learned fear responses. Using the promoters of these genes, we are generating mice in which the activity of these neurons can be reversibly suppressed using genetically encoded neuronal silencing methods developed in collaboration with Henry Lester's laboratory (Lerchner *et al*., 2007). The role of these neurons, and the circuits in which they participate in mediating responses to conditioned and unconditioned fearful stimuli, can then be assessed.

In parallel with these studies in mice, we are engaged in conceptually similar experiments in the fruitfly, *Drosophila melanogaster*. Our goal is to identify simple and robust innate behaviors, and then perform unbiased "anatomical" and genetic screens to map the neuronal circuits and identify the genes that control these behaviors. This dual approach will provide an opportunity to integrate molecular genetic and circuit-level approaches to understanding how genes influence behavior. The "anatomical" screen exploits the availability of "enhancer trap" lines, in which the yeast transactivator protein GAL4 is expressed in specific subsets of neurons, and a conditional (temperature-sensitive) neuronal silencer gene ($shibere^{ts}$) that prevents synaptic transmission. Currently, we have developed assays for an innate avoidance response triggered by an odorant mixture released from traumatized flies (Suh *et al*., 2004), as well as for aggression (Wang *et al*., 2008). In collaboration with Professor Pietro Perona in the Division of Engineering and Applied Sciences, we are developing machine vision-based methods for automated scoring of aggression in flies, which will permit high-throughput screening for mutations and circuit-level manipulations that affect this social behavior.

**109. Automated monitoring and analysis of social behavior in *Drosophila***
*Heiko Dankert, Pietro Perona, David J. Anderson*

We have developed a methodology for automated high-throughput screening for social behaviors in *Drosophila*. A system for automatic detection and classification allows us to quantify and analyze objectively and reproducibly the effects of genetic and neural circuit-level manipulations on behavior. This in turn provides important new insights into the genetic and neural control of behavior.

The technique is based on video monitoring of interacting fly pairs. Accurate and detailed information on position, shape, and movements of interacting flies are extracted by machine vision. These features are used for detection, analysis and recognition of simple and complex behaviors. Examples of simple behaviors are locomotion, chasing, circling, copulation, and wing extension. Complex behaviors are subdivided into two groups: aggression and courtship. Aggression is more variable in the laboratory, but less studied. It is analyzed by detecting lunging, tussling, and wing threat, three unique behavioral acts between male flies. Courtship is analyzed by combining simple behaviors in a stereotyped sequence of male behaviors including chasing, orientation towards the female, circling, and more complex behaviors like wing vibration (courtship "song"), and finally copulation. These behaviors are usually manually scored, which is both laborious and subjective.

The next step is the fully unsupervised discrimination between behaviors, and the detection of new behaviors. This will provide important new insights into the genetic and neural control of behavior - on a level not previously achieved.

**110. Genetic dissection of amygdala neuronal circuitry for fear and anxiety in mice**

*Prabhat S. Kunwar*

An animal's survival depends on its capacity to identify which sensory stimuli are dangerous to its existence, and its ability to produce the appropriate responses to avoid or combat harmful stimuli. The neural circuitry that controls fear, which can be learned or innate, and anxiety govern these critical judgments. In human, dysfunctions of this circuitry are thought to result in different diseases such as chronic anxiety, PTSD depression, and autism.

The brain region that is linked to these diseases, and which plays a crucial role in regulating fear and anxiety, is the amygdala, an almond-shaped structure located in the medial temporal lobe of the forebrain. Precisely identifying the structure and function of the neural circuit located in the amygdala is essential for understanding how fear and anxiety occur in the normal brain, what goes wrong in the amygdala of affected people, and how appropriate treatments can be developed against fear disorders.

Out of its various nuclei, the central amygdala (CeA) plays a central role in these fear behaviors. My goal is to precisely determine the roles of different neuronal subpopulations in the CeA and to identify the neural circuits that control these behaviors and disorders. In these experiments, I plan to use molecular genetic tools of neuronal silencing and activation to modulate electrical activities of these neurons to analyze their effects on the behaviors in mice. Furthermore, I will use the genetic methods of trans-synaptic tracers to determine the anatomical and functional relationship of CeA neuronal subpopulations, in order to achieve a circuit level explanation for the behavioral phenotypes that are caused by *in vivo* functional perturbations of these subpopulations.

**111. Neuronal control of locomotor activity in the fruitfly**

*Allan M. Wong, Michael H. Dickinson, David J. Anderson*

The fruitfly when presented with various stimuli – be it visual, olfactory, thermal or mechanical, responds with a change in behavior. These behaviors are accomplished through a coordinated set of movements by the appendages. We are interested in how and where in the brain these different stimuli are integrated and how the signals propagated to the muscles that move the appendages. In particular we wish to understand if the neurons that transmit these signals from the brain to the thoracic ganglia carry multimodal commands from prior integrated sensory information or if they carry unimodal commands that represent discrete channels for sensory to motor action.

We have developed a tethered walking fly arena in which a tethered fly walks on top of a floating Styrofoam ball. This experimental setup simulates walking behavior while keeping the fly stationary, permitting tightly controlled stimulus-response experiments that can be coupled with functional imaging and electrophysiological recordings to monitor neural activity. We also developed a preparation where we remove the cuticle from the fly head to permit two-photon imaging in the fly brain. More recently, we used Channelrhodopsin, a light activated ion channel, to activate neurons to elicit walking while the fly is on the ball. We use a pulled fiber optic cable to deliver light locally to different population of neurons in the antenna and the brain to elicit turning in the tethered fly.

**112. Connectivity and function of lateral septal Crhr2+ neurons**

*Todd Anthony, David J. Anderson*

Although several brain regions have been implicated in regulating anxiety, the specific neural circuits involved remain poorly understood. The identification and analysis of these circuits is therefore a fundamental first step towards the development of improved treatments for anxiety disorders. The lateral septum (LS) is one brain region that has long been thought to be involved in controlling anxiety. However, it is unclear whether the LS is primarily anxiolytic or anxiogenic, or perhaps comprised of multiple classes of neurons each with a unique effect on anxiety. Answering this question will require systematically manipulating defined LS neuronal populations in a precise and reproducible manner. One particular population likely to be relevant to analysis of the LS role in anxiety are neurons that express the corticotropin-releasing hormone receptor 2 (Crhr2); genetic data has demonstrated that Crhr2 modulates behavioral responses to stress and anxiety, and pharmacological studies have shown that these effects are due at least in part to Crhr2-expressing

neurons within the LS. However, despite strong evidence implicating LS Crhr2+ neurons in regulating anxiety, neither their synaptic connections nor exact function are known. We are addressing these questions using genetic tools to trace the connectivity and manipulate the firing properties of LS neurons that express Crhr2. By restricting analysis to neurons expressing Crhr2, these experiments will yield highly specific results about an anatomically and genetically defined neuronal population; such information is a prerequisite in the development of targeted anxiolytic drugs.

**113. Transcriptional control of gliogenesis and translational applications to glial based malignancies**

*Ben Deneen, Hae-Ri Song*[*]*, David J. Anderson*

A fundamental rule in the developing CNS is that neurons are generated prior to the generation of glial cells. As a means of identifying the molecular mechanisms associated with the "gliogenic switch," I utilized gene expression profiling (GEP) of prospectively isolated progenitor populations from embryonic timepoints encompassing this developmental interval. *In situ* hybridization and immunostaining indicated that Nuclear Factor I-A and B (NFIA and NFIB) are expressed throughout the dorso-ventral extent of the ventricular zone (VZ), co-incident with the induction of the glial specific marker GLAST. Gain- and loss-of function analysis in the embryonic chick spinal cord revealed that NFIA is necessary and sufficient for the specification of glial identity. Interestingly, my data also indicated that NFIA expression is required for the continued inhibition of neurogenesis, such that in the absence of NFIA there is a loss of progenitors and an extended period of neurogenesis in the VZ. These data indicate that NFIA function links the two fundamental molecular processes governing the gliogenic switch--the inhibition of neurogenesis and the specification of glial identity. Investigation of NFIA/B expression during glial cell differentiation revealed that NFIA/B are highly expressed in differentiated astrocytes. Gain- and loss-of function analysis revealed that NFIA/B is also necessary and sufficient for astrocyte differentiation. Interestingly, this pro-astrocytic function of NFIA/B is antagonized in oligodendrocyte progenitors by Olig2. Currently I'm investigating the biology surrounding NFI gene function during gliogenesis. These studies involve identifying the mechanisms that govern the specific spatial and temporal pattern of induction in the VZ at the onset of gliogenesis, the identification of NFI target genes during gliogenesis, and the nature of the cross-antagonism between NFI genes and Olig2, and how this antagonism may influence the differentiation of oligodendrocyte progenitors.

Another set of studies involves translating knowledge of the basic mechanisms that control gliogenesis to improving our understanding of glial malignancies of the CNS. Many of the markers that are normally expressed in glial cells of the CNS are also expressed in gliomas, glial derived malignancies. Given this connection between normal and malignant glial cells, I investigated whether NFIA is expressed in various sub-types and grades of gliomas. I discovered that NFIA is expressed to a varying extent in different grades of astrocytoma and is not highly expressed in oligodendrogliomas; this finding is consistent with the observation that NFIA is preferentially expressed in the astrocyte lineage over the oligodendrocyte lineage during spinal cord development. Given the difficultly in differential diagnosis of glioma subtypes and grades, these observations could prove to be of diagnostic value. Preliminary functional experiments utilizing genetically modified glioma tumor cell lines transplanted into the mouse brain have revealed that these genes play a critical role in tumorigenesis. I'm currently performing these functional experiments using a genetically defined stem cell model of glioma.

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**114. Modulation of fear behaviors in the central amygdala**

*Wulf Haubensak, David J. Anderson*

Fear is probably the most conserved emotion, underlying defensive behaviors across species, and, in turn, it is a basic, medically important, human emotion that can be addressed in experimentally tractable animal model systems. Numerous studies have pointed to a central role of medial temporal lobe structures, particularly the amygdala, in various forms of fear. However, the surgical and pharmacological methods used were too coarse to study single neuronal circuits with cellular resolution. This is especially important when investigating how fear stimuli are processed in the different amygdala subregions, and the different neuronal populations therein. Among these, neurons in the central nucleus (CeA), which relays signals to lower brain regions activating the release of stress hormones, autonomic and motor responses, can be expected to play a central role in fear processing. Here, we explore a genetic strategy in mice to analyze function and circuitry of neurons with higher resolution.

We have selected *PKC-delta*, which is strongly expressed in the CeA, as region specific gene for genetic targeting of CeA neurons. We generated *PKC-delta* transgenic mice expressing one of the two subunits of a chloride channel for silencing (Slimko *et al., J. Neurosci.* **22**:7373), and delivered the second subunit by stereotaxic injection of Adeno-associated virus into the CeA, reconstituting the functional channel only in *PKC-delta* neurons. This allowed us to selectively silence *PKC-delta* cells in a temporally defined manner and investigate their function in Pavlovian fear conditioning. We found that these neurons integrate top-down stress coping signals from the prefrontal cortex and stress signals from within the amygdala, and negatively regulate fear by inhibiting amygdala output neurons. This inhibitory gating of amygdala activity keeps emotions in check. It prevents emotional overreactions and mediates the extinction of fear

memories - which bears important biomedical implications with regard to potential treatment of stress disorders.

**115. The role of neurosecretory cells in the modulation of *Drosophila* behavior**

*Timothy Tayler, Anne Hergarden, David J. Anderson*

Animals exhibit countless complex and stereotyped behaviors such as aggression, courtship and the fight or flight response. These behaviors are generated and modulated by neural circuits. Other than a few simple reflex circuits, relatively little is known about how these circuits generate appropriate behaviors. *Drosophila* exhibits complex behaviors, but are anatomically less complicated and genetically more tractable than many vertebrate model systems. In addition, flies have a highly developed set of molecular tools that can be used to manipulate and analyze specific cell populations. Additionally, previous studies have demonstrated that *Drosophila* can be used to successfully identify neural correlates underlying complex behaviors such as courtship, olfactory aversion and learning and memory.

The goal of this project is to elucidate the connectivity, function, and modulation of circuits that underlie *Drosophila* behavior. Neuropeptides are an important class of signaling molecules that are involved in various aspects of animal physiology and behavior. To gain genetic control over neuropeptide-producing neurons we have identified the putative regulatory regions of 17 neuropeptide genes and have generated transgenic animals that express the GAL4 protein in the same pattern as these neuropeptides. The Gal4/UAS system is a genetic tool that enables us to express a large variety of transgenes in a spatially and temporally controlled manner. We have used fluorescent reporters to characterize these neuropeptide-Gal4 lines and have also validated the Gal4 expression patterns. We are currently using these newly generated tools to try to learn about the role of neuropeptidergic neurons in modulating behaviors. This is accomplished by using the neuropeptide-gal4 lines to express molecules that can either silence or activate neurons. We are now testing these circuit-modified animals in a wide variety of behavioral paradigms including feeding behavior, courtship, and circadian rhythm.

**116. Sensitization and modeling emotional responses in *Drosophila***

*T.L. Lebestky, David J. Anderson*

Emotional behaviors in humans convey a positive or negative response to a stimulus, and this response is typically expressed in highly conspicuous ways, such as stereotyped facial expressions and graded changes in levels of arousal. Although fruit flies (*Drosophila melanogaster)* do not present the richness of human emotions in their behavior, they may share fundamental molecular similarities that could allow us to dissect the way that neural circuits function to provide graded responses in arousal, as measured quantitatively. To this end, we are developing automated, high-resolution behavioral assays that will allow a reproducible characterization of behavioral responses to aversive stimuli for high-throughput genetic screens.

One such assay follows the startle effects on locomotion and escape behaviors in response to a series of air-puffs, delivered at regular intervals. We observe a reproducible escalation of locomotor activity as a function of time and puff number. The initial results suggest that the animals show a robust and sustained increase in locomotor hyper activity upon receiving a train of stimuli, but a sustained response is not observed upon the presentation of a single stimulus.

Our interpretation of the results is that the presentation of the puff stimuli raises the animals' state of acute arousal, as manifest in a high, sustained locomotor response. We have performed pilot genetic screens to isolate and characterize insertional mutants and potential neural circuits that mediate this startle behavior, and are currently sorting through putative mutations. Two of these promising mutants are alleles in a dopamine receptor and also an allele in the *Drosophila* homolog of the Tachykinin receptor.

Our acquisition of the locomotor data is dependent on a video-tracking program that was developed jointly between the Perona and Anderson labs. The first generation tracking program is capable of accurately following many flies simultaneously (over 100) and generates basic velocity and trajectory data. We are currently improving and extending the basic program to provide the orientation of the animals to each other and higher forms of interaction.

**117. Roles of Mrgprd-expressing neurons in nociception**

*Hyosang Lee, Liching Lo, Mark J. Zylka, Shannon Shields*[*]*, Allan I. Basbaum*[*]*, David J. Anderson*

In mammals, a gentle touch or painful pinch is primarily detected by specialized somatosensory neurons in the skin. Our laboratory has previously identified a family of G protein-coupled receptors called Mas-related G-protein-coupled receptors (Mrgprs), of which Mrgprd is specifically expressed in a subset of sensory neurons that detect noxious stimuli. Surprisingly, axonal tracing experiments have revealed that Mrgprd -expressing fibers terminate only in the skin (both hairy and glabrous), and nowhere else in the body. To investigate the physiological roles of Mrgprd-expressing neurons, we have generated transgenic mice that express the diphtheria toxin receptor specifically in Mrgprd-expressing neurons ($Mrgprd^{DTR}$ mice), allowing us to achieve efficient inducible ablation of Mrgprd-expressing neurons by administering diphtheria toxin to the transgenic mice. Wild-type and transgenic mice displayed normal viability, overall appearance, and body weight both before and after diphtheria toxin injection. Our histological analysis of the transgenic mice showed that peripheral and central projections of Mrgprd-expressing neurons were completely eliminated, and cell bodies in the dorsal root ganglia were virtually ablated

after toxin treatment. In preliminary behavioral tests, Mrgprd$^{DTR}$/DTX-treated mice exhibited significantly reduced mechanical sensitivity, compared to DTX-treated wild-type littermates, as measured both by the percentage of withdrawal responses and mechanical threshold, suggesting that Mrgprd-expressing neurons are essential for mediating detection of mechanical stimuli. In contrast, Mrgprd$^{DTR}$/DTX mice exhibited no deficits in their behavioral sensitivity to noxious thermal stimuli, indicating that sensitivity to noxious heat is preserved in the absence of Mrgprd-expressing neurons. Taken together, our results indicate that Mrgprd$^{DTR}$ mice exhibit a specific deficit in mechanosensation, suggesting that the Mrgprd-expressing subpopulation of sensory neurons plays modality-specific role in behaviorally relevant somtosensation.

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**118. Genetic manipulation of MrgD neural circuits**

*Liching Lo, David J. Anderson*

MrgD is expressed in a subset of non-peptidergic, IB4$^{+}$ DRG neurons. In the dorsal horn, these neurons project to a distinct lamina layer IIm. In contrast, the CGRP$^{+}$ peptidergic neurons project to lamina layer I. It is therefore important to genetically ablate or silence either population to compare their contributions to pain sensation. I have made knock-in mice with a diphtheria toxin receptor (DTR) targeted to the MrgD locus (D-DTR mice). Mice or rats are insensitive to diphtheria toxin (DTX), therefore injection of DTX to the D-DTR mice will only ablate the DTR expressing MrgD neurons. To avoid compensation for behavioral deficits from other type of neurons/mechanisms, as reported by studies using IB4-saporin conjugates, I have tested several conditions, such as, DTX dosage, frequency of injection, etc., to optimize the ablation protocol for behavioral studies. The specificity of ablation was also confirmed by double labeling using either IB4 or CGRP with anti-GFP (MrgD-DTR) antibodies on DRG, spinal cord, and epidermis. This conditional ablation was then used by Hyoang Lee on the D-DTR mice to study the behavioral consequences (see Hyosang Lee's abstract).

To understand the principles of pain information processing in MrgD neural circuits, it is important to characterize all the participating cells types and their connections. Transneuronal or transynaptic viral tracers have been used to produce controlled infections that spread in sequentially within neural networks. The H129 strain of HSV-1 (human herpes simplex virus-1) is a highly specific anterograde transneuronal marker and has been used to identify CNS regions that receive relayed viscerosensory inputs from the stomach wall. In order to trace molecularly defined neurons, such as MrgD neurons, I have constructed a derivative of H129 that replicates only when it infects a cell expressing the Cre-recominbase protein. This new H129 derivative does not express the viral thymidine kinase (*HTK)* gene and therefore should not replicate in non-mitotic cells, such as neurons. The virus encodes a "loxp-stop-loxp-td-Tomato-IRES-mouse TK1" cassette, so that when a neuron expresses Cre recombinase, mouse *TK1* is expressed and the viruses replicate and spread to all synaptically connected neurons. I am currently testing these viruses in different types of neurons.

**119. Neural pathways for reproduction and aggression in rodents**

*Dayu Lin, David J. Anderson*

Inter-male aggression and male female mating are generally considered as distinct behaviors. Nevertheless, it is increasingly recognized that these opponent behaviors may share similar neurobiological and neuroendocrine mechanisms. For example, both mating and territorial aggression are dependent upon circulating gonadal steroids. Both behaviors rely heavily on olfactory and pheromonal input. Lesions of the medial hypothalamus and medial amygdala in rodents decrease the occurrences of both mating and fighting. Taken together, these data suggest that mating and agonistic behaviors may be subserved by a common network of steroid hormone sensitive limbic areas. It is unclear how these two heavily overlapping pathways produce two opposite behavioral outcomes. One possibility is that two intermingled but distinct subpopulations of neurons mediate mating and fighting. Alternatively, the same population of neurons mediates both mating and fighting through neuromodulation. The goal of this current study is to distinguish these two signal-processing scenarios. We first performed between animal comparisons of the patterns of brain activation during mating and fighting, using c-fos analytic methods that permit rapid sampling across the entire brain. Our preliminary results indicate that mating and fighting indeed activate many similar hypothalamic and amygdalar regions in mice. However, these areas are distinct from those activated during anti-predator defense. Next, we adapted a method to compare c-fos expression induced during the two behaviors in the same animal. Our data suggest that, at least in some commonly activated regions, two largely distinct sets of neurons are likely involved in mating and fighting. Given our poor understanding of the aggression circuit in general, we decided to further investigate the functional roles of several hypothalamic regions in aggression based on our Fos results. Using reversible viral inactivation tools, we found that neurons in the VMHvl and its surrounding regions are likely to be critical for aggression initiation. Currently, we are using chronic recording in awake behaving animals to understand the physiological responses of those neurons during various episodes of aggressive behaviors.

**120. Control of neural stem-to-progenitor transition by CyclinD family members**

*Agnes Lukaszewicz, David J. Anderson*

Human embryonic stem (hES) cells are believed to soon be a key tool to repair or replace diseased or damaged tissue. For instance, replacing motor neurons (MN) degenerating in the Amyotrophic Lateral Sclerosis appears to be one of the most promising treatments for this disease. Improving the knowledge of the molecular mechanisms regulating the differentiation of ES cells into MNs is the first step crucial to achieve this goal.

Dr. David Anderson's lab has been interested for several years in understanding the molecular control of neural fate specification during development, using MN as a model. MNs are derived from a specific progenitor domain: the pMN domain of the spinal cord. While carrying out a systematic characterization of changes in gene expression in this domain, genes coding for cell cycle regulators, the CyclinDs, have been isolated as potential candidates to regulate cell fate in the developing spinal cord.

CyclinD1 and D2 are specifically expressed in distinct subsets of precursors, and dynamically regulated during the neuronal to glia transition. This led us to hypothesize that CyclinD1 may regulate neurogenesis, whereas CyclinD2 may regulate the maintenance of the neural stem cell (NSC). Over the last year, we have made progress in demonstrating the central role of CyclinD1 in modulating neurogenesis, as well as studying the role of CyclinD2 in the maintenance of neural stem cells. By modulating the level of CyclinD1 expression, we observed effects on neurogenesis in support of our hypothesis. Furthermore, we showed that CyclinD1 re-expression is sufficient for glial-restricted progenitors to regain their neurogenic potential when transplanted into a permissive environment. Hence, we demonstrated that CyclinD1, independently of any effects on the cell cycle, modulates the generation of MN. We believe this constitutes an unexpected result of great potential importance for the field. In parallel, CyclinD2 seemed to exert an opposite effects on neurogenesis. We are now interested in deciphering the molecular mechanism involved. To do so, we are analyzing and comparing the CyclinDs interactome, at different stages of the neuronal differentiation pathway.

**121. Genetic manipulation of neuronal subpopulations involved in pain and pleasure**

*Sophia Vrontou, David J. Anderson*

Studies in our lab have identified a novel murine family of G-protein-coupled receptors (GPCRs), related to the proto-oncogene MAS1, called Mas-related genes (Mrgs). Mrgs As, Bs, C and D thus far analyzed, are specifically expressed in mostly non-overlapping subpopulations of trigeminal and dorsal root ganglion (DRG) small-diameter sensory neurons, implying that they might have a potential nociceptive role. Most surprisingly it was found that the expression of MrgD is restricted exclusively to cutaneous peripheral afferents, rendering MrgD the first specific molecular marker that predicts the end-organ specificity of a subset of primary sensory neurons, and supporting the existence of a molecularly distinct subpopulation of cutaneous nociceptors. These data raise the question of: a) whether there are other molecularly distinct nociceptor subsets, innervating different targets; and b) whether the circuits they engage in follow separate pathways and up to what point they might intersect into the brain. We are looking for molecular markers for such subsets and especially for visceral nociceptors, since visceral pain is the most common but still understudied form of pain. We will use these markers to compare the circuitry of their expressing neurons with that of nociceptor subsets innervating other targets, such as the skin and also to genetically manipulate them so as to identify their function.

Most recently, anatomical analysis of MrgB4 expressing neurons revealed that these neurons constitute a rare population of small–diameter sensory neurons, innervating exclusively the hairy skin. It is also suggested that they might mark the mouse analogs of the so-called C-fiber tactile (CT) afferents in humans that respond to gentle stroking. We are interested in deciphering the circuits engaged by this specific subpopulation in the higher brain regions, and most importantly in identifying their function by measurements of their activity *in vivo* using gene targeting technology.

**122. The genetic and neural basis of aggressive behavior in *Drosophila***

*Liming Wang, David J. Anderson*

Aggressive behavior, which is critical for the survival and reproduction of many species, widely exists across animal kingdom. In previous studies, we have used the fruit flies, *Drosophila melanogaster*, as a model organism to study the genes/neural circuits underlying aggressive behavior. We noticed that social experience suppresses aggression in a reversible manner, and showed that a gene named *Cyp6a20* mediates this effect. Furthermore, *Cyp6a20* is expressed in olfactory support cells, suggesting that olfaction is involved in the effect of social experience influencing aggressiveness.

*Cyp6a20* is co-expressed with *Lush*, an odorant binding protein critical for the detection of one fly pheromone, *cis*-vaccenyl acetate (cVA). cVA is known to facilitate aggregation behavior and suppress male courtship behavior in fruit flies. We have found cVA also promotes aggressive behavior. On the other hand, *lush* mutants, in which cVA detection is abolished, exhibit reduced levels of aggressiveness. Both lines of evidence suggest that cVA detection is important for aggressive behavior in fruit flies. We propose to identify the olfactory receptors and olfactory sensory neurons that are responsible for the aggression-stimulating effect of cVA. Furthermore, we are trying to investigate the possible function of cVA in mediating the effect of social experience to suppress aggressive behavior.

**Reference**
Wang, L., Dankert, H., Perona, P. and Anderson, D.J. (2008) *Proc. Natl. Acad. Sci. USA* **105**:5657-5663.

**123. Investigation of behavior and neural circuitry underlying mechanosensation in *Drosophila melanogaster***

*Suzuko Yorozu, Azusa Kamikouchi, Kei Ito, David J. Anderson*

Mechanosensation is intricately linked to an organism's survival. For example, the ability to detect wind allows many flying insects to navigate, while other insects detect disturbances of air particles to help them locate predators and prey. Thus, a coordinated locomotor output in response to mechanical stimuli is critical for survival of many insects. The link between mechanosensation and locomotor control at the neural circuit level is currently not well understood. In my project, I am trying to understand the fly's behavioral and neuronal responses to wind stimulation using a novel behavioral assay called as Air Suppression of Locomotion (ASL). In the ASL assay, wild-type adult flies immediately slow their locomotor activity in response to wind stimulation. They remain immobile for as long as they are exposed to wind stimulation, while they immediately re-engage in locomotor activity when wind stops, as if wind stimulus acts as a neural switch for fly's locomotor activity. Interestingly, when the third antennal segment is surgically removed bilaterally, they continue to engage in locomotor activity in the presence of air flow stimulation, suggesting that detection of air current is probably mediated through sensory organs housed in the antennae. We propose to take an advantage of the simplicity, robustness, high reproducibility, and unique locomotor phenotype of ASL to obtain insights into the link between mechanosensation and locomotor control at the neural circuit level. The goal of my project is to understand where and how air currents act on the nervous system to suppress locomotor activity in the fly. To this end, we will combine behavioral genetics, electrophysiology and calcium response imaging to investigate: 1) which sensory organ mediates ASL; and 2) identify the second- and higher-order neurons that mediate the processing of wind detection and suppression of locomotor activity.

**124. Neural circuits responsible for *Drosophila* aggressive behavior**

*Kiichi Watanabe, David J. Anderson*

Aggressive behavior is one of the important behaviors for animal survival and reproduction, throughout the animal kingdom from insects to humans. With powerful molecular and genetic tools, *Drosophila* will provide great opportunities for the study of molecular- and circuit-level mechanisms of aggressive behavior. However, because of its complexity, the neural circuitry or molecules responsible for this behavior are still unclear. To approach this problem, we have been developing methods to detect specific behaviors observed during, aggression in collaboration with Dr. Dankert in Perona's lab (Electrical Engineering, Caltech). We have developed a computer-based fly tracking system to detect several kinds of behavior including "lunging" and "tussling." Using this system, I have initiated a project to understand the role of octopamine in aggressive behavior.

Octopamine, a biogenic monoamine structurally related to noradrenaline, acts as a neuromodulator in *Drosophila*, and is implicated in aggressive behavior. Although, there are some reports relating the roles of octopamine to aggressive behavior, it is still unclear which specific subsets of octopamine receptors, and receptor-expressing neurons, are involved in aggressive behavior. Firstly, we have trying to perform knockdown analysis of each octopamine receptor using relevant UAS-RNAi constructs and pan-neuronal drivers such as Elav-Gal4 and Appl-Gal4. If we can identify the specific subsets of octopamine receptor responsible for aggression, we will generate Gal4 driver lines containing the promoters of these receptors and manipulate the activity of such neurons. In collaboration with Dr. Gerald Rubin's lab (Janelia farm), we have been generating driver lines expressing Gal4 in octopamine receptor-expressing neurons, under the control of various enhancer regions of octopamine receptors. Using these Gal4 lines with various effector lines such as UAS-Kir2.1, we will screen for phenotypes affecting aggressive behavior after silencing octopamine receptor expressing neurons.

In this way, we will try to functionally dissect the *Drosophila* brain to understand how specific neural circuits control aggressive behavior.

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**Fear/Amygdala**

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**Fly Behavior**

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Wang, L. and Anderson, D.J. (2008) A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **105**(15):5657-5663, PMCID:PMC2311352.

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**Research and Laboratory Staff:** Nick Lawrence, Viveca Sapin, Rong Xiao, Rosalind Young
**Graduate Students:** Gil Carvalho, Julien Muffat

**Support:** The work described in the following research report has been supported by:
American Federation for Aging Research
The James G. Boswell Foundation
Ellison Medical Foundation
The John Douglas French Alzheimer's Foundation
Life Sciences Research Foundation
McKnight Foundation
National Institutes of Health
National Science Foundation
Retina Research Foundation

**Summary:** Our group uses *Drosophila* as a model system to identify and characterize genes involved in behavior, aging, and neurodegeneration. The high degree of homology between the fly and human genomes forms the basis of a strategy for understanding the corresponding human genes.

**125. Dual modes of lifespan extension by dietary restriction in *Drosophila***

*William W. Ja, Gil Carvalho, Brian Zid, Seymour Benzer*

Dietary restriction (DR) is a robust and widely conserved environmental intervention that leads to lifespan extension in multiple organisms including yeast, worms, flies, and mice. A question of major importance is whether DR extends lifespan in different organisms through the same pathways. In *Drosophila*, DR is most commonly achieved by total dilution of the food medium. We previously described *Drosophila* compensatory feeding, the ability of flies to sense changes in nutrient concentration and adapt their food intake accordingly. We noticed that for *Drosophila* DR studies, the food medium is simultaneously the nutrient and the water source. Hence, decreases in food intake on higher concentration foods results in significant decreases in fluid intake. We have recently found that longevity by DR, as typically performed in *Drosophila*, is completely mimicked by providing a supplementary water supply. This finding suggests that most discoveries made in *Drosophila* DR of the past 15 years may be due to or at least complicated by dehydration stress.

We have subsequently identified an alternative regime of DR that robustly extends lifespan but is not affected by hydration. This paradigm likely provides a more relevant model for the DR phenomenon in mammals and we are further characterizing this system. Our observations warrant a careful reexamination of the entire body of work of nutrient manipulation in fruit flies. Past insights should be viewed with caution, since dehydration stress may be a confounding factor. This caveat extends to all lifespan mutants isolated on DR diets, especially since desiccation-resistant strains have been shown by others to be long-lived. Given that insights originating from work done on *Drosophila* DR direct a number of costly, time-consuming studies in mammals, elucidating the mechanisms of hydration-dependent and -independent modes of DR is of utmost importance.

**126. 4E-BP modulates lifespan and mitochondrial translation upon dietary restriction in *Drosophila***

*Brian M. Zid, Aric Rogers[*], Subhash Katewa[*], Tony Au Lu, Pankaj Kapahi[*], Seymour Benzer*

We have found that in *Drosophila*, dietary restriction (DR) upregulates the translational repressor 4E-BP, the eukaryotic translation initiation factor 4E binding protein, and that this upregulation is necessary for the full lifespan extension upon DR. Overexpression of an activated 4E-BP is sufficient to extend lifespan on a nutrient-rich diet, yet not under conditions of DR. This implicates 4E-BP and translational control as a key molecular output of DR in *Drosophila*.

We, therefore, investigated the genome-wide translational changes upon DR using translation state array analysis (TSAA) and found that translationally regulated mRNAs had altered 5' untranslated region (UTR) structural properties. The 5'UTRs of translationally downregulated genes are longer with stronger secondary structure compared to the rest of the genome, while the 5'UTRs of translationally upregulated genes are shorter, with lower GC content and weaker secondary structure. Among the translationally upregulated genes, components of the mitochondria, including mitochondrial ribosomal proteins and electron transport chain subunits, were overrepresented. Mitochondrial genes were found to have short, weakly structured 5'UTRs, with low GC content in *Drosophila*. Upon investigation of the gene classes which have weak or strong 5'UTR secondary structures in both *Drosophila* and humans, it was found that mitochondrial, proteolysis and immunity genes have conserved weak 5'UTR secondary structures, while signal transduction, regulation of metabolism and development genes have conserved strong 5'UTR secondary structures.

We then investigated the role that mitochondrial 5'UTRs have on translational control in S2 cells using luciferase reporter constructs. The 5'UTRs of mitochondrial genes were sufficient to confer preferential translation during times of high 4E-BP activity in a cap-independent manner to reporter constructs. Upregulation of mitochondrial function upon DR was verified and found to be *d4E-BP*-dependent, implicating a novel mechanism for regulating mitochondrial function upon DR. These results implicate mRNA translation initiation in modulating lifespan and mitochondrial function upon DR.

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**127. Neurophysiological analysis of aging in *Drosophila***

*Shlomo Ben-Tabou de-Leon, Seymour Benzer*

Age-related changes in various behaviors of the fruit fly *Drosophila melanogaster*, such as memory and motor activity, have been previously characterized. However, little is known about the cellular and molecular mechanisms of these age-related behavioral changes. During the past year, we have used electrophysiological methods to characterize the changes in the function of the retina and the indirect dorsal longitudinal flight muscles (DLM) to investigate the molecular basis of age-related deterioration of fast phototaxis and flight behaviors.

Electroretinogram (ERG) recordings from the retinas of Berlin flies show a reduction in the amplitude of the receptor potential with age in both males and females. In both males and females, the decline rate of the receptor potential with age is much slower than the mortality rate and, even in 91-day old flies, which is close to the maximum lifespan at 25ºC, a substantial fraction of the receptor potential amplitude is still present. Similarly, the "off-response" amplitude also declines with age in both males and females. However, in contrast to the receptor potential amplitude, the off-response disappears almost completely in 91-day old males and more closely follows the survival rate of the flies.

Intracellular recordings from DLM cells show that the resting potential of the DLM cells becomes less negative, the action potential amplitude is gradually decreased, and the width of the action potential is increased with age in both males and females. In very old flies (over 80 days old at 25ºC), a failure of the action potentials to terminate causes a prolonged depolarization of the membrane potential. These results suggest that age affects the function of various membrane proteins (e.g., ion pumps, calcium channels, and potassium channels) in the DLM cells. We will study the effects of aging on the function of various candidate proteins (e.g., ion channels) expressed in DLM cells and examine the molecular mechanisms underlying the age-related behavioral changes by combining electrophysiological and molecular methods.

**128. Role of Apolipoprotein D and its orthologs in normal and pathological aging in *Drosophila***

*Julien A. Muffat, Seymour Benzer*

Human ApoD (hApoD), when expressed in *Drosophila*, can extend fly lifespan and protect against various stresses relevant to pathological processes. Flies overexpressing hApoD live 30% longer than controls and show increased survival under conditions of stress thought to model normal aging and pathological conditions. We showed, in adult flies, that such stressors strongly upregulate *GLaz*, the fly ortholog of hApoD, suggesting that *GLaz* is also part of a canonical stress response in flies. *GLaz* and *NLaz* overexpression in *Drosophila* cell cultures protect against Aβ42-induced cytotoxicity and against oxidative stress by paraquat, suggesting that the elevation of hApoD in Alzheimer's (AD) and Parkinson's (PD) diseases may play a role in salvaging neurons under oxidative stress. ApoD homologs may also protect cells in acute stress situations, providing a possible beneficial role for chronic secretion of hApoD by astrocytes in the brains of patients with AD or PD, and its acute secretion by glia in crushed peripheral nerves. Remarkably, we have shown that hApoD expressed in *Drosophila* is secreted to the circulating hemolymph and is recruited at the site of lesions to the flight muscle or the nervous system. This recruitment of hApoD is reminiscent of its accumulation in human pathologies, providing further relevance to our model. In collaboration with H. Jasper (University of Rochester, NY), we observed that flies overexpressing Nlaz are also long-lived and resistant to multiple stressors, while flies lacking Nlaz, are conversely stress-sensitive and short-lived. Interestingly, it appears that NLaz, which is upregulated by stress, is under the control of the JNK pathway, and may be a mediator in the insulin and insulin-like growth factor signaling (IIS) pathways. We are currently testing the ability of NLaz, GLaz, and hApoD to protect cells in transgenic models of AD or PD. We will test at various levels (histology, electrophysiology and behavior) whether hApoD and its homologs protect flies and delay the course of these disease models.

**129. Genetic analysis of mitochondrial complex I assembly defects in *Drosophila***

*Jaehyoung Cho, Seymour Benzer*

Mitochondrial respiratory Complex I (NADH-ubiquinone oxidoreductase), the largest of the respiratory enzymes, consists of 46 subunits and is affected by various factors including cellular redox potential and energy metabolism (1). Coordinated assembly of Complex I is crucial to its integrity and activity, and mis-assembly can result in neuromuscular degenerative disorders, including Leigh's syndrome and Leber's hereditary optic neuropathy (1). We have identified *Drosophila* mutants deficient in the Complex I assembly process.

A P-element insertion mutant has been characterized that disrupts the gene for Complex I assembly factor, *dCIA30*. Homozygous mutant flies show significant pupal lethality, and only ~0.2% of embryos reach adulthood. Adult flies live only ~20% as long as control lines. These adults have poor motor skills, exhibit sluggish movement, and cannot fly. The mitochondrial cristae-structure in their indirect flight muscles is abnormal and dilated, and the muscles show degeneration very early in adulthood.

Precise excisions of the P-element rescued the neuromuscular and behavioral defects, sensitivity to stress, and the abnormal mitochondrial ultra-structure of the homozygous mutant flies, supporting the role of the P-element in these phenotypes. We have used native 2-D gel electrophoresis to confirm the assembly state of the Complex I components, and only ~5% of Complex I is assembled in the homozygous mutant flies, compared with control lines.

The *dCIA30* mutant is the first model system for aberrant Complex I assembly, and we will be conducting drug candidate and genetic screens to find interventions that ameliorate the striking phenotypes observed in this model. Since Complex I activity is a gateway for regulating mitochondrial energy metabolism, we will also test Complex I assembly and activity upon dietary restriction.

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Ja, W.W., Carvalho, G.B., Mak, E.M., de la Rosa, N.N., Fang, A.Y., Liong, J.C., Brummel, T. and Benzer, S. (2007) Prandiology of *Drosophila* and the CAFE assay. *Proc. Natl. Acad. Sci. USA* **104**:8253-8256.

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**Research and Laboratory Staff**: Philippe P. Brieu, Heather Hein, Alex Huth, Leonard Mlodinow

**Support**: The work described in the following research reports has been supported by:
Eli and Edythe Broad Foundations
Defense Advanced Research Project Agency
Gordon and Betty Moore Foundation
The G. Harold & Leila Y. Mathers Charitable Foundation
National Geospatial Intelligence Agency
National Institutes of Health
National Institutes of Mental Health
National Science Foundation
Office of Naval Research
Rube Mettler Funds
Sony Corporation
Swartz Foundation
University of Washington/Dana Foundation

**Summary**: Research in the laboratory focuses on three areas: (1) biophysics of computation in nerve cells; (2) understanding visual selective attention and visual consciousness at the neuronal, behavioral and computational levels; and (3) develop biological-motivated vision algorithm that predict where people and animals will look in natural scenes (gaze prediction). For more details and all publications, see http://www.klab.caltech.edu.

Research carried out as part of a "Biophysics of Computation" focuses on how the electrophysiology, synaptic architecture, and dendritic morphology of groups of individual neurons subserve information processing. What can we infer about a neuron by listening to its extracellular recorded action potential? How does the constantly fluctuating extracellular membrane potential influence the intracellular membrane potential? Can the extracellular membrane potential carry information of relevance to neural computation? Analytical work, backed up by detailed computer simulations of nerve cells based on electrophysiological data from our experimental collaborators at the Hebrew University in Jerusalem (Idan Segev and Yosef Yarom) and generates experimentally verifiable predictions. With Guyri Buzsaki at Rutgers, we continue to investigate the relationship between extra- and intracellular spike waveforms and the genesis of the local field potential.

Our laboratory collaborates with the neurosurgeon and neuroscientist Itzhak Fried at UCLA, recording from 128 electrodes in the medial temporal lobe of awake patients with pharmacologically intractable epilepsy who are implanted with depth electrodes to localize the focus of seizure onset. This unique setting allows us to observe invariant recognition, imagery and representation of familiar objects and famous individuals in conscious humans by listening in on the spiking activity of many individual neurons - complemented by local field analysis. This work requires sophisticated data processing skills and the careful design of the appropriate behavioral-physiological paradigms that will work in a clinical context.

Understanding the action of selective, visual attention (both saliency-driven, bottom-up as well as task-dependent, top-down forms) requires a firm grasp of how visual object recognition in natural scenes can be solved at the computational level, and how the resulting algorithms can be mapped onto the known architecture of the visual cortex and associated cortical and sub-cortical areas. We use analytical methods, coupled with computer simulations of the appropriate circuitry in the primate visual system, visual psychophysics, eye tracking and functional brain imaging at Caltech's 3.0 T Trio scanner to investigate human attentional selection (via saliency) and object recognition in the near-absence of focal attention, in visual search, in natural scene perception and as reward is modulated. Aspects of this work are done in collaboration with Pietro Perona, Antonio Rangel and Ralph Adolphs of Caltech and with Laurent Itti at USC. Together with Tomaso Poggio at MIT, we investigate neurobiologically plausible models of both, the ventral, object-recognition and the dorsal, attention visual streams. We continue to work on understanding how neurons in regions LIP and FEF - modulated by top-down information - implement a visual saliency map.

We study the neuronal correlates of consciousness, developing a neurobiological framework to understand how subjective feelings (in particular, conscious visual perception) can arise in the mammalian forebrain. Using 'Continuous Flash Suppression' (CFS) and other techniques we have invented to hide images from conscious perception, we can show that visual, selective attention is a distinct process from visual consciousness.

Finally, the **Broad Fellows Program in Brain Circuitry** studies how ensembles of neurons create internal representations of the external world, and how such representations are decoded by other ensembles of neurons, to produce organized programs of motor output that create behavior. Within this large question are many sub-questions. For example: Are neural circuits organized in a "modular" fashion, with certain neuronal subsets dedicated to certain functions, or rather in a "distributed" fashion, such that each neuron participates in multiple ensembles that may play very different roles at different times? How do neural circuits process opposing types of sensory input, such that when an organism is faced with conflicting cues, it is able to

choose one behavior and suppress another? Fellows can focus exclusively for a few years on developing an independent research program without the usual junior faculty burden of teaching, administration, grant-writing, and the pressures of the tenure-clock. By attracting and selecting the most highly gifted individuals, willing to risk the single-minded pursuit of a goal, at the time in their careers of greatest energy, enthusiasm and drive, we hope to foster an environment in which unique discoveries and advances in knowledge are the norm. At the moment, three fellows are in residence, Sotiris Masmanidis, Andrew Steele, and Daniel Wagenaar, with a fourth fellow, Guangying Wu, joining in early 2009. For more information, see http://www.broadfellows.caltech.edu

**130. fMRI activation to visible and invisible faces and houses using continuous flash suppression with a confidence rating task**

*J. Dubois, N. Tsuchiya, R. Adolphs, Christof Koch*

The neuronal correlates of conscious awareness of visual stimuli have been extensively studied, yet the relationships between physical stimuli, subjects' confidence about their own visual awareness, and the neuronal activities in various areas of the brain remain unclear. Here, we asked this question using the Continuous Flash Suppression (CFS) technique (Tsuchiya and Koch, 2005). We manipulated the contrast of Mondrian maskers to modulate the visibility of face and house stimuli, while we fixed the contrast of the faces and houses throughout the experiment. We performed functional imaging in a 3T Siemens scanner (TR = 2 sec, 3x3x3 mm, 32 slices) while subjects viewed visual stimuli through MRI compatible goggles. Each run consisted of multiple trials. Each trial, lasting ~10 sec, consisted of a sequence of six images projected at the fovea for 800 ms each and masked with CFS. We presented the Mondrians flashed at 10Hz to the dominant eye and either faces, houses, or a blank screen to the non-dominant eye. In each trial, we fixed the contrast of Mondrians and the category of the stimuli presented to the non-dominant eye. Across trials, we varied the contrast of the mask, using four levels. Subjects' arousal and fixation were controlled by a fixation task. After each trial, subjects reported: 1) whether faces or houses were presented (2AFC); and 2) the visibility of the reported category of the stimuli, with a rating (1 for nothing; 2 for guess; 3 for parts visible; and 4 for clearly visible). Using a general linear model, we estimated the BOLD responses in various regions of interest (V1, FFA, STS, OFA, and PPA), which were localized in separate localizer runs.

***RESULTS***: As expected, the BOLD response in V1 increased as the contrast of Mondrians increased. The response in V1 did not saturate even at the highest contrast for the mask; there was a constant increase in the response when the faces or houses were presented regardless of the contrast of the Mondrians. The responses in high-level visual areas (FFA, STS, OFA, and PPA) were not modulated by the contrast of the Mondrians. The responses increased as the rating for each region's preferred stimuli increased. Interestingly, this seems also true even when no stimuli were presented to the non-dominant eye (the blank screen trials), following the subjective confidence rating.

**131. Dynamics of selective single neurons in the human MTL in a visual working memory task**

*Florian Mormann[1], Rodrigo Quian Quiroga[2], Alexander Kraskov[3], Moran Surf[1], Itzhak Fried[1,4], Christof Koch*

Single unit recordings in the human medial temporal lobe (MTL) have revealed neurons with selective, sparse, and invariant responses to different pictures of a given, familiar individual. We here used a working memory paradigm involving serial presentations of pictures at different presentation rates to investigate the dynamics of these neuronal responses.

We analyzed unit activity of MTL neurons from two patients undergoing monitoring for resective epilepsy surgery. Signals were recorded from microwires implanted in the MTL while patients performed a modified Sternberg task comprising a total of eight different pictures. Each trial consisted of a baseline period, an encoding period with presentation of a series of four images, a maintenance period, and a recall task in which the patient had to decide which one of two images he/she had seen in the previous series. Presentation rates of the picture series was varied from 1- 5 Hz using four different inter-stimulus intervals (ISIs). In total 192 trials were randomized and balanced to yield six presentations for each combination of image, ISI, and position within series. Evaluation of peri-stimulus time histograms of neurons responding selectively to only one of the images yielded two types of responses. One group (10 neurons) showed early responses (with a latency of approx. 200 – 400 ms) independent of presentation rates. In another group (2 neurons), responses occurred later (approximately 400 - 700 ms after stimulus onset) and were substantially diminished for fast presentation. Response dynamics did not appear to be influenced by the position of images within the presented series. For both groups there was no apparent correlation with task performance, but neurons in the second group tended to show selective activity during the maintenance period, as well.

Results indicate distinct types of neurons with respect to their dynamical responses. The dynamics observed in the first group of neurons indicate an entirely stimulus-driven serial feed-forward processing for the investigated presentation rates, while those in the second group could reflect interference of consecutive processing for higher presentation rates, possibly caused by lateral inhibition. The activity of neurons during the maintenance period in the second group indicates an involvement of these MTL cells in working memory processing, which has previously been attributed primarily to prefrontal brain regions.

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**132. Predicting human gaze using low-level saliency combined with face detection**

*Neural Information Processing Systems*
*Moran Cerf, December 2007*

Under natural viewing conditions, human observers shift their gaze to allocate processing resources to subsets of the visual input. Many computational models try to predict such voluntary eye and attentional shifts. Although the important role of high level stimulus properties (e.g., semantic information) in search stands undisputed, most models are based on low-level image properties. We here demonstrate that a combined model of face detection and low-level saliency significantly outperforms a low-level model in predicting locations humans fixate on, based on eye movement recordings of humans observing photographs of natural scenes, most of which contained at least one person. Observers, even when not instructed to look for anything particular, fixate on a face with a probability of over 80% within their first two fixations; furthermore, they exhibit more similar scanpaths when faces are present. Remarkably, our model's predictive performance in images that do not contain faces is not impaired, and is even improved in some cases by spurious face detector responses.

**133. Using semantic content as cues for better scanpath prediction**

*Eye Tracking Research and Application*
*Moran Cerf, April 2008*

Under natural viewing conditions, human observers use shifts in gaze to allocate processing resources to subsets of the visual input. There are many computational models that try to predict these shifts in eye movement and attention. Although the important role of high-level stimulus properties (e.g., semantic information) stands undisputed, most models are based solely on low-level image properties. We here demonstrate that a combined model of high-level object detection and low-level saliency significantly outperforms a low-level saliency model in predicting locations humans fixate on. The data is based on eye-movement recordings of humans observing photographs of natural scenes, which contained one of the following high-level stimuli: faces, text, scrambled text or cell phones. We show that observers - even when not instructed to look for anything particular, fixate on a face with a probability of over 80% within their first two fixations, on text and scrambled text with a probability of over 65.1% and 57.9%, respectively, and on cell phones with probability of 8.3%. This suggests that content with meaningful semantic information is significantly more likely to be seen earlier. Adding regions of interest (ROI), which depict the locations of the high-level meaningful features, significantly improves the prediction of a saliency model for stimuli with high semantic importance, while it has little effect for an object with no semantic meaning.

**134. Decoding what people see from where they look: Predicting visual stimuli from scanpaths**

*Workshop on Attention Processing and Computer Vision*
*Moran Cerf, May 2008*

Saliency algorithms are applied to correlate with the overt attentional shifts, corresponding to eye movements, made by observers viewing an image. In this study, we investigated if saliency maps could be used to predict which image observers were viewing given only scanpath data. The results were strong: in an experiment with 441 trials, each consisting of two images with scanpath data - pooled over nine subjects - belonging to one unknown image in the set, in 304 trials (69%) the correct image was selected, a fraction significantly above chance, but much lower than the correctness rate achieved using scanpaths from individual subjects, which was 82:4%. This leads us to propose a new metric for quantifying the importance of saliency map features, based on discriminability between images, as well as a new method for comparing present saliency map efficacy metrics. This has potential application for other kinds of predictions, e.g., categories of image content, or even subject class.

**135. Subjects' inability to avoid looking at faces suggests bottom-up attention allocation mechanism for faces**

*Society for Neuroscience*
*Moran Cerf, May 2008*

Subjects looking at images with faces were shown to have very distinct scanpaths. Faces were looked at in the very early fixations, regardless of the size, location, and number in the images, and independent of the task which was presented to subjects. Bottom-up saliency models are typically used to model subjects' attention. We have shown that adding a face channel (based on the Viola and Jones face detection algorithm) to a commonly used saliency model yields a higher accuracy in the prediction of subjects' scanpaths.

In this study, we test the extent to which faces act as attractors and examine the mechanism by which this occurs. We use eye-tracking data from subjects viewing images that contained faces, text, and cell phones (high-level semantic entities, as opposed to low level ones such as color, intensity and orientation). Subjects were performing a search task where they were looking for a target cross in images presented for 2s in each trial. Subjects were instructed to answer y/n whether the target was found in the image. There were four blocks in the experiment; in three of the four a hint was given with respect to the location of the target in the image. While in the 1st block no hint was given, in the 2nd block the hint indicated that the target would not appear on a face area in the image. In the 3rd block the target would not be located on a text region, and in the 4th block the target would not be located on a cell phone. Each block contained 40 images. The difficulty of the task was modified such that the cross was blended in the background so that subjects' success rate in training trials

was 75%. As this task was fairly hard subjects were encouraged to follow the hint and avoid looking at the faces, text and cell phone, respectively, in order to succeed. The ability to avoid looking at an entity is informative of the extent to which we are drawn to that entity despite our incentive to avoid it. We quantified the ability to avoid looking at an entity by measuring the fraction of first fixations falling on the entity's region, and compared this fraction it to the baseline set in the first block where no avoidance is necessary. Results suggest that subjects are able to avoid looking at cell phones and text when hinted to do so over ten times more than they can for faces. While subjects were able to avoid looking at cell phones in 93% and text in 89% of trials, they were able to avoid looking at faces in only 12% of the trials in which they were instructed to do so. This suggests that faces are indeed stronger attractors than other entities and may even be part of a very early bottom-up mechanism in the brain.

**136. Unsupervised category discovery in images using sparse neural coding**

*Steve Waydo*

We present an unsupervised method for learning and recognizing object categories from unlabeled images. Motivated by the existence of highly selective, sparsely firing cells observed in the human medial temporal lobe (MTL), we apply a sparse generative model to the outputs of a biologically faithful model of the primate ventral visual system. In our model, a network of nonlinear neurons learns a sparse representation of its inputs through an unsupervised expectation-maximization process. In recognition, this model is used in a maximum-likelihood manner to classify unseen images, and we find units emerging from learning that respond selectively to specific image categories. A significant advantage of this approach is that there is no need to specify the number of categories present in the training set. We present classification accuracy using three different evaluation metrics.

**Reference**

Waydo, S. and Koch, C. Unsupervised category discovery in images using sparse neural coding, British Machine Vision Conference, September 2007.

**137. Unsupervised learning of individuals and categories from images**

*Steve Waydo*

Motivated by the existence of highly selective, sparsely firing cells observed in the human medial temporal lobe (MTL), we present an unsupervised method for learning and recognizing object categories from unlabeled images. In our model, a network of nonlinear neurons learns a sparse representation of its inputs through an unsupervised expectation-maximization process. We show that the application of this strategy to an invariant feature-based description of natural images leads to the development of units displaying sparse, invariant selectivity for particular individuals or image categories much like those observed in the MTL data.

**Reference**

Waydo, S. and Koch, C. (2008) *Neural Comput.* **20**:1165-1178.

**138. Cortical topography of intracortical inhibition explains speed of decision making**

*Claudia Wilimzig, Hubert R. Dinse*[*]

To address the functional consequences of the topographical arrangement of cortical maps of the body surface we used a tactile multiple-choice reaction time (RT) task requiring the selection of a given finger out of all ten fingers of both hands (1). The distribution of RTs displays a striking inverted U-shape, with significantly slower RTs for the middle fingers of each hand. This effect is restricted to the condition of selection among all fingers as middle fingers may be equally fast than the small finger or the thumb when tested in a dual choice task. To investigate a role of cortical topographies in the speed of the selection decision, we applied tactile coactivation (2-7), an unattended Hebbian learning protocol to induce changes of the topographic hand representation through cortical plasticity. Indeed, changes in topography influences RTs as after coactivating the right middle finger, RTs on the stimulated middle finger were significantly shortened resulting in the inverted U-shape distribution to almost disappear. Our data demonstrate that early stages of somatosensory cortical areas are involved in this task and topographies play an important role in determining the speed of selection processes.

As an approach to cortical population activation (8,9) we use neural fields with Mexican-hat type interaction to account for our experimental results. Simulations show the inverted U-shape distribution as an emergent consequence of the lateral inhibition within cortical representational maps indicating a crucial role of interaction in the process of decision making. By weakening the strength of inhibition (10) we can model the influence of coactivation both on the level of changes of cortical topography and its influence on reaction time. Our model thus attributes both the emergence of the inverted U-shape distribution and its modification through learning to lateral inhibition within cortical topography.

While the role of cortical topographies for localization tasks is straightforward, we provide evidence that interaction processes within cortical maps are also crucial for establishing selection and thus functions related to decision making.

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## 139. A new masking technique for natural scenes reveals the saliency of an image

*Claudia Wilimzig, Rufin VanRullen[*] Christof Koch*

Experiments on temporal integration show that for two differently colored lights flashed subsequently, people report a blended version of both stimuli as if the process underlying perception integrates over both stimuli [1]. We use the same experimental technique for natural scenes: briefly flashing (e.g., 10 ms) a natural scene followed by a brief flash of a negative version of the same scene. The stimulus and its negative version are inversely related like a photo and its negative and can be obtained by subtracting the stimulus from the maximum palette entry in each respective color channel (see Fig. 1). Since this reduces the visibility of the natural scene, this may be considered a masking technique, albeit with very specific timing.

If the visual system perfectly averaged over stimulus and mask, subjects would report seeing a uniform gray patch. Contrariwise, in some areas of the picture, subjects reported deviations from mere gray perception.

We show (1) that these areas are systematically related to predictions of computational approaches to saliency; [2] e.g., such that our masking technique masks everything but the most salient regions of the image; (2) that saliency accounts better for our results than intensity of the image alone; (3) that this effect is weakened but not abolished in a dichoptic version of the experiment showing that retinal mechanisms may contribute but are not sufficient to explain this effect; and, (4) that we can directly correlate subject's percepts with predictions derived by computational algorithms [2].

This paradigm shows that temporal integration is non-linear and this non-linearity results in a percept that approximates the saliency map of an image. Thus, the brain may compute an estimate ("snapshot") of saliency for presentations as short as 6.3 ms for natural scenes and their negatives, based on a small number of action potentials. These results have significant implications for the neuronal coding of saliency and for computational approaches to saliency.

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Figure 1: Christof's syncretic photo (left) would be masked by the image in the middle. Adding the two pictures results in a perfectly grey image (right; except for all skin hues that remain unchanged for aesthetic reasons).

**140. Scalable biologically inspired neural networks with spike time based learning**

*Lyle N. Long,*
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The human brain is the most complicated system in the known universe. It has roughly $10^{11}$ neurons and $10^{15}$ synapses. The storage capacity is roughly 10 times larger than the data in the Library of Congress (500 terabytes). It would be extremely difficult to simulate this on even the largest current supercomputer. Even more daunting, however, is the "wiring diagram." Each neuron can be connected to thousands of other neurons, and there are feedback connections. This paper describes the software and algorithmic issues involved in developing very large scalable biologically inspired spiking neural networks, with spike-time based learning. The software uses leaky integrate and fire neurons. The network has distinct layers, and allows recurrent connections. Each layer contains a 2-D array of neurons, so the neurons in each layer can be addressed using i, j indices; and the layer can be addressed with an index k. So each neuron can connect to a rectangular patch of neurons in any other layer. This offers a great deal of flexibility in defining "wiring diagrams" with minimal memory. Hebbian learning (and forgetting) based on spike timing is also implemented very efficiently here. If a neuron fires, then the learning method is called for that neuron. Since we know which presynaptic neurons fired between the last two firings of the postsynaptic neuron, these are the neurons that that are strengthened. Any neuron that has not contributed to the postsynaptic neuron firing has its weight decreased. This approach is spike-time dependent, but it is much more scalable and efficient than STDP. The other key to a robust and stable biological or computational spike-time learning algorithm is homeostatic behavior, which is also implemented here. These neural networks will be useful in object recognition and signal processing tasks, but will also be useful in simulations to help understand the human brain. The software is written using object oriented programming and is very general and usable for processing a wide range of sensor data.

*Invited paper for IEEE Symposium on Learning and Adaptive Behavior in Robotic Systems, Edinburgh, Scotland, August 2008.*

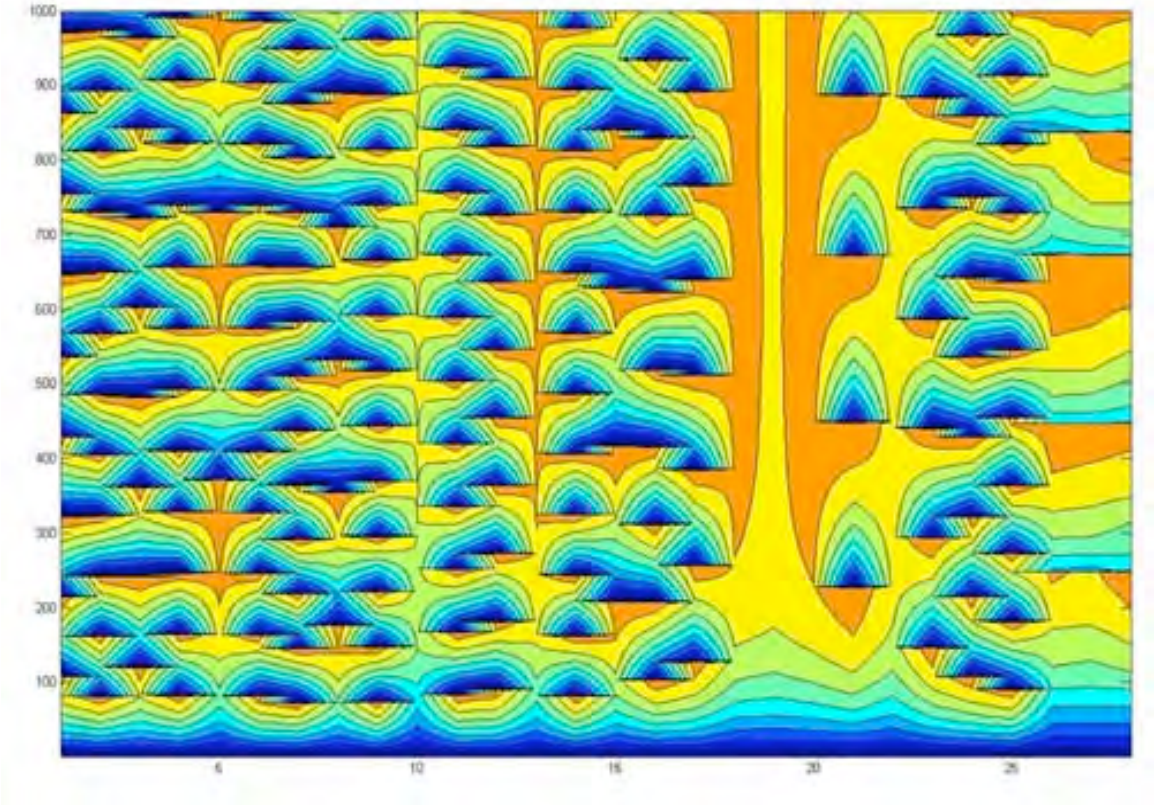


Figure 1. This shows the voltages in 28 neurons over 1000 times steps. The dark blue colors indicate high voltages (spikes). The large vertical yellow band indicates a neuron that has not fired. This image appears on the website for the American Physics Society (www.aps.org), courtesy of Lyle N. Long.

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**Support:** The work described in the following research reports has been supported by:
Beard Charitable Foundation
Bing Chair Funds
Whitehall Foundation

***Fast signal discrimination in the owl's auditory system***

We have been studying how barn owls use hearing to localize prey in total darkness. We now know all the brain areas that process the acoustic cues for sound localization, which are the time (ITD) and the intensity (IID) differences between the ears. Neurophysiologists usually count the number of nerve impulses that a neuron produces in response to a specific stimulus. They present the same stimulus 10 or more times to a single neuron to obtain the mean discharge rate for each neuron. Our early behavioral studies showed that owls could localize the source of a single burst of sound as short as 65 msec. We chose neurons that respond selectively to ITDs to find out if they might be able to detect the ITD they prefer in response to a single stimulus of 100 msec in duration. We immediately established that midbrain neurons could detect their preferred ITD in response to a single burst of sound. However, this finding did not exclude the possibility that the fast response is transmitted from an earlier stage in the pathway. Postdoctoral fellow (Brian Fischer) showed that the fast response occurs in the station before the midbrain but not at the station where ITDs are first computed. Our next question is how the fast detection of ITD is achieved. We think that each neuron in the first site of fast responses receives input from multiple neurons. We have to perform intracellular recordings to see how the synaptic potentials due to multiple inputs are processed to achieve the fast detection. Also, it is important to show anatomically that a single fast responding neuron receives inputs from multiple pre-synaptic neurons.

***Cross-correlation by coincidence detector neurons in the owl's auditory system***

In 1948 when little was known about how nerve cells work, Lloyd Jeffress, a well known psychologist, proposed a model to explain how people localize sound sources by computing time differences in the arrival of sounds in the two ears. Barn owls provide a neural system in which we can test his hypothesis. One of the predictions of the Jeffress model is that the neurons that compute ITD should behave as cross-correlators. Whereas cross-correlation-like properties of the ITD-computing neurons have been reported, attempts to show that the shape of the ITD response function is determined by the spectral tuning of the neuron, a core prediction of cross-correlation, have been unsuccessful. Using reverse correlation analysis, we demonstrate in the barn owl that the relationship between the spectral tuning and the ITD response of the ITD-computing neurons is that predicted by cross-correlation. Moreover, we show that a model of coincidence detector responses derived from responses to binaurally uncorrelated noise is consistent with binaural interaction based on cross-correlation. These results are thus consistent with one of the key tenets of the Jeffress model. Our work sets forth both the methodology to answer whether cross-correlation describes coincidence detector responses and a demonstration that in the barn owl, the result is that expected by theory.

***The brain song control system of birds***

Songbirds and parrots are unique among non-human animals, because they learn vocal signals. Young birds raised in isolation develop very abnormal songs. Other avian species such as chickens and even songbirds that belong to a group called the sub-oscine develop normal vocalizations even when they cannot hear their own voice. Special brain areas evolved in birds groups that learn or mimic sound signals. The main thrust of the present project is to explore new areas and connections between them.

**Publications**

Christianson, B. and Peña, J.L. (2007) Preservation of spectrotemporal tuning between the nucleus laminaris and the inferior colliculus of the barn owl. *J. Neurophysiol*. **97**:3544-3553.

Fischer, B. and Konishi, M. (2008) Variability reduction in interaural time difference tuning in the barn owl. *J. Neurophysiol.* **100**:708-715.

Fischer, B., Christianson, B. and Pena, J.L. (2008) Cross-correlation in the coincidence detectors of owls. *J. Neurosci.* **28**:8107-8115.

Fischer, B., Peña, J.L. and Konishi, M. (2007) Emergence of multiplicative auditory responses in the midbrain of the barn owl. *J. Neurophysiol*. **98**:1181-1193.

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**Postdoctoral Scholars**: Stijn Cassenaer, Stephen Huston, Mala Murthy, Ingmar Riedel-Kruse
**Graduate Students**: Cindy N. Chiu, Anusha Narayan*, Maria Papadopoulou, Kai Shen, Sina Tootoonian
**Research and Laboratory Staff**: Astrid Hoffius
*Joint with Professor Paul Sternberg, Division of Biology, Caltech*

**Support:** The work described in the following research reports has been supported by:
Beckman Fellowship
Jane Coffin Childs Foundation
Ferguson Fellowship
Lawrence A. Hanson Jr. Professorship of Biology
Della Martin Fellowship
Betty and Gordon Moore Foundation
National Institute on Deafness and Other Communication Disorders
National Institutes of Health
National Science Foundation
Swartz-Sloan Foundation
Helen Hay Whitney Foundation

**Summary:** We are interested in information coding in the brain and in the design principles of circuits involved in processing sensory information. We are particularly interested in understanding the role of time, circuit dynamics, synchronization and oscillations in information coding and in relating the biophysical properties of neurons and synapses to the function of the networks in which they are embedded. We therefore study the cellular, synaptic and network aspects of neural processing. We continued to focus our research this year on the olfactory system of insects (antennal lobes and mushroom bodies, circuits analogous to the vertebrate olfactory bulbs and anterior/posterior piriform cortices), using locusts, *Drosophila* and crickets as primary model systems, and of zebrafish (with focus on target regions of the olfactory bulb in the ventral telencephalon). Our work combines experimental (behavioral, electro-physiological and two-photon imaging) and modeling techniques and aims at understanding functional aspects of brain circuit design, such as the coding and learning rules used by the nervous system.

## 141. The consequences of STDP in the locust mushroom body

*Stijn Cassenaer*

The insect mushroom body (MB) has served as a model system to address several questions of general neurobiological interest including sensory discrimination, multi-modal integration, the control of complex behavioral repertoires, as well as learning and memory [1]. Among these, the role of the MB in olfaction and memory has received considerable attention. A number of studies have addressed how olfactory information reaches the MB and how it is transformed along the way [2-5]. Broad activation of the AL by an olfactory stimulus gives rise to oscillatory population activity and diverging trajectories of projection neuron (PN) activation. Different points along these trajectories can be thought of as representing different aspects of the odor stimulus, and cells that decode PN activity in the MB, Kenyon cells (KCs), respond sparsely at specific time-points along the PN trajectories [6]. Previous work suggests that individual oscillation cycles are meaningful units for the encoding and decoding of olfactory information by PNs and KCs [4-8] and this appears to be the case also for extrinsic neurons in the mushroom body beta-lobe (βLNs), which decode the KCs' sparse responses.

We have examined transmission and plasticity of KC-βLN synapses with intracellular recording. These synapses are excitatory and undergo Hebbian spike-timing dependent plasticity (STDP) on a timescale similar to the 20-Hz oscillatory population discharge [9]. As a consequence, STDP has a homeostatic effect on the phase of βLN firing, which facilitates the synchronous flow of olfactory information, and maintains the segregation between oscillation cycles. We have found an additional component that contributes to this segregation, namely lateral inhibition among βLNs. We implement this in a network model to evaluate the consequences of the interaction between STDP and the competition among βLNs due to this phase-locked inhibition. Considered within the context of the circuit in which the KC-βLN network is embedded, the modeling results suggest a mechanism for learning different aspects of the odor, after they are extracted and formatted as a function of oscillation cycle in the AL.

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**142. Active sampling in locust olfaction**

*Stephen J. Huston*

I am studying how the processing of sensory information changes when an animal actively investigates its environment. We know a lot about how the locust nervous system encodes olfactory information. This knowledge comes from experiments on locusts with restrained antennae (the main olfactory sense organs), but during normal function locusts move their antennae – possibly to actively sample odors. I have begun experiments to determine how these antennal movements affect olfactory processing. For these experiments I have developed equipment to automatically record the direction of walking and track the 3D antennal movements of a tethered locust while it is presented with spatially localized odors.

I have discovered that, when presented with an odor, locusts change the location (Figure 1a-d) and frequency (Figure 1e) of antennal movements. This behavioral strategy leads to an increase in the frequency at which the locusts are able to sample odors.

My ultimate aim is to record neural activity from the locust olfactory system while the locust is free to move its antennae. Can we predict the neural responses to olfactory stimuli from previous experiments on restrained locusts, or does neural processing in the olfactory system change as the locust moves its antennae to investigate the environment? I will also determine the relationship between the well-characterized dynamics of the olfactory system's neural responses and the dynamics of the antennal sampling behavior. Hopefully this work will help us to understand how behavioral and neural coding strategies interact.

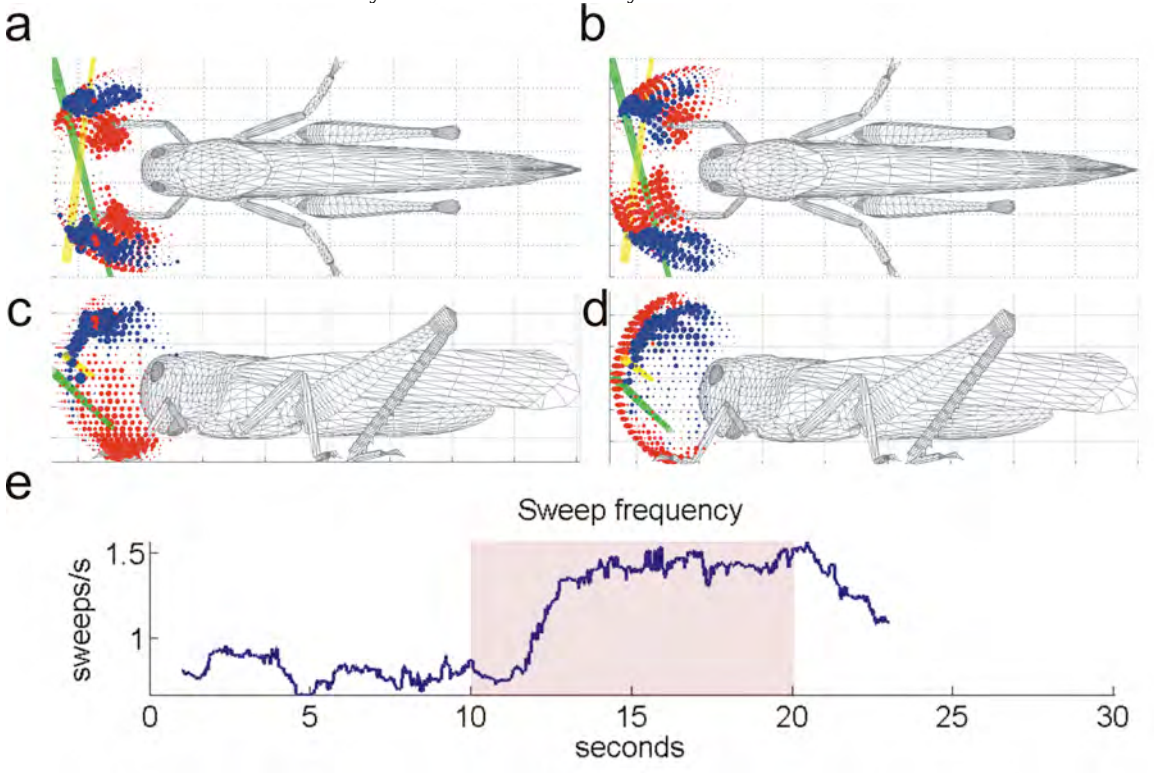


**Figure 1.** (**a-d**) Difference between the distribution of antennal tip positions before (blue) and during (red) odor presentation. (**a**,**c**) Wheat grass odor was added to an air-stream originating on the locust's left, represented by the green tube (**a** - above view, **c** - side view). In **(b**,**d)** the same odor was added to the yellow air-stream originating from the locust's right (**b** - above view, **d** - side view). (**e**) Change in median antennal sweeping frequency with the presentation of wheat grass odor (red box).

**143. Sensory coding and perception in *Drosophila***

*Mala Murthy*

Over the past year, I have investigated 1) the logic of neuronal connectivity in the *Drosophila* mushroom body, a higher order brain center required for complex processes such as learning and memory (1), and 2) the neuronal basis for species-specific preferences to courtship songs in *Drosophila.*

1) The mushroom body is required for olfactory learning in flies (2-4). In adults, the principal neurons of the mushroom body, the Kenyon cells (KCs), form a large cell population usually divided into three classes (α/β, α'/β', and γ) based on anatomical criteria. Both neuronal populations from which their olfactory input derives, the antennal lobe projection neurons (PNs) and the presynaptic partners of the PNs, the olfactory sensory neurons, can be individually identified by their glomerular projections, by the expression of specific genes, and by their responses to odors (5-10). I have investigated whether or not Kenyon cells are similarly individually identifiable across flies – this issue is important because it relates to the specification of sensory network connectivity in general: at what level (if at all) does the order that exists in early sensory circuits break down, such that connections and cellular properties become specific to each individual animal?

To record from potential KC functional replicates across flies, I used *in vivo* physiology methods for *Drosophila* developed in the Laurent lab and a GAL4 line with restricted expression in a small subset of ~23 α/β KCs (11). If KCs are identifiable neurons, with recordings from one labeled KC each in 27 flies, at least 13 replicates (non-singletons) should occur in our dataset with $p>0.99$. Odor response profiles (both spiking and subthreshold activity) across recordings from GAL4-labeled KCs provided no evidence for obvious repeats. Further, across-animal responses were as diverse within the genetically-labeled subset as across all KCs in a larger sample. However, identifying functional repeats, supposing that they exist, required some knowledge of expected inter-individual variability. For this reason, I recorded from the presynaptic partners of the KCs, the PNs, under the same stimulus conditions. In collaboration with Dr. Ila Fiete (Caltech Broad Fellow), we used these data as inputs to a KC model: different KC types were generated by summation of odor responses from PNs of different glomerular types, and different KC individual responses of one type were generated based on the measured inter-fly variability across responses from PNs of a single glomerular type. In the model, regardless of the convergence factor between PNs and KCs, KC responses could easily be clustered by type and across individuals. We applied clustering thresholds derived from this model to the experimental KC data, and in several thousand runs of the simulations, the thresholds identified an average of 14 non-singletons among the model KCs, but fewer than three among the real data. Our data and analyses collectively indicate that measured PN response variability combined with stereotyped PN-to-KC connectivity across individuals cannot account for the variability of KC responses we observe.

Our result, that one-to-one connections between PNs and KCs may vary substantially from animal to animal, is surprising given the genetic, anatomical and functional stereotypy observed with most *Drosophila* neurons studied to date, and in particular, with the olfactory inputs to the mushroom body. However, the lack of precise circuit specification in the mushroom body may result from two features of this structure: (i) mushroom bodies are required for olfactory learning and recall; thus, inputs to KCs may be plastic. (ii) KCs are very numerous (~2,500 in *Drosophila*); this makes the precise specification of pairwise PN-to-KC connectivity a significant mechanistic challenge. Further, functional stereotypy across animals is not a prerequisite for memory formation. In a system in which associative memories are stored as patterns of synapses between KCs and their targets, those memories could be defined without a priori specification. However, these target neurons may need to respond to odors in more stereotyped ways than KCs, in order to generate reproducible learned behaviors. It will thus, be of great interest to determine if and how identifiably reemerges downstream of the Kenyon cells.

2) During courtship, virgin females display preferences for males of their own species; following courtship and persisting for several hours to days, mated females reject conspecific males, suppressing courtship behavior in favor of egg-laying. While the importance of auditory cues in mating in *Drosophila* has been well documented (12-15), how courtship songs are encoded within the auditory pathway and how the perception of song leads to different behaviors depending on the song (conspecific or heterospecific) and the state of the female (mated or unmated) is unknown. Songs are produced by courting males via wing vibration, and males of each species (there are >1700 *Drosophila* species) sing a unique courtship song, consisting of low frequency pulses with species-specific pulse carrier frequencies and inter-pulse intervals (16-18). I have chosen to take a comparative approach to understanding the neural coding of courtship song by presenting auditory stimuli from and performing electrophysiological recordings in several of these species.

In *Drosophila*, sounds, like odors, are received by the antenna – there they are transformed into neuronal signals in stretch receptor neurons, which connect with second-order neurons in the brain (19, 20). Our preliminary studies have involved: 1) analyzing sound traces of male courtship singing, in order to build a behaviorally-relevant stimulus set; and 2) conducting electrophysiological recordings from primary and genetically-labeled secondary neurons in response to both synthetic and recorded courtship song. These studies suggest a basis for species-specific auditory tuning at the level of the antenna. However, antennal tuning curves are broad, indicating that song preference is mostly encoded downstream. Future characterization of the system will involve more detailed song analysis, continued patch

recordings, genetic silencing and activation of neuronal subsets, and cross-species comparisons, in order to decipher how flies distinguish between conspecific and heterospecific courtship songs, and how this distinction leads to reproducible song-elicited attraction or avoidance behaviors.

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### 144. Dynamic coding in an olfactory system

*Ingmar Riedel-Kruse*

I wish to understand how the architecture of neuronal networks determines its dynamics ("firing patterns"), and how such dynamics constitutes computational and coding tasks. This is a central question in systems neurobiology. Specifically, I study how the sense of smell is processed in insects in a brain structure called the antenna lobe. In this lobe the neurons have been shown to exhibit synchronized, oscillatory firing patterns. I use multi-unit recording techniques ("tetrodes") to measure these firing patterns of a large number of neurons at the same time. In order to increase the number of simultaneously recorded neurons I collaborate with Sotiris Masmanidis and Jiangang Du to develop and test new tetrodes (Reference 8). The analysis of my data has revealed so far that these firing patterns become more reliably over the time course an odor is encountered. In collaboration with Ila Fiete, Ila Varma and Joshua Milstein we use information theoretical approaches to quantify the information content in these firing patterns. I hope that my results will lead to new conceptual insight into olfactory processing and, more generally, into the role of the widely observed oscillatory activity in other neuronal networks.

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### 145. Decoding of multiplexed odor representations by mitral cell downstream target neurons

*Cindy N. Chiu*[*]

Previous work in the Laurent lab has demonstrated that the zebrafish olfactory system is a useful model for understanding how brains can perform complex computational tasks such as stimulus categorization and identification. Odors can evoke synchronous action potentials among stimulus-specific subsets of mitral cells (MCs), the principal output neurons of the olfactory bulb. MCs can also respond dynamically to odors without synchrony. Friedrich *et al.* (2004) have shown that during an odor response, patterns of synchronous MC spikes evoked by related stimuli converge whereas patterns of non-synchronous MC spikes diverge. These observations led to the proposal that two types of complementary information about stimulus quality can be carried within a single output stream (i.e., the population of spiking MCs): information about odor category is conveyed by converging patterns of synchronous spikes while information about odor identity is conveyed by diverging patterns of non-synchronous spikes.

Our goal here is to address how brain areas downstream of the olfactory bulb decode the previously characterized MC activity and what types of computations they might perform in turn. Mitral cells project to at least several downstream targets in the fish telencephalon,

including a ventral nucleus in the ventral telencephalon (Vv) and a posterior nucleus in the dorsal telencephalon (Dp). We predict that these or other downstream regions use different decoding strategies, namely using different levels of temporal sensitivity to integrate synaptic input, to extract information about stimulus quality.

Towards these goals, we are studying the morphological, biophysical, and odor response properties of MC target neurons to characterize their role in processing of olfactory information, with particular interest in their sensitivity to synchronous input. Additionally, several methodological developments are ongoing. We have conducted pilot experiments using ballistic sparse neural tracing with the aim of characterizing anatomical properties of MC target regions. We have also built a prototype microfluidic device with automated pneumatic valve control for the purpose of delivering precise aqueous odor stimuli to the zebrafish olfactory epithelium.

[*]*Cindy Chiu is funded by the Gordon and Betty Moore Foundation*

**146. Synaptic transfer function in *C. elegans***

*Anusha Narayan*[*]

The neural circuits of the nematode *C. elegans* have been characterized based on anatomical connectivity. There is currently very little known about the dynamic functioning of these cells, and, more fundamentally, how information is transmitted across a *C. elegans* synapse. We intend to characterize a synaptic transfer function in *C. elegans*, to explore how the dynamic range of the post-synaptic response is set, and to understand the mechanisms for integration and gain control in this system.

In order to address these questions, one needs a controlled, precise way of stimulating the presynaptic cell. Channelrhodopsin (ChR2), a light-activated cation channel has been used to obtain genetically targeted optical control of neural activity, with a timescale of milliseconds [1]. In *C. elegans*, light activation of neurons and muscles expressing ChR2 has been shown to evoke behavioral responses [2]. Our plan is to use cell-specific promoters to express ChR2 in the presynaptic cell and gfp in the postsynaptic cell, and monitor cellular activity using whole-cell patch clamp. We selected the AFD-AIY synapse based on criteria including the following: a demonstrated behavioral relevance of the synapse, an absence of feedforward connections and direct feedback, and the presence of unique cell-specific promoters. AFD is a thermosensory neuron that measures the difference between ambient temperature and preferred temperature (temperature of cultivation). Imaging studies from AFD [3] indicate that AFD can phase-lock its calcium dynamics to oscillatory thermosensory inputs. AIY is an interneuron that is the primary postsynaptic partner of AFD. It also receives input from chemosensory neurons ASE, AWA and AWC. AIY is thought to mediate locomotor responses to chemosensory and thermal stimuli transduced though AFD, ASE and AWA [4]. Preliminary results indicate that the AFD-AIY synapse shows reliable tonic release.

Since ChR2 allows millisecond-timescale, targeted control of stimulus, with electrophysiology, it should be possible to obtain temporally precise information about the dynamics of the synaptic transfer function between AFD and AIY. This is the first step towards understanding the functional dynamics of neural sub-circuits in *C. elegans*.

[*]*Joint with Paul Sternberg, Professor, Division of Biology, Caltech*

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**147. Feedback inhibition in the mushroom body and gain control**

*Maria Papadopoulou*

The giant GABAergic neuron (GGN) is a single, paired, non-spiking neuron that arborizes extensively in the mushroom body (MB) [1], where it overlaps with the dendrites and the axons of Kenyon cells (KCs). KCs are the intrinsic neurons of the MB and are thought to be required for learning and memory [2]. We are interested in understanding the function of GGN in olfactory processing: in particular, its pattern of arborization makes it an attractive candidate for controlling or modulating KC responses to odors, with potential implications for learning and recall. Physiological recordings of KCs in locust show that these neurons respond sparsely to odors, by contrast with their excitatory input from the antennal lobe (projection neurons or PNs) [3]. Inhibition appears to be critical to control KC response threshold, probability and duration during odor stimulation [3]. We have shown that there exists a feedback loop whereby KCs provide excitatory input to GGN and this cell because of its GABAergic output contributes to the inhibitory control of KC excitability. As such, this neuron could act to control the gain of PN-to-KC information transfer and normalize KC-population output, making it independent of input strength. Using electrophysiological techniques, we are studying the properties and modes of action of GGN in locust.

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**148. Sparse and invariant representations of odor mixtures in the mushroom body**

*Kai Shen*

Principal neurons (PNs) of the locust antennal lobe (AL) exhibit odor-specific dynamic responses. And because not all PNs express the same patterns at the same time, the state of the AL network is dynamic, carried by an assembly of neurons that evolves in a stimulus-specific manner over time. These neuronal responses can be described geometrically as stimulus-specific trajectories reflecting the state of the AL network. How do these trajectories change with changes in the stimulus? Are there general rules to how these trajectories evolve? We addressed this question by first probing the PN network with small changes to the stimulus that varied progressively along a continuum. By varying the ratio of concentrations of two components within a binary mixture, we effectively morphed one unique odor to another. We observed a progressive rather than abrupt transformation from one odor-specific trajectory to another; thus, the PN network optimizes its encoding space to fully complement the stimulus parameter space.

To observe transformations across a more broad range of stimulus space, we systematically increased the complexity of the mixture by adding single odorants in a stepwise manner from 2 to 8 (e.g., AB, ABC, ABCD, → ABCDWXYZ) and presenting different combinations that overlap by varying amounts (e.g., ABCD, ABCX, BCWX, BDWX, DWYZ, WXYZ). To test the linearity of mixture representation, we estimated the ensemble PN response vectors to odor mixtures from the mixture components using a simple linear model. We then examined the degree of deviation between these estimated response vectors and the experimentally-observed response vectors. We find that for binary mixtures, there is good approximation between the estimated and experimental response vectors, but this approximation degrades rapidly when more odorants are added to the mixture. As expected, the PN ensemble output (as represented by stimulus-specific trajectories) due to single odor components (e.g., A), are very different to that of multi-component mixtures (4-, 5-, 8-, e.g., ABCDW).

Interestingly, when we recorded from the Kenyon cells (KCs), - the downstream targets of the PNs in the mushroom body, we find many KCs that are invariant across odor mixtures. For example, many KCs respond invariantly to mixtures containing A, for e.g., to A, AB, ABC, ABCD, ABCDW, etc., but to no other single components, like B, C, D. How is it possible that any downstream decoder can do this? We gain some intuition by analyzing the many PN ensemble subspaces that each downstream KC sees. We suspect that there exist many such subspaces within the AL, where the neural representations of odor components and more complicated mixtures overlap. In addition, and in collaboration with Anusha Narayan and Sina Tootoonian, we are building a simple feed-forward model of the PN-KC network, with minimal assumptions and nonlinearities. By comparing the statistics of the output of this model (model KCs) to our real experimental KCs, we hope to elucidate the essential mechanisms that give rise to such rich computations.

**149. Computational models of locust olfaction, and analysis of *Drosophila* courtship song**

*Sina Tootoonian*

I have worked on three projects over the past year. In the first, I calculated the probability of the observation of spontaneous local field potential (LFP) oscillations in the locust antennal lobe and found that, assuming the projection neurons (PNs) therein have Poissonian baseline firing statistics, spontaneous oscillations should be extremely rare. Hence under this assumption the 'spontaneous' oscillations observed during experiments are due to extra-experimental odors.

The PNs project to the lateral horn interneurons (LHIs), and to the Kenyon cells (KCs) of the mushroom body, a structure implicated in learning. The KCs fire much more sparsely than the PNs and can show odor- and odor-and-concentration-specificity. A computational model of the PN-LHI-KC interaction has been developed in the lab over the last few years to help determine the aspects of the network that produce the observed KC behavior. I have converted this model to C to improve run-times and to allow the simulation of larger networks. In collaboration with Anusha Narayan and Kai Shen, we are currently evaluating the performance of the model to ascertain whether additional experimentally observed features need to be incorporated into it to reproduce observed KC behavior.

The males of many *Drosophila* species will 'sing' to females during courtship by extending and vibrating their wings. These species-specific 'fly songs' modulate the success of courtship. I have been working with Mala Murthy, a post-doctoral scholar in the lab, on the analysis and characterization of fly songs that she has recorded from males of several *Drosophila* species. The main goals of the analysis are to allow quantitative comparisons of songs across species, and the synthesis of realistic fly song for playback to females whose auditory neurons are being recorded from. I have written software that uses simple wavelet techniques to automatically extract fly song from the audio recordings, and we are presently in the early stages of song analysis.

**Publications**

Du, J., Cassenaer, S., Lubenov, E.V., Laurent, G., Roukes, M.L., Siapas, A. and Masmanidis, S.C. (2008) Microelectrode arrays fabricated from ultra-thin si substrates for three-dimensional neural recording applications. *J. Microelectromechan. Sys.* Submitted.

Du, J., Riedel-Kruse, I.H., Nawroth, J.C., Laurent, G.J., Roukes, M.L. and Masmanidis, S.C. (2008) Three-dimensional extracellular recording of neuronal activity with multifunctional microelectrode arrays. Submitted.

Jayaraman, V. and Laurent G. (2007) Evaluating a genetically-encoded optical sensor of neural activity using electrophysiology in intact adult fruit flies. *Front. Neural Circuits*, doi:10.3389/neuro.04.003.2007.

Moreaux, L and Laurent, G. (2008) A simple method to reconstruct firing rates from dendritic calcium signal. *Front*. *Neurosci.* In press.

Moreaux, L. and Laurent G. (2007). Estimating firing rates from calcium signals in locust projection neurons *in vivo*. *Front. Neural Circuits*, doi:10.3389/neuro.04.002.2007.

Murthy, M., Fiete, I. R., and Laurent G. (2008) Testing odor response stereotypy in the *Drosophila* mushroom body. *Neuron* **59**:1009-1023.

Rabinovich, M., Herta, R. and Laurent, G. (2008) Transient dynamics for neural processing. *Science* **321**:48-50.

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**Support**: The work described in the following research reports has been supported by:
Alzheimer's Association
American Heart Association
Amgen, Inc.
G. Louis Fletcher
Della Martin
Moore Foundation
Michael J. Fox Foundation
National Heart, Lung and Blood Institute
National Institute on Aging
National Institute on Alcohol Abuse and Alcoholism
National Institute on Drug Abuse
National Eye Institute
National Institutes of Health
National Institute of Mental Health
National Institute of Neurological Disorders and Stroke
National Alliance for Research on Schizophrenia and Depression
Neurion Pharmaceuticals
Philip Morris External Research Program
Targacept, Inc.
University of California, Tobacco-Related Disease Research Program

**Summary**: Our lab studies the Cys-loop receptor family. These are channels that respond to acetylcholine, serotonin, GABA, glycine, and (among invertebrates) glutamate. Especially for nicotinic acetylcholine receptors, we study a range of topics: the genes, the receptor proteins, the effects on neurons, the organization of neurons in circuits, the resulting behavior of animals, and even neural events in humans. For instance, with Professor Dennis Dougherty's group in Caltech's Division of Chemistry and Chemical Engineering and Professor Sarah Lummis of Cambridge University, we apply new types of chemistry to understand how Cys-loop receptors transduce the binding of agonists into the opening of the channels.

We are describing the neural events that occur when an animal is chronically exposed to nicotine. We hypothesize that this set of responses underlies the pathophysiology of nicotine addiction, the world's largest preventable cause of death. We also hypothesize that the same processes underlie the benefits of two inadvertent therapeutic effects of smoking (the inverse correlation between smoking and Parkinson's disease, and the preventive effect of nicotine in autosomal dominant nocturnal frontal-lobe epilepsy). These complex neural processes involve events at all the levels that we study.

Several lab members are describing the most fundamental molecular / biophysical aspect of the process. We have been using the acronym S-CHARNS, for "selective chaperoning of acetylcholine receptor number and stoichiometry." We hypothesize that S-CHARNS is a thermodynamically-driven process leading to the classical observation that chronic exposure to nicotine causes "upregulation" of nicotinic receptors. If the hypothesis is proven, S-CHARNS is the molecular mechanism that shapes an animal's response to chronic exposure to nicotine. Over the next few years, we hope to produce cellular movies depicting first 24 hours of nicotine addiction—thought to be the most crucial stage in the process, especially for adolescents. We think we can literally display the spread of newly chaperoned, fluorescent receptors as they travel from the endoplasmic reticulum to the cell membrane.

Other lab members are generating mice with genetically modified nicotinic receptors—gain of function, not knockouts. Some mice have a hypersensitive subunit; in such mice, responses to nicotine represent selective excitation of receptors containing that subunit. Other mice have a fluorescent subunit, so that we can quantify and localize upregulation of receptors containing that subunit.

The human end of the scale is represented by our collaboration with Professor Johannes Schwarz of the University of Leipzig. Deep brain stimulation for Parkinson's disease often requires an initial session of microelectrode-based single unit recording, in order to localize the stimulating electrodes. We're analyzing the effects of nicotine in these single-unit recordings.

With Professor David Anderson, we are engineering channel variants in order to manipulate neurons. This project also uses our knowledge of Cys-loop receptors. We introduce *C. elegans* GluCl receptor channels into vertebrate neurons. Because the GluCl channels are activated by miniscule concentrations of the anthelmintic ivermectin and its derivatives, the neurons can be selectively silenced—and eventually, we hope, selectively activated.

Several of our projects lead naturally to drug discovery procedures. We have a drug discovery collaboration with Mike Marks and his group at the University of Colorado, Boulder; and with Targacept, Inc.

We also have interests in new techniques at the intersection of biophysics, single-molecule imaging,

chemistry, mouse genetics, and neuroscience. We're delighted to host visitors in our lab the third floor of the Kerckhoff Laboratory.

**150. Ligand-receptor interactions in the D2 dopamine receptor**

*Kiowa S. Bower[*], Henry A. Lester, Dennis A. Dougherty[*]*

G protein-coupled receptors play an important role in many aspects of human biology, including vision, olfaction, taste, memory, drug addiction, and cardiac function. Much important work has been done elucidating the structure and function of this class of receptors. This work has utilized structural, pharmacological, and homology studies, as well as conventional site directed mutagenesis. Although these investigations have successfully identified residues that are important for agonist binding and receptor activation, there are many specific aspects of ligand binding that are not understood. In this study we adapt the unnatural amino acid methodology to the GPCR system, and focus our investigations on the D2 dopamine receptor. The use of unnatural amino acids allows us to probe the function of important binding site residues in ways not possible with conventional mutagenesis. Our first goal is to determine whether dopamine forms a cation-π interaction with one of the conserved aromatic residues that form the ligand-binding site. Using a series of fluorinated analogues, we can incrementally reduce the cation-π binding ability of aromatic residues and definitively identify which residue makes this important interaction.

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**151. Specific *in vivo* activation of midbrain dopamine neurons via sensitized, high-affinity nicotinic acetylcholine receptors**

*Ryan M. Drenan, Sharon R. Grady[1], Paul Whiteaker[1,5], Tristan McClure-Begley[1], Sheri McKinney, Julie Miwa[2], Sujata Bupp[2], Nathaniel Heintz[2], J. Michael McIntosh[3], Merouane Bencherif[4], Michael J. Marks[1], Henry A. Lester*

α6-containing (α6*) nicotinic ACh receptors (nAChRs) are specifically expressed in dopamine (DA) neurons and participate in cholinergic transmission. We generated and studied mice with gain-of-function α6* nAChRs, which isolate and amplify cholinergic control of DA neuron activity. In contrast to gene knockouts or pharmacological blockers, which show necessity, we show that activating α6* nAChRs and DA neurons is sufficient to cause locomotor hyperactivity. $\alpha6^{L9'S}$ mice are hyperactive in their home cage and fail to habituate to a novel environment. Specific activation of α6* nAChRs with low doses of nicotine, by stimulating DA but not GABA neurons, exaggerates these phenotypes and produces a hyperdopaminergic state *in vivo*. Experiments with additional nicotinic drugs show that altering agonist efficacy at α6* provides fine-tuning of DA release and locomotor responses. α6*-specific agonists or antagonists may, by targeting endogenous cholinergic mechanisms, provide a new method for manipulating DA transmission in Parkinson's disease, nicotine dependence, or attention deficit hyperactivity disorder (ADHD).

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**152. Demonstration of functional α4-containing nicotinic receptors in the medial habenula**

*Carlos Fonck[1], Raad Nashmi[2], Ramiro Salas[3], Chunyi Zhou, Qi Huang, Mariella De Biasi[3], Robin A.J. Lester[4], Henry A. Lester*

The medial habenula (MHb) exhibits exceptionally high levels of nicotinic acetylcholine receptors (nAChRs), but it remains unclear whether all expressed nAChR subunit mRNAs are translated to form functional receptors. In particular α4 subunits have not been reported to have any functional role, despite strong α4 mRNA expression in the ventrolateral MHb. We studied a strain of knock-in mice expressing fluorescent α 4* nAChrs (α4YFP), as well as a knock-in strain expressing hypersensitive α4* nAChRs (α4L9'A). In α 4YFP mice, there was strong fluorescence in the ventrolateral MHb. In hypersensitive α4L9'A mice, injections of a low dose of nicotine (0.1 mg/kg) led to strong c-fos expression in only the ventrolateral region of the MHb, but not in the MHb of wild-type (WT) mice. In MHb slice recordings, ventrolateral neurons from α4L9'A mice, but not from WT mice, responded robustly to nicotine (1μM). Neurons in the medial aspect of the MHb had >10-fold smaller responses. Thus, α4*-nAChRs contribute to the selective activation of a subset of MHb neurons. Subunit composition analysis based on gain-of-function knock-in mice provides a useful experimental paradigm.

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**Acknowledgements**: This work was supported by PHS grants NS31669, DA17173, DA17279, California Tobacco-Related Disease Research Project, and the Philip Morris External Research Fund. R.N. was supported by a NARSAD Young Investigator Award.

**153. Single-channel recordings corresponding to the biphasic dose-response curve of a mutant nAChR**

*Shawnalea J. Frazier, Jai A.P. Shanata[*], Kristin Rule Gleitsman[*], Henry A. Lester*

Muscle nicotinic acetylcholine receptors are ligand-gated ion channels allosterically regulated by neurotransmitter binding events. Attempts at understanding the mechanism of allosteric transduction between the binding site and putative channel gate some 60 Å away have led to the categorization of particular extracellular domain residues as having specific functional roles in either ligand binding or channel gating events. One such residue, γW55/δW57, which participates in formation of the 'aromatic binding box', i.e., the site at which agonist binds, has been reported to elicit greater effects on the channel gating equilibrium than agonist affinity when conventionally mutated. Introduction of a backbone mutation at this site, accomplished by incorporating the unnatural amino acid α-hydroxy tryptophan to form an ester rather than an amide peptide bond, exhibited anomalous biphasic dose-response behavior when combined with another known gating mutation in the channel pore, βL9'S. TIRF imaging studies and stochastic simulations of this αβ9'γW55WahδW57Wah receptor confirm that the biphasic response is produced by a single receptor population. To date, single-channel recordings obtained at a variety of concentrations contain two main types of channel behavior, one with a high probability of channel opening and one with a low probability of channel opening. These two types likely contribute to the two components of the macroscopic dose-response curve. The dependence of the predominating type on agonist concentration may lead to further hypotheses that mono- and di-liganded openings are responsible for the lower and upper biphasic components, respectively. The possibility of modal gating is also currently being explored. Meanwhile, one of the initial hypotheses, that a significant concentration-dependent change in single-channel conductance could account for the two components, has been legitimately ruled out. Altogether, the recordings exhibit a trend in $P_{open}$ that follows that of the macroscopic response.

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**154. Characterizing long-range interactions in the nAChR**

*Kristin R. Gleitsman[1], Jai A.P. Shanata[*], Shawnalea J. Frazier, Henry A. Lester, Dennis A. Dougherty[*]*

The muscle nicotinic acetylcholine receptor (nAChR) is a pentameric ligand-gated ion channel (LGIC) with stoichiometry $\alpha_2\beta\gamma\delta$. Using two-electrode voltage clamp, we have discovered several sites in the extracellular domain (ECD) of the muscle nAChR which exhibit coupling to the pore domain, specifically to a mutation termed βL9'S. Introduction of the βL9'S mutation into wild-type (wt) muscle nAChRs results in a gain-in-function mutant with an $EC_{50}$ that is 40-fold lower (1.2 μM versus 50 μM for wt)—an effect which has been attributed to a significant increase in open channel lifetime. From the whole-cell data alone, this long-range interaction between the ECD and channel pore manifests itself as a decrease in the extent to which βL9'S causes gain-in-function.

In order to further characterize this apparent long-range interaction, we have performed single-channel recording (in the cell-attached configuration) using acetylcholine at $EC_{50}$ on six total mutants—three ECD mutants with and without the βL9'S. Our goal was to determine how this coupling is manifest in terms of the probability that a channel is open, $P_{open}$. Each of the ECD mutants tested shows a dramatic decrease in $P_{open}$, as well as a recovery of $P_{open}$ upon addition of the βL9'S mutation. These results are generally consistent with the whole-cell data, which indicate that $I_{max}$ increases greatly upon addition of the βL9'S mutation. Taken together, our whole-cell and single-channel recordings indicate that there is a long-range interaction between these ECD residues and the channel pore. We have also obtained data at a range of concentrations for one mutant with the goal of doing detailed modeling to determine the gating equilibrium constant.

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**155. A stereochemical test of a proposed mechanism for nicotinic receptor gating**

*Ariele P. Hanek[*], Henry A. Lester, Dennis A. Dougherty[*]*

Understanding the gating mechanism of the nicotinic acetylcholine receptor (nAChR) and similar channels constitutes a contemporary challenge in chemical neurobiology. In the present work, we use a stereochemical probe to evaluate a proposed pin-into-hydrophobic socket mechanism for the αVal46 side chain of the nAChR. Utilizing nonsense suppression methodology, we incorporated isoleucine (Ile), O-methyl threonine (Omt) and threonine (Thr), as well as their side chain epimers (the allo-counterparts). Surprisingly, our results indicate that only the Pro-S methyl group of the αVal46 side chain is sensitive to changes in

hydrophobicity, consistent with the precise geometrical requirements of the pin-into-socket mechanism.

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**156. Ezrin mediates tethering of the γ-aminobutyric acid transporter GAT1 to actin filaments via a C-terminal PDZ-interacting domain**

*P.I. Imoukhuede[1], Fraser J. Moss, Darren J. Michael[2], Robert H. Chow[2], Henry A. Lester*

A high density of neurotransmitter transporters on axons and presynaptic boutons is required for efficient clearance of neurotransmitter from the synapse. Therefore, regulators of transporter trafficking (insertion, retrieval, and confinement) can play an important role in maintaining the transporter density necessary for effective function. We determine interactions that confine GAT1 at the membrane by investigating the lateral mobility of GAT1-YFP8 expressed in neuroblastoma 2a (N2a) cells. Through fluorescence recovery after photobleaching (FRAP) we find that a significant fraction (~50%) of membrane-localized GAT1 is immobile. The mobility of the transporter can be increased by depolymerizing actin or by interrupting the GAT1 PDZ-interacting domain. Microtubule depolymerization, in contrast, does not affect GAT1 membrane mobility. We also identify ezrin as a major GAT1 adaptor to actin. Förster resonance energy transfer (FRET) determines that the distance between GAT1-YFP8 and ezrin-CFP is 64-68 Å. This distance can be increased by disrupting the actin cytoskeleton. Additionally, the disruption of actin results in a >3-fold increase in GABA uptake, apparently via a mechanism distinct from the PDZ-interacting protein. Our data reveal that actin confines GAT1 to the plasma membrane via ezrin, and this interaction is mediated through the PDZ-interacting domain of GAT1.

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**157. The influence of selective silencing the insular cortex on nicotine induced-seizures in a genetic mouse model of epilepsy**

*Herwig Just, Bruce N. Cohen, Henry A. Lester*

Traditionally, neuronal function in the central nervous system has been studied by introducing lesions into the brain region of interest. These techniques rely on the destruction of neuronal circuitry and are irreversible. We have established a method to reversibly inactivate neuronal function using a glutamate-gated chloride channel (GluCl) from *C. elegans* that is heterologously expressed in mammalian neurons. Activation of GluCl with the allosteric activator ivermectin (IVM) elicits a current that clamps the neurons to the resting potential of chloride, thus inhibiting action potentials ('silencing') and allows the study of neuronal function in the context of an intact circuit. The transgenic mouse carrying the V262L mutation in the nicotinic acetylcholine receptor (nAChR) β2 subunit serves as a model of human autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE) caused by an orthologous human mutation (nAChR β2 V287L). These transgenic mice exhibit nicotine-induced seizures that are hypothesized to originate in the insular cortex. To inhibit nicotine-induced seizures adeno-associated virus encoding GluCl α and β subunits were injected stereotaxically into the insular cortices of V262L mice. The animals were administered either saline or 10 mg/kg IVM 24 h before the experiment which was initiated with an intraperitoneal injection of 2 mg/kg nicotine. The resulting behavioral responses and the seizure severity were recorded. Unexpectedly, animals injected bilaterally with virus and given IVM displayed a significant increase in seizure severity. Nicotine-induced clonic convulsions and a loss of balance in all IVM-treated animals, but not in the saline treated controls. Animals pretreated with saline displayed only dystonic arousal complex (stereotypical head movements, forelimb dystonia) characteristic for these mice at this nicotine dose. Sectioning and visualization of GluCl channel expression by immunofluorescence confirmed that the virus had mediated specific expression of the silencing channels in the insular cortices. Further control experiments eliminated the possibility that IVM directly affects mouse behavior. Pretreatment of mutant (V262L) mice with IVM that were not injected with the GluCl adenovirus did not significantly affect the response to 2 mg/kg nicotine.

**158. Mutating glutamate-gated chloride channel to a cation-selective channel**

*Herwig Just, Sindhuja Kadambi, Bruce N. Cohen, Henry A. Lester*

The *C. elegans* glutamate-gated chloride channel (GluCl) belongs to the Cys-loop superfamily of ligand gated ion channels. Other members of this family include nicotinic acetylcholine receptors (nAChRs), glycine receptors, GABA receptors and the 5-$HT_3$ serotonin receptors. We are using the chloride-selective GluCl channel as a tool to selectively inactivate ("silence") neuronal activity in specific regions of the central nervous system *in vivo*. Selective activation of specific neuronal populations would also be a useful tool in the study of neuronal function in an intact circuit. Conversion of GluCl from anion-selective to cation-selective channel would achieve this goal. The GluCl channel consists of α and β-subunits that assemble into a heteropentamer. The subunits contain four transmembrane domains (TM) with an extracellular amino and carboxy terminus. Ion selectivity is determined by amino acids within TM2. Changing ion selectivity has been achieved for several ion channels in this superfamily: The cation selective nAChR α7 channel and 5-$HT_3$ receptors have been mutated to anion-selective channels. The inverse switch from an anion to a cation-conducting channel--as we intend for the GluCl channel--has been achieved with the glycine receptor and with an invertebrate serotonergic chloride

channel MOD-1. To modify GluCl from an anionic to a cation-selective channel, we have introduced mutations into the M2 domain of the GluCl α- and β -subunits. In the GluCl α-subunit, the -2' proline residue has been deleted (P304Δ) and the adjacent alanine has been replaced by a negatively charged glutamate (A305E). Position 13' has been modified from an alanine to a valine (A319V). In the β -subunit, we have mutated the -1' glycine residue to glutamate (G276E) and threonine in the 13' position to valine (T290V). GluCl α (triple mutant) and β (double mutant) cRNAs are transcribed to co-express the mutated GluCl subunits in *Xenopus* oocytes and perform two electrode voltage-clamp recordings. Shifts in reversal potential are measured by recording the membrane current ramping the membrane potential from -70 to +50 mV in the absence or presence of 1 μM IVM. Half of the NaCl in the bath solution is replaced by mannitol to measure changes in the ion selectivity that is determined by the direction of the shift of the reversal potential.

**159. Block of α4(L9'A)β2 nAChRs by quinpirole results in Parkinson's disease-like symptoms in knock-in mice**

*Sindhu Kadambi, Bruce Cohen, Andrew Tapper, Herwig Just, Outi Salminen, Ryan Drenan, Henry A. Lester*

A Leu to Ala mutation (L9'A) in the M2 transmembrane domain of the α4 nicotinic acetylcholine receptor (nAChR) subunit renders α4β2 nAChRs ~50-fold more sensitive to agonists (ACh and nicotine). Homozygous knock-in mice expressing this mutation demonstrate several distinct drug-induced behaviors, allowing for the study of α4* nAChRs in nicotine addiction and other neural disorders. Injecting 0.5-10 mg/Kg of the dopamine D2-like receptor (D2R) agonist quinpirole (i. p.) induces greater transient Parkinsonian symptoms (tremors, rigidity, and akinesia) in L9'A than in wild-type (WT) mice. Nicotine injections (0.015 mg/Kg) reverse the effects of quinpirole. In microdialysis experiments, quinpirole inhibits striatal dopamine release equally in L9'A and WT mice. We hypothesized that direct inhibition of the L9'A mutant receptors by quinpirole, or indirect inhibition via D2R activation, mediates the effects of quinpirole on L9'A mice. To test this, we expressed WT α4 β2 or L9'A nAChRs in *Xenopus* oocytes with, or without, the human D2R. At [ACh] equal to the mutant and WT $EC_{50}$, 5 μM quinpirole blocked the peak L9'A ACh response, regardless of D2R co-expression. The mean fractional response to 20 nM ACh of the $(\alpha4L9'A)_3(\beta2)_2$ subtype was 0.73 ± 0.036 SEM (16 oocytes). This block varies with subunit stoichiometry: mean fractional response to 600 nM ACh of $(\alpha4L9'A)_2(\beta2)_3$ receptors was 0.99 ± 0.040 (12 oocytes). Near-saturating ACh concentrations prevented quinpirole block of the $(\alpha4L9'A)_3(\beta2)_2$ receptors, suggesting competitive inhibition of these receptors by quinpirole. The results suggest that a direct block of the L9'A receptors by quinpirole mediates its effects on the motor behavior of the L9'A mice. The mechanism is under study; work on L9'A mouse brain slices demonstrate that quinpirole disinhibits striatal cholinergic interneurons.

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**160. Optimizing ivermectin-activated anion channels for neuronal silencing**

*Sindhu Kadambi, Henry A. Lester*

Our lab seeks to modulate neuronal excitability by employing ion channels that are activated pharmacologically rather than by gene activation. We are currently developing a $GABA_A$-like silencing strategy that utilizes a glutamate-activated chloride channel (GluCl) cloned from *C. elegans* to respond exclusively to the anti-parasitic drug ivermectin (IVM). GluCl receptors are pentameric Cys-loop receptors formed by α and β subunits around a central pore. We have modified the subunits for our purposes: A Tyr182Phe mutation has been introduced into the GluCl β subunit to eliminate glutamate responses. Both subunits have been codon-optimized for enhanced mammalian expression and labeled with fluorescent proteins for visualization. Previous proof-of-principle experiments have demonstrated the advantages and limitations of the GluCl/IVM system. We seek to improve the GluCl-IVM neuronal silencing technique by altering the properties of the expressed channels. Site-directed mutagenesis will be employed to enhance the potency of IVM to activate GluCl and increase the single-channel conductance of GluCl. Mutations will be introduced in the M2 region, a domain that has been shown to be important in modulating agonist sensitivity of other Cys-loop receptors. Mutations will be introduced within the large cytoplasmic loop of the receptor [the HA-stretch] to increase single channel conductance. The functional mutant constructs will be expressed in neuronal cells and "silencing" the firing of transfected neurons by application of IVM will be tested using electrophysiology techniques. These modifications will improve the GluCl-IVM by: (1) reducing the IVM dose required to activate GluCl *in vivo* and thereby minimizing non-specific effects and (2) increasing the effectiveness of the GluCl channel in hyperpolarizing the cell membrane, which "silences" the neurons.

**161. Confirming the revised C-terminal domain of the MscL crystal structure**

*Joshua A. Maurer[1,2], Donald E. Elmore[2,3], Daniel Clayton, Li Xiong[2], Henry A. Lester, Dennis A. Dougherty[3]*

The structure of the C-terminal domain of the mechanosensitive channel of large conductance (MscL) has generated significant controversy. As a result, several structures have been proposed for this region: the original crystal structure (1MSL) of the M. tuberculosis homologue (Tb), a model of the *E. coli* homologue (Ec), and most recently a revised crystal structure of Tb-MscL (2OAR). In order to understand which of these structures represents a physiological conformation, we have measured the

impact of mutations to the C-terminal domain on the thermal stability of Tb-MscL using circular dichroism and performed molecular dynamics simulations of the original and the revised crystal structures of Tb-MscL. Our results imply that this region is helical and adopts an α-helical bundle similar to that observed in the Ec-MscL model and the revised Tb-MscL crystal structure.

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**162. hERG $K^+$ channel drug block underlying acquired long-QT syndrome probed with natural and unnatural amino acid mutagenesis in mammalian cells**

*Fraser Moss, Shawna Frazier, Mark Nowak[1], Elisha Mackey[1], Nima Shiva[1], Dennis Dougherty[2], Henry A. Lester*

Through systematic mutagenesis of critical residues in the hERG pore to other natural and/or unnatural amino acids (UAAs) we aim to guide the rational redesign of molecules to reduce their hERG block. We perform our studies in mammalian cells because a major limitation in *Xenopus* oocytes is that the test substance accumulates in the oocyte yolk, resulting in significant inconsistencies in potency estimates. To validate the methodology, we examined the effect of the known hERG blockers risperidone, amperozide and sparfloxacin on the conventional hERG mutants T623S, S624A, S624T and Y652F when expressed in CHO cells. The wild-type $IC_{50}$ for each drug was compared to its $IC_{50}$ for each mutant and used to calculate ΔΔG, the difference between the Gibbs free energy of association between the drug and the wild-type channel versus the mutant channel. The ΔΔG's indicate the type of interaction between the drug and channel pore at each position. More precise probing of drug-channel interactions requires UAA mutagenesis of hERG. We identified a bacterial tyrosine aminoacyl-tRNA synthetase (bTyrRS)/tRNA (bTyr-$tRNA_{CUA}$) pair that rescue the expression of a hERG Y652TAG mutant. The voltage dependence and waveforms of rescued currents were identical to those of wild type, within experimental error. In control experiments, if either the bTyrRS or the bTyr-$tRNA_{CUA}$ were omitted from the transfection, no hERG expression was detected. Directed evolution of the bTyrRS binding site has generated a mutant that specifically aminoacylates our bTyrt$RNA_{CUA}$ with the UAA, cyclohexylalanine (CHA). Specific incorporation of CHA in Y652TAG and F656TAG hERG mutants expressed in mammalian cell lines is presently under investigation.

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**163. Quantitative and non-saturating methods for investigating oligomerization and trafficking of the γ-aminobutyric acid transporter, GAT1**

*Fraser J. Moss, P. I. Imoukhuede[1], Jia Hiu[2], Joanna L. Jankowsky, Michael W. Quick[2], Henry A. Lester*

To investigate the protein-protein interactions, trafficking, and oligomerization of mGAT1, the mouse γ-aminobutyric acid (GABA) transporter, we generated nineteen different fusions of mGAT1 with cyan, yellow or green fluorescent protein (CFP, YFP or GFP; collectively XFP). We investigated the function of these constructs in a quantitative, non-saturated [$^3$H]GABA uptake assay when expressed in mouse neuroblastoma 2A cells (N2a, a neuron-like cell line). We propose this system as a model transient expression system for the characterization of all transiently expressed mutant neuronal transporters. Nine of these fluorescent mGAT1 transporters were functionally identical to wild-type mGAT1. To function like wild-type mGAT1, the mGAT1XFP C-terminal fusions required their final three residues to constitute a naturally occurring PDZ-type II interacting motif. Additionally, a construct in which the XFP moiety was fused between residues 577 and 578 within the mGAT1 C-terminus was determined to be the first example of a fluorescent SLC6 transporter with wild-type function, trafficking or assembly where the XFP has been fused within the transporter coding sequence. We studied the assembly of the fluorescent mGAT1s using two different Förster resonance energy transfer (FRET) techniques; donor recovery after acceptor photobleach (DRAP) and pixel-by-pixel based analysis of sensitized FRET normalized to relative intracellular fluorophore expression (NFRET). The versatility of the NFRET technique was demonstrated by determining and comparing the oligomerization efficiencies of each fluorescent mGAT1 construct and by resolving differential NFRET in various subcellular compartments for a particular fluorescent mGAT1 construct. We determined that oligomerization is a prerequisite for expression mGAT1 at the plasma membrane. Furthermore, for cells cotransfected either with mGAT1CFP8/mGAT1YFP8 or mGAT1$^{577}$CFP$^{578}$CT/mGAT1$^{577}$YFP$^{578}$CT, which both function as wild-type mGAT1, the NFRET value at the plasma membrane was approximately double that measured in the perinuclear endoplasmic reticulum where newly synthesized mGAT1 protomers assemble. Therefore, the probability of an mGAT1 protomer being part of an oligomerized complex in the plasma membrane is approximately twice as likely as it being oligomerized in the perinuclear ER. The strong FRET signals from the constructs described in this study coupled with sensitized emission pixel-by-pixel NFRET analysis make them suitable to study critical events in transporter modulation such as substrate-mediated dissociation of transporter oligomers.

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**164. Single-molecule imaging of a fluorescent unnatural amino acid incorporated into nicotinic receptors**

*Rigo Pantoja, Erik A. Rodriguez*, Mohammed I. Dibas, Dennis A. Dougherty*, Henry A. Lester*

We report the first successful detection of a fluorescent unnatural amino acid (fUAA), Lys(BODIPYFL), incorporated into a membrane protein (the muscle nicotinic acetylcholine receptor (nAChR)) in a living cell. *Xenopus* oocytes were injected with a frameshift suppressor tRNA aminoacylated with Lys(BODIPYFL) and nAChR (α:β19'GGGU:γ:δ) mRNAs. We measured fluorescence from oocytes expressing the nAChR β19'Lys(BODIPYFL) using time-resolved total internal reflection fluorescence (TIRF) microscopy. Under conditions of relatively low receptor density (<0.1 receptors / $\mu m^2$), we observed puncta with diffraction-limited profiles that were consistent with the point-spread function of our microscope. Furthermore, diffraction-limited puncta displayed step decreases in fluorescence intensity, consistent with single-molecule photobleaching. The puncta densities agreed with macroscopic ACh-induced current densities, showing that the fUAA was incorporated and the receptors were functional. Dose-response relations for the nAChR β19'Lys(BODIPYFL) receptors were similar to those for wild-type (WT) receptors. We also studied nAChR β19'Lys(BODIPYFL) receptors labeled with α-bungarotoxin mono-conjugated with Alexa488 (αBtxAlexa488). The nAChR has two αBtx binding sites and puncta containing the Lys(BODIPYFL) labeled with αBtxAlexa488 yielded the expected three discrete photobleaching steps. We also performed positive control experiments with a nAChR containing enhanced green fluorescent protein in the γ subunit M3-M4 loop, which confirmed our nAChR β19'Lys(BODIPYFL) measurements. Thus, we report the cell-based single-molecule detection of nAChR β19'Lys(BODIPYFL).

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**165. Unnatural amino acid replacement scanning in *Xenopus* oocytes**

*Erik A. Rodriguez*, Dennis A. Dougherty*, Henry A. Lester*

Unnatural amino acids (UAAs) are becoming commercially available for detection of protein-protein interactions and detection of newly translated proteins. The UAAs are heterogeneously incorporated into all translated proteins in eukaryotic cells, but little is known about the effect on protein function and expression. We have heterogeneously incorporated UAAs into the mouse muscle nicotinic acetylcholine receptor (nAChR) expressed in *Xenopus* oocytes and determine changes in function and expression by the sensitive assay of electrophysiology, a technique termed unnatural amino acid replacement scanning (UAARS). UAARS with many UAAs caused alteration in function of the nAChR, including loss of function and gain of function phenotypes. The commercially available UAAs, photo-leucine and photo-methionine, were incorporated into the nAChR and were detected by increased molecular weight bands on a Western blot after UV cross-linking of the nAChR subunits. UAARS with many UAAs also resulted in alteration in functional expression of nAChR on the surface of *Xenopus* oocytes as determined by electrophysiology, but Western blots shows no loss in protein translation or increased protein degradation. UAARS will be useful to detect global alteration in protein function at a specific amino acid, create a large and chemically unique population of proteins, and to assess whether the protein translational machinery of eukaryotic cells accepts an UAA.

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**166. The role of cation-π interactions in muscle nicotinic receptor activation**

*Jai A. P. Shanata*, Bruce N. Cohen, Dennis A. Dougherty*, Henry A. Lester*

Our previous results show that a cation-π interaction between the agonist acetylcholine (ACh) and a tryptophan residue (αW149) in the extracellular domain (ECD) of the muscle nicotinic acetylcholine receptor (nAChR) dramatically enhances the ability of ACh to activate this receptor. To activate a receptor, the agonist must first bind to it and then induce conformational change(s) leading to activation. To determine which of these steps the cation-π interaction at residue αW149 primarily affects, we expressed wild-type (wt) and mono-fluorinated mutant (5-F-W149) nAChRs in *Xenopus* oocytes and recorded their single-channel activity in cell-attached configuration with 300 μM ACh. We previously found that modifying the cation-π interaction at residue αW149 by fluorinating this residue (5-F-W149) reduces the ACh sensitivity (i.e., increases the $EC_{50}$) of the muscle nAChR six-fold. Analyses of our single-channel kinetic data show this mutation reduced the ACh gating equilibrium constant (Θ) less than two-fold, far short of the 36-fold decrease required to account for the six-fold increase in $EC_{50}$ ($EC_{50} \sim \Theta^{-1/2}$). In contrast, fitting the wt and 5-F-W149 mutant single-channel data to a four-state kinetic model with three agonist-bound closed states suggests that the 5-F-W149 mutant reduced the apparent forward rate constant for ACh binding seven-fold, enough to account for the effects of tryptophan fluorination on the $EC_{50}$, but this rate constant could also reflect rapid desensitization of the receptor rather than agonist binding.

Our experiments using ACh did not allow resolution of the fastest rate constant (channel opening). Two different methods will be employed in order to verify that the binding rate, as opposed to Θ, is being diminished by fluorination. (1) The partial agonists tetramethylammonium and choline, which are known to have channel opening rates that are approximately 20 and 1000 times slower than ACh, respectively, will be used in

order to directly measure the channel opening rate. (2) Patches will be obtained at multiple ACh concentrations in order to estimate the opening rate from the effective opening rate by fitting the data across concentrations to various models.

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**167. Mutations causing autosomal dominant nocturnal frontal lobe epilepsy alter subunit stoichiometry of mouse α4β2 nicotinic acetylcholine receptors**

*Cagdas D. Son, Fraser J. Moss, Raad Nashmi[*], Bruce N. Cohen, Henry A. Lester*

The relationship between autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) and α4β2 neuronal nicotinic acetylcholine receptors (nAChRs) has been strongly reinforced by the report of five distinct nAChR mutations. Three of these mutations (S247F), (S252L) and (776ins3) are in the channel-lining domain (M2) of the α4 subunit, while two mutations (V287L) and (V287M) are in a similar region of the β2 subunit. To understand how these mutations alter nAChR function, we analyzed the effects of each mutation on the subunit stoichiometry by using Förster resonance energy transfer (FRET). The mutant subunits were expressed in mouse neuroblastoma (N2a) cells with fluorescently-tagged complementary wild-type subunits and imaged using a Nikon C1 laser-scanning confocal microscope system equipped with spectral imaging capabilities and a Prior remote-focus device. These images were later analyzed by two independent FRET analysis methods: i) donor recovery after acceptor photobleaching (DRAP); and ii) pixel based sensitized emission (PixFRET). Due to the distance dependency of FRET efficiency and the subunit organization in assembled nAChRs, observed FRET efficiencies from receptors that have three fluorescently-tagged subunits are significantly higher than the FRET efficiencies from receptors that carry only two tagged subunits. Our results showed that all five mutants tested had 10-20% higher $(\alpha4)_3(\beta2)_2$ stoichiometry compared to the wild-type controls. In this study we also generated fluorescently-tagged (YFP and CFP) subunits containing each of the five ADNFLE mutations. These tagged mutant subunits confirmed our findings with the untagged subunits and showed a similar shift towards a mostly $(\alpha4)_3(\beta2)_2$ receptor population.

We are now investigating the effects of chronic nicotine on this shift in subunit stoichiometry induced by the ADNFLE mutations. Initial studies with V287L mutation showed that chronic nicotine exposure reverse the subunit stoichiometry back to a wild-type distribution. This observation might help us partly explain the reported effects of nicotine on ADNFLE patients. It has been shown that the use of nicotine patches or tobacco significantly decreases seizure frequency in ADNFLE patients with nAChR point mutations. Investigating nAChR receptor changes in ADNFLE at the molecular level can thus, lead to the rational development of preventive or/and therapeutic drugs for this, as well as other similar disorders that result from rearrangements in nAChR subunit stoichiometry.

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**168. Development of fluorescence-based assays to study mechanisms of nAChR upregulation**

*Rahul Srinivasan, Rigo Pantoja, Henry A. Lester*

Our laboratory has characterized fluorescently-tagged neuronal nicotinic acetylcholine receptor (nAChR) subunits that are readily visualized using fluorescence microscopy and possess similar pharmacological properties as wild-type receptors. These XFP-tagged receptors (where X = cyan (C), green (G), or yellow (Y)) can be employed to study the mechanisms underlying nAChR upregulation. Fluorescence-based assays such as Förster's resonance energy transfer (FRET) and total internal reflection fluorescence microscopy (TIRFM) have been utilized to respectively detect intracellular receptor assembly and expression at the plasma membrane. The α4 and β2 nAChR subunits were re-engineered to contain fluorophores that are superior to the first generation constructs. Several monomeric enhanced XFP (meXFP) variants including meYFP, mvenus, mcerulean, meGFP and mcherry were cloned into α4 and β2 nAChR subunits and tested for receptor assembly using FRET in transiently transfected mouse neuroblastoma (N2a) cells. Specific pairs tested were mcerulean/mvenus, mcerulean/meYFP and meGFP/mcherry. These experiments revealed that the α4-mcherry and β2-meGFP nAChR FRET pair displayed optimal properties such as a wide spectral separation of excitation and emission maxima, increased donor photostability and a significant reduction in FRET variability. Following incubation with 1 μM nicotine for 48 h, α4-mcherry/ β2-meGFP transfected N2a cells displayed robust increases in intracellular FRET, indicative of increased receptor assembly in the presence of nicotine. In addition, TIRFM showed that nicotine incubation significantly increased trafficking of α4-mcherry/β2-meGFP nAChRs to the cell surface. 48 h co-incubation of cells with nicotine (1 μM) and the competitive α4β2 nAChR antagonist, dihydro-beta-erythroidine (1 μM) prevented nicotine-induced increases in FRET, pointing to a putative role of nAChR activation in receptor upregulation. Future experiments to dissect the role of nAChR activation in upregulation will involve the co-incubation of nicotine with non-competitive nAChR antagonists such as mecamylamine and the use of membrane impermeable agonists such as tetramethylammonium. The FRET efficiency following each drug or drug pair treatment will be determined. These experiments will provide insight into the contribution of surface nAChR activation to receptor upregulation.

**169. Role of β2 subunits in nicotine-induced nAChR upregulation**

*Rahul Srinivasan*, Rigo Pantoja*, Henry A. Lester*

We are investigating the role of β2 neuronal nicotinic acetylcholine receptor (nAChR) subunits in nicotine-induced α4β2 receptor upregulation. To delineate the effect of β2 on α4 nAChR trafficking in the absence of nicotine, mouse neuroblastoma (N2a) cells were transiently transfected with either α4-meGFP/wildtype β2 or α4-meGFP/wildtype β4 subunits (m = monomeric; e = enhanced). Cells were imaged at 48 h post-transfection by total internal reflection fluorescence microscopy (TIRFM), a technique that allows plasma membrane (PM) visualization. To set TIRFM parameters, cells were co-transfected with the pCS2-mcherry plasmid, which served as a reference probe. pCS2-mcherry expresses mcherry with a lyn kinase membrane localization signal, allowing visualization of the PM using red emission from mcherry. The α4-meGFP reporter was used to detect receptor expression at the PM. Results showed that the α4-meGFP/wildtype β2 receptors trafficked to the PM in ~10 % of the cells while ~90 % of imaged cells displayed α4-meGFP/wildtype β4 at the PM. In the presence of nicotine (1 μM for 48 h), α4-meGFP/wildtype β2 transfected N2a cells displayed a clear increase in receptor trafficking to the PM when visualized using TIRFM. These results point to a modulatory role of β2 subunits in α4β2 nAChR trafficking and possibly, nicotine-induced receptor upregulation. The β2 nAChR subunit possesses a putative endoplasmic retention (ER) reticulum motif (RXR) in the intracellular loop between M3 and M4 domains. In future experiments, we will engineer chimeric β2 and β4 subunits in which the M3-M4 domains of β2 and β4 are exchanged such that the β2 contains the β4 M3-M4 loop (β2- β4). A reciprocal construct with β4 containing the β2 M3-M4 will also be created (β4- β2). N2a cells transfected with either α4-meGFP/ β2- β4 or α4-meGFP/β4-β2 will be examined by TIRFM for PM trafficking of receptors in the presence or absence of chronic nicotine exposure. These experiments will pave the way for detailed mechanistic investigations of chronic nicotine effects on β2 subunit-mediated ER retention of α4 β2 nAChRs.

**These authors contributed equally to this work.*

**170. Imaging the organizational structure and composition of neuronal receptors**

*Lawrence A. Wade, Scott E. Fraser, Henry A. Lester*

Nicotinic acetylcholine receptors (nAChRs) are found in many central nervous system and nerve-skeletal muscle postsynaptic membranes. The nAChR is a (pseudo)symmetric pentameric structure comprised of homologous subunits. This straightforward structure is complicated by there being five major groups of subunits that can be arranged in a variety of permutations: α, β, γ, δ, and ε. Furthermore the α subunits exist in at least ten different subtypes (α 1 through α 10) and the β subunits exist in at least four subtypes (β1 through β4). Therefore, a remarkable variety of specific assemblages are possible with a corresponding variety of chemical reaction rates.

We've initiated an effort that combines several novel techniques to directly image the microorganization of nAChRs expressed in *Xenopus* oocytes. By expressing different color XFPs in specific AChR α and β subunits, we expect to resolve individual receptors. Furthermore, we hope to discretely identify individual receptors over the field-of-view. This will enable us to simultaneously characterize the large-scale distribution of such receptors, to identify local structures within an imaged membrane surface, and to assay variation of receptor subtypes.

The techniques being combined in this experiment include a novel method for repeatedly aligning the atomic force microscope probe and excitation laser with specific substrate locations to within a few 10's of nm, and an ~10 nm resolution, single-molecule sensitive near-field optical microscope. Receptor subunit imaging is enabled by the Lester group's development of specific XFP-labeled AChR subunits.

By developing the ability to directly image each subunit of each AChR expressed in the membrane of *Xenopus* oocytes, we lay the groundwork for future studies of transmembrane proteins, receptors and membrane-embedded proteins in many different types of cells. In particular we will substantially advance our ability to image the microorganization of nicotinic acetylcholine receptors at neuronal synapses.

**Support**: JPL R&TD and BioNano programs, NASA-PRSGC IDEAS-ER grant, Caltech President's fund, NIH, NSF and Pharmagenomix grants, NS-11756, DA-17279, and Philip Morris External Research Program.

This work has been supervised by Professor Scott Fraser in collaboration with Professor Henry Lester.

**171. α4β2* and α7 nicotinic receptors modulate medium spiny neuron in the dorsal striatum**

*Cheng Xiao, Raad Nashmi*, Haijiang Cai, Purnima Deshpande, Chunyi Zhou, Henry A. Lester*

Medium spiny neuron (MSN) is the principal and projection neuron in the dorsal striatum, which is implicated in motor control. Nicotinic acetylcholine receptors (nAChRs) are among the promising targets for the treatment of movement disorders, while it remains unclear how nAChRs modulate the activity of MSN. To address this issue, we did patch-clamp whole-cell recordings in the dorsal striatum of acute brain slice. Somatodendritic responses to puffed nicotinic agonists were detected in a considerable portion of striatal neurons, including MSN and interneurons. A total of 27 out of 28 nicotinic responses were primarily produced by α7 nAChRs. The α7 nAChRs could not be activated, but was

desensitized by 1 μM nicotine. These receptors can be desensitized by nicotine (1 μM). Nicotine (1 μM) had no effect on spontaneous inhibitory postsynaptic currents onto MSNs. In contrast, nicotine enhanced evoked excitatory postsynaptic currents (EPSCs), and the frequency, but not amplitude, of spontaneous EPSCs (sEPSCs). Surprisingly, both desensitization and blockade of nAChRs enhanced sEPSC frequency, and occluded nicotine enhancement of sEPSC frequency. Similarly, both nicotine and DHβE enhanced EPSP-spike coupling in MSN. Interestingly, the blockade of $D_2$ receptor mimicked and attenuated the facilitation of sEPSCs by nicotine and DHβE, suggesting a significant contribution of the dopamine system. In brain slices of α4 nAChR subunit knock-out mice; however, nicotinic antagonists did not change sEPSC frequency, and nicotine did not change EPSP-spike coupling, in MSN. Taken together, the data show a neural circuit underlying nicotine modulation of MSN excitability in the dorsal striatum. That is, somatodendritic α7 nAChRs in MSN respond to higher concentrations of nicotine; endogenous ACh continually activates α4β2* receptors on dopaminergic axon terminals to enhance the release of dopamine; dopamine acts on the $D_2$ receptors of corticostriatal glutamatergic terminals, inhibiting glutamate release; smoked concentrations of nicotine desensitization of α4β2* nAChRs eliminates this process, producing enhanced MSN activity.

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**172. Chronic nicotine enhances GABAergic inhibition to dopaminergic neurons**

*Cheng Xiao, Sheri McKinney, Henry A. Lester*

The degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) is a hallmark of Parkinson's disease (PD) pathology. Accumulating evidence shows an inverse correlation between smoking and PD, suggesting that nicotine confers neuroprotection in PD. To address the underlying mechanism, we subjected mice to continuous infusion of nicotine (2 mg/kg/h) or vehicle (saline) for 10-14 days. We recorded nicotinic currents (3 – 1000 μM ACh) from voltage-clamped SN neurons in brain slices. Also we recorded spontaneous firing from SN neurons using either current-clamp recordings in brain slices or *in vivo* single unit recordings. In chronic nicotine-treated mice, ACh (3, 10, 30, and 1000 μM) induced larger nicotinic currents in SNr GABAergic neurons than in vehicle controls, but there was no difference in nicotinic currents in SNc DA neurons between nicotine- and saline-treated mice. In addition, the frequency of spontaneous inhibitory postsynaptic currents, recorded from SNc DA neurons, is significantly higher in chronic nicotine-treated mice than chronic vehicle-treated mice. Interestingly, (1) in wild-type mice, chronic nicotine suppressed SNc DA neuronal activity, but enhanced SNr GABAergic neuronal activity, while in α4 knock-out mice, chronic nicotine did not change the activity of either SNc DA or SNr GABAergic neurons; (2) in chronic nicotine-treated mice, the $GABA_A$ receptor blocker (SR95531, 10 μM) induced a stronger excitation of current-clamped SNc DA neurons. In the presence of 10 μM SR 95531, the activity of SNc DA neurons became the same in chronic nicotine- and saline-treated mice. In conclusion, chronic nicotine cell-specifically increases both the number and function of α4* nAChRs in SNr GABAergic neurons. Furthermore, chronic nicotine amplifies α4* nAChR responses to ambient ACh in SNr GABAergic neurons, which enhances the activity of those neurons and provides a stronger inhibition to SNc DA neurons. Here, we provide a possible mechanistic framework for neuroprotection of SNc DA neurons: synaptic interactions are altered within a neuronal circuit through cell-specific upregulation of nAChRs with chronic nicotine.

**173. Nicotine binding to brain receptors requires a strong cation-π interaction**

*Xinan Xiu[*], Nyssa L. Puskar[*], Jai A.P. Shanata[*], Henry A. Lester, Dennis A. Dougherty[*]*

Nicotine addiction begins with high-affinity binding of nicotine to acetylcholine (ACh) receptors in the brain. The consequence of this chemical interaction is >4,000,000 smoking-related deaths worldwide and the largest source of preventable mortality in developed countries. Pleasure, stress reduction, improved cognition, and other CNS effects are dominant effects of smoking, in part because closely related ACh receptors at neuromuscular junctions throughout the body are relatively insensitive to nicotine. Despite much pharmacological, functional, and structural information for ACh receptors, the differential action of nicotine on brain vs muscle ACh receptors has not been determined. Here we show that the high affinity of nicotine for α4β2 brain receptors is the result of a strong cation-π interaction, as well as an enhanced hydrogen bond interaction, between nicotine and a specific aromatic amino acid of the receptor, TrpB. Although the immediate binding site residues, including the key TrpB, are identical in the brain and muscle receptors, subtle differences within or near the binding site apparently influence the strength of the cation-π interaction for nicotine, eliminating it entirely for the muscle receptor and rationalizing the low affinity of nicotine. A recently released smoking cessation drug, varenicline, targets α4β2 Ach receptors; and ACh receptors are also established therapeutic targets for Alzheimer's disease, schizophrenia, Parkinson's disease, pain, attention deficit-hyperactivity disorder, epilepsy, autism, and depression. Our results provide guidance for ongoing efforts to develop drugs that target specific types of nicotinic receptors.

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**174. Increased sensitivity to nicotine and altered sleep-wake pattern in β2 V287L ADNFLE knock-in mice**

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Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is characterized by brief motor seizures that predominantly occur during sleep. Mutations in genes coding for the neuronal nicotinic acetylcholine receptor (nAChR) α4 and β2 subunits are strongly associated with ADNFLE. In the present study, we generated and studied a knock-in mouse carrying a β2 subunit V287L mutation, a missense mutation found in human ADNFLE patients. Mutant β2 V287L mice are born at an expected Mendelian ratio and appear normal in many respects. However, we found a high rate of sudden deaths in homozygous mutant β2 V287L mice (HOM). Although spontaneous epilepsy is rare in mutant animals, both heterozygous (HET) and HOM mutants are hypersensitive to several aspects of nicotine-induced behavior changes including seizures, dorsiflexion, hypothermia and immobility. There are no detectable electroencephalographic signals associated with the nicotine-induced seizures. Synaptosomes from β2 V287L mice show markedly decreased agonist-induced $^{86}Rb$ influx and a shift to higher agonist sensitivity, a ~3-fold lower overall $EC_{50}$ for nicotine-induced [$^{3}$H]dopamine release, but only modest changes in [$^{125}$I]epibatidine binding. Activity-rest patterns are extensively altered in mutant mice, suggesting that sleep-wake disruption may underlie symptoms in ADNFLE patients. The β2 V287L mice share several major characteristics with ADNFLE mice previously constructed with α4 subunit mutations, just as ADNFLE patients have similar symptoms whether their mutations are linked to α4 or β2 subunits. Mouse models for ADNFLE, therefore, provide a valuable set of strains for investigating the pathophysiology of epileptogenesis in this disease.

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**Support:** The work described in the following research reports has been supported by:
Anne P. and Benjamin F. Biaggini Chair in Biological Sciences
Autism Speaks Foundation
California Institute of Regenerative Medicine
Caltech Brain Imaging Center Discovery Grant
Foundation Blanceflor Boncopagni-Lu Dovisi
Hereditary Disease Foundation
John Douglas French Alzheimer's Foundation
McGrath Foundation
McKnight Neuroscience of Brain Disorders Award
National Institute of Aging
National Institute of Mental Health
National Institute of Neurological Disease and Stroke
National Research Service Award, National Institutes of Health
Simons Foundation
Weston Havens Foundation

**Summary:** Much of the research in this laboratory involves the study of interactions between the nervous and immune systems. Using knockout mice and over-expression *in vivo* with viral vectors, we are exploring the role of the neuropoietic cytokine leukemia inhibitor factor (LIF) in regulating neural stem cell proliferation and fate in the adult brain. In the context of neuroimmune interactions during fetal brain development, we are investigating a mouse model of mental illness based on the known risk factor of maternal influenza infection. Huntington's disease (HD) is another focus, where we are investigating potential therapies using intracellular expression of antibodies (intrabodies) and also manipulating NFκB activity.

Cytokines are diffusible, intercellular messengers that were originally studied in the immune system. Our group contributed to the discovery of a family that we have termed the neuropoietic cytokines, because of their action in both the nervous and hematopoietic/immune systems. We demonstrated that one of these cytokines, LIF, can coordinate the neuronal, glial and immune reactions to injury. Using both delivery of LIF *in vivo* and examination of the consequences of knocking out the LIF gene in mice, we find that this cytokine has a powerful regulatory effect on the inflammatory cascade. Moreover, LIF can regulate neurogenesis and gliogenesis. LIF is a critical regulator of astrocyte and microglial activation following stroke, seizure and trauma, and this cytokine also regulates inflammatory cell infiltration, neuronal and oligodendrocyte death, gene expression, as well as adult neural stem cell renewal. These results highlight LIF as an important therapeutic target. We are currently examining the role of LIF in a chemical model of multiple sclerosis, where exogenous LIF can increase oligodendrocyte number and stimulate remyelination.

Cytokine involvement in a model for mental illness is also being investigated. This mouse model is based on findings that maternal infection can increase the likelihood of schizophrenia or autism in the offspring. We are using behavioral, neuropathological, molecular and brain imaging methods to investigate the effects of activating the maternal immune system on fetal brain development and how this leads to altered behavior in young and adult offspring. Recent results indicate that the cytokine IL-6 is key in mediating the effects of maternal immune activation on fetal brain development.

We are utilizing intracellular antibody expression to block the toxicity of mutant huntingtin (Htt), the protein that causes HD. We have produced single chain intrabodies that bind to various domains of Htt, and these can either exacerbate or alleviate Htt toxicity in cultured cells, acute brain slices, and in *Drosophila* HD models. Current work is evaluating the efficacy of viral delivery of intrabodies in several mouse models of HD. Promising results have recently been obtained in one of the mouse models. We have also implicated the NFκB signaling pathway in the pathogenesis of HD, and identified several steps in this signaling cascade as potential therapeutic targets.

### 175. Interleukin-6 mediates many of the effects of maternal immune activation on fetal brain development

*Stephen Smith, Paul H. Patterson*

Maternal infection by several different organisms has been implicated in the pathogenesis of schizophrenia. Maternal influenza infection or maternal immune activation (MIA) with the double-stranded RNA, poly(I:C), or with bacterial lipopolysaccharide (LPS) in rodents causes behavioral, histological and transcriptional changes in adult offspring. This indicates that MIA, rather than a specific pathogen, is responsible for the increased risk of mental illness in the offspring of mothers with infections during pregnancy. In investigating the possibility that cytokines may mediate the effects of MIA, we find that the cytokine interleukin-6 (IL-6) is essential for the manifestation of a variety of abnormalities in the adult offspring of poly(I:C)-treated mothers. Pregnant mice given a single injection of IL-6 on E12.5 show deficits in pre-pulse inhibition of the acoustic startle response (PPI) as well as deficits in latent inhibition (LI). Pregnant mice given an injection of poly(I:C) on E12.5

also show PPI, LI, exploratory and social interaction deficits, as previously reported. Co-administration of an anti-IL-6 neutralizing antibody with the poly(I:C) prevents all of these deficits, while co-administration of anti-interferon-γ or anti-IL-1β does not. Anti-IL-6 also prevents maternal poly(I:C)-induced changes in gene expression in the adult frontal cortex. Finally, maternal injection of poly(I:C) in IL-6 knockout mice does *not* cause behavioral deficits in the offspring. Thus, IL-6 is necessary for MIA to produce autism- and schizophrenia-like behaviors that are manifested in the adult offspring. Current research is exploring the site(s) of IL-6 action.

**176. Identifying the sites of interleukin-6 action following maternal immune activation**

*Elaine Hsiao, Paul H. Patterson*

Maternal infection increases the risk for schizophrenia and autism in the offspring. In rodents, maternal influenza infection or maternal immune activation (MIA) with the double-stranded RNA, poly(I:C) causes behavioral, histological and transcriptional changes in adult offspring that are consistent with those seen in schizophrenia and autism. This indicates that MIA, rather than a specific pathogen, is responsible for the increased risk of mental illness in the offspring of mothers with infections during pregnancy. In investigating the possibility that cytokines may mediate the effects of MIA, it was determined that the cytokine interleukin-6 (IL-6) is essential for the manifestation of a variety of abnormalities in the adult offspring of poly(I:C)-treated mothers. Therefore, localizing the site of IL-6 action may illuminate the anatomical and molecular pathways through which MIA alters fetal brain development. Towards that end, experiments are underway to identify the sites of IL-6 receptor activation as well as the tissues where genes regulated by IL-6 are being altered following MIA. The candidate target areas are the maternal immune system, the placenta and the fetal brain.

**177. Maternal influenza infection alters fetal brain development**

*Limin Shi, Doris Tse[*], Paul H. Patterson*

Epidemiological studies have shown that maternal infection can increase the risk for mental illness in the offspring. In a mouse model of maternal respiratory infection with influenza virus, the adult offspring display striking behavioral, pharmacological and histological abnormalities. In addition to a spatially localized loss of Purkinje cells that is very similar to the common cerebellar pathology in autism, we find delayed migration of granule cells (GCs) in lobules VI and VII. During development, GCs are born in the external granular layer (EGL) and migrate through the molecular layer (ML) to their final position in the internal granular layer (IGL). On P17, a time at which the EGL is disappearing in control mice, the EGL is significantly thicker in the offspring of infected mothers. This effect is most pronounced in lobules VI and VII, consistent with the localized deficit in PCs. The abnormally persistent EGL is eventually lost, however, as Nissl staining in adult animals reveals the normal absence of an EGL in both control and exposed offspring. To determine if the thicker EGL is due to a migrational delay, BrdU was injected at P11 to label newly generated GCs, and the mice sacrificed at P17. We find significantly more BrdU+ GCs in the ML of lobule VII of exposed mice, suggesting a spatially localized migrational delay in exposed animals. No GCs are found in the ML of adult animals, however. These findings in the mouse model are relevant to the pathology and behavioral abnormalities that have been linked to cerebellar pathology in autism and schizophrenia.

[*]*UC Riverside student*

**178. Interaction between genes and environment in a mouse model of mental illness**

*Catherine Bregere, Paul H. Patterson*

Although it is recognized that both environmental and genetic factors are involved in the pathogenesis of autism and schizophrenia, their respective contributions to these disorders have been investigated independently. It is now possible to model an environmental risk factor, which has both face and construct validity for these disorders, and apply it to mouse models of several newly identified candidate genes, and study possible synergistic interactions. The environmental risk factor model involves maternal immune activation (MIA), using respiratory infection or injection of the viral mimic, poly(I:C). The offspring of MIA mice display behaviors and neuropathologies reminiscent of autism and schizophrenia. We have now initiated a study to assess whether mutant mice carrying a candidate gene are more vulnerable to MIA. Disrupted in schizophrenia-1 (DISC1) is a gene that is linked to mental illness, and several different lines of mice with mutant DISC1 display behavioral deficits consistent with schizophrenia. Preliminary evidence from mating wildtype female mice with heterozygous DISC1 males suggests that heterozygous DISC1 fetuses display increased sensitivity to polyIC. Additional mutant mouse lines relevant to mental diseases, including urokinase-type plasminogen activator receptor (uPAR) knockout mice will be similarly evaluated in the near future.

**179. Information processing in the hippocampus of the offspring of immune-activated mothers**

*Hiroshi Ito, Stephen Smith*

Maternal immune activation by injection of the dsRNA, poly(I:C), causes the offspring to display a series of behavioral abnormalities that are consistent with those seen in schizophrenia and autism. Several of these behaviors, including increased responses to low doses of amphetamine, as well as disrupted latent inhibition, suggest altered function of hippocampal and dopamine systems. To examine this possibility, we made hippocampal slices from adult mice born to control or immune-activated mothers, and measured the electrophysiological responses of CA1 pyramidal neurons. The offspring of poly(I:C)-treated mice show increased amplitude and decreased frequency of spontaneous

miniature excitatory post-synaptic currents, suggesting abnormal synaptic structure or function. Dopamine sensitivity in CA1 is of interest because dopamine is known to depress excitatory responses at temporoammonic-CA1 synapses. Compared to controls, we find that dopamine-induced excitatory depression is significantly larger in the slices from offspring of poly(I:C)-treated mothers, indicating a higher sensitivity to dopamine. Taken together, our physiological data suggest that the offspring of poly(I:C)-treated mothers exhibit abnormal information processing in the hippocampus.

**180. Maternal immune activation impairs extinction of the conditioned eyeblink response in the adult offspring**

*Ka Hung Lee[1], Stephen Smith, Soyun Kim[1], Paul H. Patterson, Richard F. Thompson[1]*

We are investigating classical eyeblink conditioning, a behavior that is abnormal in autistic subjects, in the adult offspring of poly(I:C)-injected mice. Pregnant mice are injected poly(I:C) or saline on embryonic day 12.5, and adult offspring are tested in a delay eyeblink-conditioning paradigm. Compared to the saline group, the offspring of the poly(I:C)-activated mothers show impaired extinction of the conditioned response. Auditory brainstem responses and tail-flick latency are similar in both groups, indicating normal auditory and somatosensory functions. The impaired extinction in the poly(I:C) offspring suggests that perseverative motor behavior, a characteristic of autism, is induced in the offspring by maternal immune activation.

[1]*University of Southern California*

**181. The effect of maternal immune activation on early behavioral development of mouse offspring**

*Natalia Malkova, Paul H. Patterson*

We are investigating the neurobehavioral development of mouse pups born to mothers whose immune systems were activated at mid-gestation. Tests include the rate of ultrasonic vocalizations, which is important for mother–infant social interaction, maturation status of neuromotor reflexes, and neurogenesis. We find that injection of double-stranded RNA (poly(I:C)), which evokes an inflammatory response in the mother similar to that induced by influenza virus, alters the behavior of the offspring. Compared to controls, 10-day-old C57BL/6J pups born to mothers given poly(I:C) on E12.5 have lower rates of ultrasound calling when separated from their mothers. Analysis of temporal organization of pup ultrasonic vocalizations shows that, compared to controls, pups born to poly(I:C)-treated mothers emit more single calls than calls in bouts. We also monitored developing motor reflexes such as negative geotaxis, righting and grasping, and used body weight as an indicator of general health. No difference is found in the physical abilities and general health between the control and experimental groups. Thus, the deficit in the social behavior of pups born to mothers with an activated immune system is not due to a delay in physical development. The absence of detectable differences in maternal responsiveness towards the pups also suggests that the fewer distress calls emitted by pups are the result of a reduced sensitivity to isolation.

It is known that FOXP2 is a genetic factor in the pathogenesis of speech-language disorder and Foxp2 knockout mice have deficit in ultrasonic vocalization. Therefore, studies are in progress to analyze whether FOXP2 expression is affected in the pups born to mothers whose immune systems were activated.

**182. IκB kinase complex regulates cleavage of huntingtin protein**

*Ali Khoshnan, Jan Ko*

Cleavage of huntingtin (Htt) protein plays a key role in the pathogenesis of Huntington's disease (HD). However, the environmental signals and molecular pathways that regulate this event are not well characterized. One potential factor is the accumulation of DNA damage that is known to occur in HD neurons. We have discovered that the DNA damaging agent etoposide stimulates cleavage of endogenous Htt in cultured human neurons, generating N-terminal fragments of ~85 kDa. Etoposide also stimulates cleavage of full-length mutant Htt, which could lead to accumulation of neurotoxic fragments. Moreover, we find that etoposide-induced Htt cleavage is regulated by the IκB kinase β (IKKβ). Silencing IKKβ using a small hairpin RNA, or small molecule inhibition of its activity, blocks Htt cleavage and promotes neuronal survival. In terms of mechanism, IKKβ phosphorylates the pro-survival protein Bcl-xL, promotes its degradation and leads to the activation of caspase-3 and subsequent Htt cleavage. Inhibition of IKKβ prevents etoposide-stimulated degradation of Bcl-xL. Moreover, neurons engineered to express elevated levels of Bcl-xL resist etoposide-induced caspase-3 activation and Htt cleavage. These data indicate that IKKβ regulates stress-induced Htt cleavage and is therefore a potential target for regulating Htt turnover.

**183. Validation of IKKβ as a therapeutic target for HD**

*Ali Khoshnan, Jan Ko*

The role of the IβB-kinase complex (IKK) in neuronal survival and degeneration is not well understood. In non-neuronal cells, IKK regulates the activity of the transcription factor NF-βB. The core components of the IKK complex include two serine-threonine kinases, IKKα (IKK1) and IKKβ (IKK2), and a regulatory, non-catalytic module, IKKγ (NEMO). IKKα and IKKγ also have NF-κB-independent functions. We previously showed that binding of mutant Htt activates IKKβ in neuronal and animal models of HD (Khoshnan *et al*., 2004). Moreover, IKKβ promotes stress-induced cleavage of full-length Htt, which could result in build up of oligomeric neurotoxic fragments. Therefore, Htt cleavage and IKKβ activation can form a positive feedback loop that could perpetuate neuronal degeneration. We are testing the hypothesis that

reduction of IKKβ expression could ameliorate HD pathology. To this end, we have deleted its expression in the CNS and are crossing these knockout mice with an HD mouse model.

**184. Characterization of the neuroprotective functions of IKKα**

*Ali Khoshnan*

Our recent studies indicate that IKKα expression in neurons promotes survival and imparts protection against DNA damage induced by genotoxic stress. Further investigation of IKKα in neurons have led to discovery that IKKα also promotes BDNF expression and enhances neurite sprouting. IKKα appears to regulate the activity of MeCP2, and CREB binding proteins, which are important modulators of BDNF expression. We also find that IKKα regulates expression of microRNAs that have neuroprotective properties. Studies are in progress to identify key regulatory targets of IKKα in neurons, dissect its role in BDNF expression, and understand how it promotes neuroprotection in stressed neurons.

**185. Intrabodies binding the proline-rich domains of mutant huntingtin increase its turnover and reduce neurotoxicity**

*Amber L. Southwell, Ali Khoshnan, Paul H. Patterson*

While expanded polyQ repeats are inherently toxic, causing at least nine neurodegenerative diseases, the protein context determines which neurons are affected. The polyQ expansion that causes Huntington's disease (HD) is in the first exon (HDx1) of huntingtin (Htt). However, other parts of the protein, including the 17 N-terminal amino acids (AA) and two proline (polyP) repeat domains, regulate the toxicity of mutant Htt. The role of the P-rich domain that is flanked by the polyP domains has not been explored. Using highly specific intracellular antibodies (intrabodies), we tested various epitopes for their roles in the toxicity, aggregation, localization and turnover of HDx1. Three domains in the P-rich region (PRR) of HDx1 are defined by intrabodies: MW7 binds the two polyP domains, and Happs1 and 3, two new intrabodies, bind the unique, P-rich epitope located between the two polyP epitopes. We find that the PRR-binding intrabodies, as well as $V_L$12.3, which binds the N-terminal 17 AA, decrease the toxicity and aggregation of mutant Htt, but they do so by different mechanisms. The PRR-binding intrabodies have no effect on Htt localization, but they cause a significant increase in the turnover rate of mutant Htt, which $V_L$12.3 does not change. In contrast, expression of $V_L$12.3 increases the nuclear localization of Htt. We propose that the PRR of mutant Htt regulates its stability, and that compromising this pathogenic epitope by intrabody binding represents a novel therapeutic strategy for treating HD. We also note that intrabody binding represents a powerful tool for determining the function of protein epitopes in living cells. Preliminary *in vivo* studies in adult mice suggest that intrastriatal injection of a $V_L$12.3-expressing adeno-associated virus (AAV) reduces aggregation of mHtt and ameliorates the reduction of neuronal size caused by injection of mHtt-lentivirus. AAV-$V_L$12.3 also improves the amphetamine-induced rotation bias seen with unilateral mHtt lentivirus injection.

**186. Exogenous LIF stimulates oligodendrocyte progenitor cell proliferation and remyelination**

*Benjamin E. Deverman, Sylvian Bauer*

The development of therapies that enhance the repair capabilities of the adult brain by stimulating the proliferation of endogenous neural stem and progenitor cells, and/or directing their subsequent differentiation into functional neurons and glia, is a relatively neglected area of current stem cell research. We found that injection of an adenovirus expressing leukemia inhibitory factor (LIF) into the adult mouse brain promotes neural stem cell (NSC) self-renewal and stimulates the proliferation oligodendrocyte progenitor cells (OPCs). Based on these findings, in particular the effect of LIF on OPCs, we hypothesized that if LIF could enhance the OPC response in the context of chronic demyelination it may, in turn, promote the generation of new oligodendrocytes and aid remyelination. To test this, we feed mice a diet containing cuprizone for 12 weeks, a course of treatment that induces demyelination in the corpus callosum (CC), hippocampus, and cortex, and inject the mice with either a LIF- or a lacZ-expressing adenovirus (Ad-LIF or Ad-lacZ) in the lateral ventricle. Three weeks after adenovirus injection and removal of cuprizone from the diet, mice that received Ad-LIF exhibit increased numbers of proliferating OPCs in the demyelinated hippocampus. Many of these OPCs survive and differentiate so that by 6 weeks after removal of cuprizone, the number of mature oligodendrocytes in LIF-treated mice is restored to near normal numbers in the CA3 region of the hippocampus, where LIF-induced Stat3 activation is the greatest. Remarkably, remyelination in the CA3 region is much more extensive in LIF-treated mice than in Ad-lacZ-treated mice, and the new myelin is accompanied by the reformation of nodal, paranodal and juxtaparanodal domains on a subset of axons. Our findings that LIF can promote oligodendrocyte generation and remyelination *in vivo*, taken together with its known ability to protect oligodendrocytes from death in mouse models of MS and spinal cord injury, suggest that LIF has multiple activities that could be of therapeutic benefit.

**187. New epitopes for intracellular antibodies for Huntington's disease**

*Charles W. Bugg, Ali Khoshnan, Paul H. Patterson*

Mutant huntingtin (mHtt) causes Huntington's disease (HD), a progressive and fatal neurodegenerative disorder. Intracellular antibodies (intrabodies) directed to different epitopes within exon 1 of mHtt have a variety of effects on the toxic protein. They can increase its turnover (intrabodies to the multiple proline domain), exacerbate its aggregation and toxicity (intrabodies to the polyglutamine

region), or alter its cellular localization (intrabodies to the N-terminus). We are attempting to isolate intrabodies to novel epitopes of mHtt and characterize the role that these epitopes play in the cellular function and toxicity of mHtt. To this end, we have selected a panel of 30 intrabodies by phage display and are in the process of determining to which epitopes they bind. After determination of the epitopes, the contribution of the epitopes to mHtt toxicity will be characterized.

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**Support:** The work described in the following research reports has been supported by:
Damon Runyon Cancer Research Fund
The German Academy of Natural Scientists Leopoldina
Gimbel Discovery Fund
Howard Hughes Medical Institute
Huntington Hospital Research Institute
Johnson & Johnson
Moore Fund
National Institute of Mental Health
National Institutes of Health, USPHS
University of California Tobacco-Related Disease Research Program
Weston Havens Foundation

**Summary:** Synapses, the points of contact and communication between neurons, can vary in their size, strength and number. The ability of synapses to change throughout the lifetime of the animal contributes to the ability to learn and remember. We are interested in how synapses are modified at the cellular and molecular level. We are also interested in how neuronal circuits change when synapses change their properties. We conduct all of our studies in the hippocampus, a structure known to be important for memory in both humans and animals. We use molecular biology, electrophysiology and imaging to address the questions detailed below.

A major focus of the lab concerns the cell biological mechanisms that govern modifications at individual synaptic sites. In particular, we are interested in the idea that dendritic protein synthesis and degradation may contribute to synaptic plasticity. We are also interested in mRNA and protein trafficking during synaptic plasticity. We develop microfluidic platforms to optimize the stimulation and visualization of cell biological processes at synapses.

We are also examining the role of the cadherins family of cell adhesion molecules in synaptic plasticity. Several labs have shown that cadherins are localized to synapses in the hippocampus. Earlier, we demonstrated that function-blocking cadherin antibodies or peptides could prevent long-term potentiation, without interfering with basal synaptic transmission. We hypothesize that cadherin bonds may be sensitive to local fluxes in extracellular calcium imposed by action potential activity. We are now examining the molecular mechanisms by which cadherins influence synaptic strength and the involvement of cadherins in the formation and maintenance of synapses, using fluorescence resonance energy transfer and endocytosis assays.

A unique endeavor in the lab involves the recording of single neuron activities in the medial temporal lobe of human epilepsy patients. In these studies, we are able to correlate single neuron responses with behavioral experience and performance. These studies should elucidate some fundamental mechanisms of brain coding and representation. In addition, we conduct experiments in hippocampal slices to examine how activity and neuromodulators influence synaptic transmission and plasticity.

**188. Investigating local processing in dendrites using a microfluidic perfusion chamber**

*Anne M. Taylor, Daniela C. Dieterich, Erin M. Schuman*

Synaptic plasticity involves local and coordinated changes in both the presynaptic and postsynaptic regions of the synapse. In particular, recent evidence shows that local protein synthesis in dendrites is required for some forms of synaptic plasticity. The underlying molecular and structural mechanisms of synaptic plasticity are the focus of extensive studies using a variety of different neuronal culture systems. Dissociated hippocampal neurons provide an important tool for investigating local changes in synaptic activity with particular relevance to learning and memory. The recent use of microfluidic devices provides an important improvement for the organization and manipulation of dissociated hippocampal neurons that extend processes long distances and with random orientations. Here we develop and use a microfluidic device to investigate local processing in dendrites. Using this microfluidic device we are able to spatially orient dendrites and axons in a highly structured/organized manner, while enabling both pharmacological and genetic manipulations. This microfluidic device consists of a series of microgrooves that align dendrites and axons in parallel orientation. We show that dendrites and axons form synaptic contacts in these microgrooves. The use of a perfusion channel running perpendicular to the microgrooves allows a stable and locally restricted pharmacological agent to a distinct dendritic region in these devices with a resolution of 30 μm. The concentration of the perfusion stream reaches equilibrium within 1 min and can be washed out within this same timeframe. A sequential series of molecules can be perfused numerous times. Further characterization work includes the diffusion of molecules through the dendrite during the perfusion. This work provides a significant technical improvement for investigating local processing at both pre- and postsynaptic regions.

*This work is supported by HHMI and NIH.*

**189. Monitoring global and local identities and fates of activity-induced proteomes using non-canonical amino acid tagging (BONCAT and FUNCAT)**

*Daniela C. Dieterich, Jennifer J.L. Hodas, John Ngo, David A. Tirrell, Erin M. Schuman*

Both global and local protein synthesis have been implicated in different forms of synaptic plasticity. However, the selective identification and visualization of a newly synthesized proteome has been hindered by the basic fact that all proteins, new and old, share the same pool of amino acids and thus are chemically indistinguishable. Recently, we developed bioorthogonal non-canonical amino acid tagging (BONCAT) for the specific identification of newly synthesized proteins. This approach is based upon the co-translational introduction of small bioorthogonal chemical groups via the incorporation of the non-canonical amino acids Azidohomoalanine (AHA) or Homopropargylglycine (HPG) into proteins and the chemoselective tagging of labeled proteins with an affinity tag via [3+2] click chemistry. Now we demonstrate the extension of this approach to visualize newly synthesized proteins using fluorescent tags (FUNCAT) in cultured hippocampal neurons.

Incorporation of modified amino acids is unbiased, non-toxic and does not increase protein degradation. While abundant signal is detected in neurons treated with the modified amino acids, no FUNCAT signal is detected in protein synthesis inhibitor-treated cultures, confirming that this procedure labels newly synthesized proteins with high specificity. Strikingly, newly synthesized proteins in the somata cultured hippocampal neurons can be detected as early as 10 min after AHA- or HPG incubation; a steady increase in protein synthesis is observed over time. Signal in proximal dendrites can be detected after a 20 min incubation with AHA or HPG. To examine the effects of synaptic activation on protein synthesis, we conducted experiments using the neurotrophin BDNF. After bath application of BDNF (50 ng/ml) for 60 min, we are able to detect a 1.6-fold increase in the signal of newly synthesized proteins in proximal segments of BDNF-treated dendrites when compared to vehicle-treated controls. Moreover, we demonstrate that local application of AHA and HPG can be used to evaluate the extent and fate of locally synthesized proteins using micromanipulator-assisted local perfusion and microfluidic chambers. Finally, a pulse-chase-like application of AHA and HPG allows monitoring of proteins synthesized in two sequential time periods. In conclusion, FUNCAT makes it possible to address the temporal and spatial characteristics of newly synthesized proteomes under basal, as well as elevated, levels of synaptic activity.

*This work is supported by HHMI, the NIH and NIDA.*

**190. Identifying and visualizing the hippocampal dopaminergic subproteome using BONCAT and FUNCAT**

*Jennifer J.L. Hodas, Daniela C. Dieterich, David A. Tirrell, Erin M. Schuman*

Both synaptic and behavioral plasticity require new protein synthesis. Dopamine is a critical neuromodulator, and abnormalities in dopaminergic regulation underlie disorders like Parkinson's disease, Alzheimer's disease, and schizophrenia diseases that impair the ability to form and retrieve memories. The stimulation of D1/D5 dopaminergic receptors in the hippocampus is thought to be critical for protein synthesis-dependent long-term potentiation (LTP), a process important for long-term synaptic plasticity and memory. Despite considerable effort, the proteins synthesized upon activation of dopaminergic pathways, the dopaminergic subproteome, are still largely unknown.

Using bioorthogonal noncanonical amino acid tagging (BONCAT), we are able to specifically identify components of the hippocampal dopaminergic proteome in an unbiased, non-toxic manner. Moreover, we utilize a related technique, fluorescent noncanonical amino acid tagging (FUNCAT), to visualize the effect of D1/D5 dopaminergic receptor stimulation in regulating global and local protein synthesis in the hippocampus. Both techniques employ a methionine surrogate, azidohomoalanine (AHA), which is conjugated to an azide-bearing tag via [3+2] copper-catalyzed click chemistry. Upon stimulation with a D1/D5 dopamine receptor-specific agonist, there are significantly increased levels of FUNCAT signal present in dendrites when compared to unstimulated dendrites. Since our interests also extend to dendritic protein synthesis, we have combined the use of FUNCAT with a Transwell culture system and have observed protein synthesis in both the somatic and dendritic compartments. Therefore, we demonstrate the application of BONCAT and FUNCAT to probe one of the protein synthesis-dependent functions in the hippocampus.

**191. Assessing cadherin-cadherin binding dynamics in living cells using Förster Resonance Energy Transfer (FRET)**

*Sally A. Kim*[*]*, Chin-Yin Tai*[*]*, Eric A. Mosser, Anh H. Pham, Erin M. Schuman*

Cadherins are a major family of calcium-dependent cell-cell adhesion molecules that are found at cellular junctions and neuronal synapses. Classic cadherins interact via homotypic adhesion in a *cis*-dimer interaction within each membrane, as well as a *trans*-dimer (across junction) interaction between the *cis*-dimers. To monitor directly the binding dynamics, we have developed a Förster Resonance Energy Transfer (FRET) reporter system for visualizing homophilic interactions of cadherin in living cells. We created N-cadherin fusion proteins with an intramolecular fluorescent protein insertion (cerulean, donor and venus, acceptor) using a transposon-mediated insertion method. We generated fluorescently-labeled

N-cadherin that is functional, exhibits fluorescence of sufficient intensity for FRET, mimics the cellular localization of endogenous N-cadherin, and interacts with the correct binding partner, β-catenin. In heterologous cells, we can probe within cell or across junction cadherin interactions when individual cells are co-transfected and express both constructs (*cis*) or neighboring cells express either the donor or acceptor (*trans*). The dynamics of $Ca^{2+}$-dependent cadherin associations can be monitored by manipulating extracellular $Ca^{2+}$. Since cadherins play a critical role in synaptic plasticity, we will use the FRET reporter system in neurons to directly test whether synaptic activity regulates the adhesive strength of cadherin interactions across synapses. FRET will be used to detect cadherin interactions between pre- and postsynaptic cells during synaptic activity and varying extracellular $Ca^{2+}$concentrations. These experiments will test our hypothesis that cadherins act as extracellular calcium detection system to coordinate synaptic plasticity across the synapse.

[*] *These authors contributed equally.*

**192. Activity-dependent regulation of ribosomes in rat hippocampal neurons**

*Young J. Yoon, Oliver Losón, Michael Zobel, Erin M. Schuman*

A requirement for new protein synthesis has long been associated with synaptic plasticity, as well as learning and memory. In addition, the presence of ribosomes and polyribosomes in dendrites strongly suggests that translation occurs locally to modulate nearby synapses in response to synaptic activity. To investigate whether ribosomes can translocate in and out of synaptic compartments, we have analyzed postsynaptic densities (PSD) prepared from acute hippocampal slices from rats. We observed that under basal conditions, numerous ribosomal proteins were associated with PSDs in the Triton-resistant fraction, suggesting a specific interaction. Immunofluorescence studies using antibodies to ribosomal protein S17 and PSD-95 revealed increased colocalization between S17 and PSD-95 upon exposure to BDNF. Subsequently, we have analyzed the area of colocalization and we observed increase in the intensity and a decrease in the area of overlap between S17 and PSD-95 upon depolarization. The observed changes in ribosome with respect to the PSD suggest that the translational machinery is dynamic and can be modulated near synaptic compartments in response to changes in synaptic activity.

**193. Visualizing associative learning in juvenile zebrafish *(Danio rerio)***

*Mark Aizenberg, Erin M. Schuman*

There is ample evidence that stabilization of long-term memories depends on coordinated activity of large networks of neurons, sometimes in distant brain areas. However, deciphering of the mechanism underlying memory formation is challenged by the enormous complexity of such networks in the mammalian brain and our restricted ability to access activity of large neuronal populations. Zebrafish is emerging as an animal model that circumvents these problems, as young zebrafish possess a rich behavioral repertoire despite its relatively simple brain architecture. In addition, 7-10 day-old juveniles are transparent enabling optic monitoring of cell activity.

We developed a visual learning paradigm in which juvenile zebrafish learn the association of a brief light flash with aversive stimulus (e.g., mild electric shock). In combination with fluorescent dyes that change their signal intensity in response to different activity levels of the injected cells, we will monitor the activation states of neuronal networks in behaving fish. With intact fish embedded in agarose gel, we will visualize brain activity using 2-photon microscopy. Using this approach, we intend to dissect neural circuits in living zebrafish and, thus, reveal principles governing neural circuit function during memory formation.

**194. Protein synthesis in neural circuits during learning and memory in zebrafish (*Danio rerio*)**

*Flora Hinz, Daniela C. Dieterich, Erin M. Schuman*

For survival, animals need to acquire and store information about their environment. Different kinds of learning involve changes in distinct neural circuits. One way information is stored in circuits is by changing synaptic strength - by altering the proteins present at individual synapses via regulated protein synthesis and protein degradation. Determining what neural circuits and proteins are involved in encoding memories are long-standing central goals in neuroscience. I believe we can now approach this goal as protein synthesis, shown to underlie long-term memory formation, can be visualized *in vivo* using a new technique – Fluorescent Non-Canonical Amino Acid Tagging (FUNCAT). FUNCAT is a novel fluorescent labeling strategy that labels all newly synthesized proteins and is thereby able to provide a global analysis of protein synthesis. During FUNCAT, non-canonical amino acids are used to introduce azide groups into newly synthesized proteins, which can then be coupled to fluorescent alkyne-bearing groups using 'click chemistry.' This novel tagging technique has been successfully used in dissociated hippocampal culture, organotypic hippocampal slices and acute hippocampal slices. Therefore, I hope that by monitoring new protein synthesis using FUNCAT in the dorsal telencephalon of the larval zebrafish *(Danio rerio)* before and after conditioning, I will be able to locate hotspots of protein synthesis indicating the cells/neural circuits involved in storage of the memory trace. To test this hypothesis, I have begun work to: (1) develop a robust associative conditioning paradigm for larval zebrafish; (2) monitor protein synthesis in larval zebrafish using FUNCAT; and then combine the two to (3) monitor protein synthesis in larval zebrafish during olfactory conditioning to visualize formation of memory traces.

**195. Homeostasis of synaptic balance in dendrites by A-type potassium channels**

*Hiroshi T. Ito, Erin M. Schuman*

The observation that synaptic strength is dynamically modulated by activity-dependent synaptic plasticity poses the problem as to how synaptic number and efficacy are maintained from proximal to distal regions of dendrites. For the maintenance of synaptic balance, homeostatic synaptic scaling by miniature synaptic transmissions is likely to play an important role. Here, we demonstrate that CA1 pyramidal neurons in hippocampal slices incubated for 12 hours with tetrodotoxin (TTX) together with an A-type potassium channel blocker, 4-aminopyridine (4-AP), exhibit significantly higher miniature excitatory postsynaptic current (mEPSC) frequency, compared with neurons incubated with TTX alone. This synaptic scaling was still observed with low extracellular calcium and was not blocked by NMDA receptor antagonist, APV, indicating that it is a calcium-independent process. Furthermore, using dendritic recordings from the stratum radiatum in area CA1, we did not observe significant differences in miniature excitatory synaptic transmissions between 4-AP treated and untreated slices, suggesting that observed synaptic scaling was locally restricted to regions near the soma and that A-type potassium channel blockade disrupted dendritic synaptic balance. Taken together, our data indicates that A-type potassium channels play an important role in maintaining the synaptic balance in dendrites, independent of action-potential-triggered synaptic transmission, which may underlie proper dendritic computation in the neuron.

**196. Single unit activity in the human hippocampus during declarative memory retrieval - dissociation between memory response and decision**

*Ueli Rutishauser, Adam N. Mamelak, Erin M. Schuman*

Episodic memories allow us to remember not only that we have seen an item before (familiarity) but also where and when we have seen it (context). Sometimes, we are confident that an item is familiar but cannot recollect where or when it was seen. Thus, the two components of episodic recall, familiarity and recollection, can be behaviorally dissociated. It is well known that the medial temporal lobe (MTL) plays an important role in this process. It is not clear, however, whether these two components of memory are represented separately by distinct brain structures or different populations of single neurons in a single anatomical structure. We recorded single neurons in the hippocampus and amygdala of patients implanted with depth electrodes for the purpose of localizing epileptic seizure origins. Previously, we identified a class of neurons that changed its firing rate to the second presentation of a previously novel stimulus (single-trial learning). Here we investigate the activity of the same subpopulation of neurons during episodic memory retrieval. We find that the spiking activity of single neurons in the human hippocampus and amygdala contain information about both components of memory. We found that the neuronal activity evoked by the presentation of a familiar stimulus (during retrieval) distinguishes stimuli that will be successfully recollected from stimuli that will not be recollected. At the same time, neuronal activity also predicts whether the stimulus is novel or familiar (familiarity). The response was strongest for stimuli that are both recollected and recognized, intermediate for items that were only recognized, and weakest for items that are novel. The responses to items which have been seen before but were not recognized as such (forgotten), as well as false positives (false memory), were larger than for truly novel items but less than for recognized items. Importantly, the neural responses are memory responses and do not correspond to the decision about the memory. They thus represent the input into the decision making process rather then its output. We demonstrate this fact by using a simple decoder that outperforms the patient in making new/old decisions. We conclude that human medial temporal lobe neurons contain information about both components of memory. These data support a continuous strength of memory model of MTL function: the stronger the neuronal response, the better the memory.

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**Support**: The work described in the following research reports has been supported by:
Human Frontier Science Program
Japan Science and Technology Agency
National Institutes of Health

**Summary:** While we continue to examine the dynamic/adaptive nature of human visual perception - including its crossmodal, representational, sensory-motor, developmental, emotional, and neurophysiological aspects - we put more emphasis on the ERATO (Exploratory Research for Advanced Technology) Shimojo "Implicit Brain Functions" project (supported by JST, Japan Science and Technology Corporation, officially started in October '04), with its emphasis on implicit cognitive processes, emotional decision making, and their neural correlates. Vigorous collaborations are conducted among our psychophysics laboratory here and the ERATO Japan site located at NTT Communication Science Laboratories, Atsugi, Kanagawa, Japan, as well as Harvard MGH, Boston University, Gordon College London, National Academy of Science Austria, and Decode Inc. Germany.

Using a variety of methods including eye tracking, high-density EEG, fMRI and MEG, we examine how exactly peripheral sensory stimuli, neural activity in the sensory cortex, and the mental experience of perception are related to each other. As for objectives of the ERATO project, we aim to understand implicit, as opposed to explicit or conscious, somatic and neural processes that lead to, and thus predict, conscious emotional decision such as preference. As a more overarching general theme including both the perceptual psychophysics and the ERATO studies of emotional decision making, we are in particular interested in the intriguing interactions between *predictive* processes (prior to and thus predicting the mental event or behavior) and *postdictive* processes (posterior) to understand conscious experience of perception, sensory-motor learning, memory, and emotional decision modulated by internal and external factors.

In extensions of our core interest in visual preference decision making, we collaborated with neuroeconomists on campus to investigate neural correlates of choice decision in more realistic real-world situations (such as purchasing snacks).

There are also a couple of technical developments in the project, i.e., fMRI-based neural conditioning and a video-based auditory-visual data collection/analysis system.

(1) We continue our work applying TMS (Transcranial Magnetic Stimulation) to the visual cortex of alert normal subjects, to reveal neural mechanisms underlying conscious visual perceptual experience. In our earlier finding of TMS-triggered "visual replay" effect, we had demonstrated that when a simple visual stimulus is presented followed by a dual-pulse TMS (which typically yields an illusory light field in the contralateral visual field, called "phosphene"), the stimulus "reappears" perceptually, mainly within the area of phosphene. In our new study, we found that: (a) natural scenes often generate a vivid perceptual replay; (b) the phenomenology of replay varies across observers, from a photopic to filling-in-like to contours alone; and (c) the replay often goes beyond the spatial range of phosphene (e.g., bilateral, symmetrical percepts). The results revealed new aspects of visual cortical traces and dynamics that lead to conscious perception. More recently, we found that after repeated paired presentation of the visual transient stimulus and the dual-pulse TMS (say 10 trials or more), the TMS alone can trigger the replay without visual stimulation ("entrainment effect"). We are currently searching for evidence that the replay is not just a "phenomenological illusion" but rather executing real effects on visual information encoding. And on the technical side, we developed our own switchbox that enables us to give a better sham stimulation by controlling the current direction separately for each half of an 8-shaped coil. Our results showed that it is successful in providing a reliable sham condition.

(2) Emotional decision making has been the central focus of our ERATO project (JST.ERATO Shimojo Implicit Brain Function Project). We have investigated behavioral and neural correlates of such preference decision in both the human and the monkey. In particular, we addressed the issue of memory-preference relationship, in order to resolve the seeming conflict between the Familiarity and the Novelty principles in the literature. (a) We found a surprise segregation between these two principles across object categories. In a two-alternative forced choice preference task (old vs. new), the Familiarity dominates across trials in faces, whereas the Novelty dominates in natural scenes. Geometric figures falls somewhere in between. We had additional, though preliminary, evidence that similarity across stimuli as well as excessive experience matters more for preference decision in geometric figures, less in natural scenes, and least in faces, a result that is intriguing from a biological/ecological perspective. (b) We examined what

aspect of the repeated experience is critical for such memory-based preference. Mere exposure turned out to be sufficient for the Familiarity preference in faces, but some objective task/choice was necessary for the Novelty preference in natural scenes. (c) We recorded the OFC (Orbito-Frontal Cortex) neurons' activity via multiple electrodes, while the monkey was engaged in either the classical reward task (i.e., visual discrimination for juice reward) or just watching videos without reward. We found three types of reward-related neurons, those who responded only to the juice, those responded only to the videos, and those responded to both.

(3) Animal and human behavior can be modified by a procedure called Instrumental conditioning. We aimed to extend this traditional approach in order to directly shape neural activity instead of overt behavior. Using real-time fMRI feedback, we were indeed successful in conditioning hand and foot movement-related areas in the human motor cortex separately. The results were two-fold - the activity of the area that is feedback is enhanced, while that of the other area was suppressed. We also showed that such modulation of neural activity is indeed reflected in behavior, i.e., reaction times. This potentially provides us with a very powerful tool, both in scientific and clinical situations. We do also have a partial success in modifying the OFC activity.

(4) It has been known that Pavlovian conditioning affects the animal's choice under Instrumental conditioning situation ("Pavlovian-Instrumental Transfer" or PIT), and several brain areas including NAC (Nucleus Accumbense) shell are identified as a part of the neural mechanism. While people have speculated that the PIT may be a part of the underlying mechanism for various commercial effects in the human, but no direct evidence was reported so far. We did demonstrate that PIT indeed occurs in the human, and it affects its choice of drink. Our fMRI data suggest that activity in a subcortical area called septum (allegedly the human homolog of NAC shell in the animal) seems to be responsible.

(5) "Chronostasis" is a phenomenon where duration of an oddball visual stimulus is subjectively elongated relative to the others. We applied this effect to investigate dynamics of cross-modal integration. We found that: (a) qualitatively the same effect can occur in the auditory domain; and (b) the auditory duration perception can be distorted due to contingent visual stimulation, but not vice versa, thus asymmetry in the direction of visual dominance. Several other cross-modal results are coming along.

(6) We investigated how the probability and the reward structures affect arm-reaching trajectory. As the difference in value between the targets increases, subjects' biases also increase at a rate that closely matches the maximum-point predictions. Moreover, changes in biases across trials are better predicted by recent experience, rather than global experience. Together, this suggests that people learn value structure through recent experience, and this knowledge is used to guide reach planning.

(7) Implicit non-verbal communication not only assists explicit communication but rather provide the basis for it. To fully understand, we need an inclusive behavioral recording system that can record both voices and bodily gestures of communicators simultaneously, and make it easy for researchers to analyze both interpersonal and intrapersonal synchrony or coherence. Based on MATAB codes, we have created such a system and are preliminarily analyzing data of verbal and non-verbal communication.

(8) In the extension of our previous work on the flash lag and the generalized flash-lag phenomena, we further extended applicability of the theory (i.e., "the brain is equipped with a compensation mechanism against its own neural delay in perception") to static, geometric illusions. It demonstrated amazing power of applicability and accountability across a very wide range of geometric illusion. This grand theory of illusion drew lots of mass-media attention (including Scientific American and NY Times, for instance).

Other research outputs include various aspects of auditory-visual interaction particularly in the domain of speech, and perceptual resolution of ambiguity/bistability. Also in the out-reaching domain, I published my first paper on "perceptual illusions for science museum and art exhibitions."

**197. Transcranial magnetic stimulation (TMS) consolidates and retrieves a percept from short-term memory**

*Hsin-I Liao[1], Daw-An Wu[2], Hsiu-Yu Yeh[3], Shinsuke Shimojo*

Transcranial magnetic stimulation (TMS) to visual cortex dynamically interacts with retinal input and thus alters conscious perceptual experience. When a visual stimulus is presented and followed by a dual-pulse TMS, an "instant replay" of the visual stimulus is observed (Halelamien, *et al.*, VSS '07; Wu *et al.*, VSS '01, '02, '04). While following this finding, we found that repeatedly pairing the same visual stimulus with TMS can lead to a state where the replay percept can be retrieved by TMS alone without any visual stimulus presentation.

The experiment consisted of two phases, the training phase and the test phase. In the training phase, a geometric pattern (a disk or a line) was presented for 100 ms, followed by a dual-pulse TMS (50 ms inter-pulse interval) with 300 ms delay. After repeated trials (10 trials or more), TMS alone was delivered without any visual stimulus presentation (the test phase). The replay percept was often retrieved in the area of TMS-triggered phosphene. Among 18 subjects we have tested, five reported no replay percept either in the training phase or in the test phase; another four reported a replay percept only in the training phase; the remaining nine reported a replay percept both in the training phase AND in the test phase. Increasing the delay between visual stimulus and TMS during the training phase substantially weakened the vividness of the replay percept, with complete abolition at 3 sec in most subjects. In such cases, no replay was seen in the test phase, either.

That the replay precept can be entrained and then retrieved by TMS alone not only provides evidence for the existence of a neural representation for the "replay," but also implies a Hebbian-like associative learning mechanism that can be a basis of conscious perceptual experience.

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**Citation**

Liao, H.I., Wu, D.A., Yeh, H.Y. and Shimojo, S. (2008) Transcranial magnetic stimulation (TMS) consolidates and retrieves a percept from short-term memory. Poster presented at ASSC (Association for the Scientific Study of Consciousness), 12th Annual Meeting, Taipei, Taiwan.

**198. Retrieval of visual percept by paired association of a visual stimulus and transcranial magnetic stimulation (TMS): Effect of TMS delay**

*Hsin-I Liao*[*]*, Shinsuke Shimojo*

TMS to visual cortex dynamically interacts with retinal input, altering conscious perception. When a brief visual stimulus is followed by a dual-pulse TMS, an "instant replay" of the percept could be observed. After repeating the pairs, the replay percept could be retrieved even by TMS alone without accompanying visual stimulus (Liao *et al*., ASSC '08). We manipulated the delay between the visual stimulus and TMS to further investigate how the paired association leads to the TMS-triggered perceptual retrieval. When the delay increased to 3 sec., the replay was no longer observed, nor the retrieval when TMS alone was delivered. It implies that pairing the visual stimulus and TMS without inducing the replay is not sufficient to cause the retrieval. The most vivid replay and the retrieval were both observed with delay of 300 ms. It may be expected from "cortical coincidence," considering the neural conductance delay from retina plus cortical processing time required for conscious perception. In line with it, we also found a positive correlation between the strength of replay and that of retrieval later. The replay percept may be directly consolidated and then retrieved. Alternatively, there may be a common mechanism underlying the replay and the retrieval.

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**Citation**

Liao, H.I. and Shimojo, S. (2008) Retrieval of visual percept by paired association of a visual stimulus and transcranial magnetic stimulation (TMS): Effect of TMS delay. Poster presented at Asia-Pacific Conference on Vision, Brisbane, Australia.

**199. Electronically switchable sham Transcranial Magnetic Stimulation (TMS) System**

*Fumiko Hoeft*[1]*, Daw-An Wu*[2]*, Arvel Hernandez*[1]*, Gary H. Glover*[3]*, Shinsuke Shimojo*

Transcranial magnetic stimulation (TMS) is increasingly being used to demonstrate the causal links between brain and behavior in humans. Further, extensive clinical trials are being conducted to investigate the therapeutic role of TMS in disorders such as depression. Because TMS causes strong peripheral effects such as auditory clicks and muscle twitches, experimental artifacts such as subject bias and placebo effect are clear concerns. Several sham TMS methods have been developed, but none of the techniques allows one to intermix real and sham TMS on a trial-by-trial basis in a double-blind manner. We have developed an attachment that allows fast, automated switching between Standard TMS and two types of control TMS (Sham and Reverse) without movement of the coil or reconfiguration of the setup. We validate the setup by performing mathematical modeling, search-coil and physiological measurements. To see if the stimulus conditions can be blinded, we conduct perceptual discrimination and sensory perception studies. We verify that the physical properties of the stimulus are appropriate, and that successive stimuli do not contaminate each other. We find that the threshold for motor activation is significantly higher for Reversed than for Standard stimulation, and that Sham stimulation entirely fails to activate muscle potentials. Subjects and experimenters perform poorly at discriminating between Sham and Standard TMS with a figure-of-eight coil, and between Reverse and Standard TMS with a circular coil. Our results raise the possibility of utilizing this technique for a wide range of applications.

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**Publication**

Hoeft, F., Wu, D-W., Hernandez, A., Glover, G., and Shimojo, S. (2008) Electronically switchable sham transcranial magnetic stimulation (TMS) system. *PLoS ONE* **3**(4):e1923.

**200. Familiarity for faces and novelty for natural scenes in preference: Does similarity matter?**

*Eiko Shimojo, Junghyun Park, Makio Kashino*[*]*, Shinsuke Shimojo*

Memory is obviously critical for visual preference, but how precisely is yet unclear. Two seemingly inconsistent principles, Novelty and Familiarity, have been proposed. We reported (VSS'07) a surprising segregation of these principles across object categories: familiarity preference monotonously increased in faces (FC), whereas novelty preference increased in natural scenes(NS), over

repeated 2AFC preference judgment between a new and an old stimulus. Geometric figures (GF) did not show any bias. While the segregation may indicate intrinsic biological differences among object categories, it may also be due to differences in similarity/variability within category between FC and NS. To examine it, we ran two experiments. First, we reused the same FC and NS stimuli but presented them upside down in the same 2AFC preference task, to see if the critical factor is orientation-specific and holistic. The results faithfully duplicated the pattern of the original results, i.e., Familiarity in FC and novelty in NS. Second, we directly manipulated variability within object category; i.e., to introduce more variability within FC by including cartoons, and natural face-like patterns, etc., and more similarity within NS. As a result, the familiarity preference disappeared in FC. In NS, however, the same increasing bias towards novelty preference persisted. In addition, we ran two sets of GF; one with high similarity within, and the other with low similarity. We found an increasing bias towards familiarity preference in the high similarity set, but no such bias in the low. The overall pattern of results is consistent with a notion that increasing similarity in local features shifts preference from novel to familiar stimuli, in close relation to the averageness hypothesis of attractiveness and the efficient coding theory of perception. The persistence of novelty preference in NC with more similarity is an exception that requires more investigation.

[*]*JST.ERATO Shimojo Implicit Brain Function Project, and Human and Information Science Laboratory NTT Communication Science Laboratories NTT Corporation*

This project is supported by the Japanese Science and Technology Agency.

**201. Novelty vs. familiarity principles in preference decision: Task context of memory matters**

*Hsin-I Liao[*], Shinsuke Shimojo*

Whereas, memory obviously affects preference, how precisely is yet unknown. Shimojo *et al.,* VSS '07, reported a segregation of the two principles, novelty and familiarity, across object categories: familiarity preference for faces and novelty preference for natural scenes. However, the results are partly inconsistent with the "mere exposure" effect (Zajonc, '68), i.e., repeated exposure to a visual object, regardless of the object category, leads to an increase in preference. The inconsistency may be partly due to what exact task was repeatedly performed during the experience phase: preference decision, selection of any kind, or mere exposure. To address this issue in relation to object category, we conducted the same two-alternative force-choice preference judgment as Shimojo *et al.* (VSS '07) in which the same old stimulus was presented always with a new stimulus following either kinds of experience phases: passively viewing all the paired pictures, or performing an objective judgment on the paired stimuli (on roundness of face, color temperature of natural scene, or complexity of geometric figure). The results showed that after passive viewing the old stimulus was preferred significantly more frequently in faces, i.e., mere exposure effect, but not in natural scenes or geometric figures. In the objective judgment task during the experience phase, the novel stimulus was chosen significantly more frequently in both geometric figures and natural scenes, but not in faces, even though the old stimulus was the median in pre-rating of color temperature or complexity. It possibly indicates a habituation at the selection level for certain object categories. The overall results further confirm the segregation of novelty and familiarity principles across object categories, indicate uniqueness of face as a special kind of stimulus, and suggest that different tasks lead to memory effects at different levels such as perception and selection.

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**Citation**

Liao, H.I. and Shimojo, S. (2008) Novelty vs. familiarity principles in preference decision: Task-context of memory matters. *J. Vision* 8, 522a. Poster presented at the Vision Science Society, 8th Annual Meeting, Naples, Florida.

**202. Overlapping representation of juice and video rewards in primate OFC**

*Michael Campos, Kari Koppitch, Richard A. Andersen[*], Shinsuke Shimojo*

Vision can be inherently rewarding. The reward circuitry in the brain supports an animal in identifying and obtaining rewards from its environment. The orbitofrontal cortex (OFC) is known to encode the subjective value of different juice reward options, and therefore, supports decisions based on preferences in the context of appetitive rewards. It is unclear, however, whether the brain circuitry supporting the appetitive rewards is the same, distinct, or overlapping with that supporting non-appetitive rewards, which are important to modern human life. To investigate this issue we used a self-initiated free-choice paradigm in which a monkey pressed buttons to receive either the presentation of a 5 sec video clip in the video-watching period ("leisure"), or a drop of juice in a separate period ("work"). The leisure and work periods were run in separate blocks of 20 minutes each, while we simultaneously recorded 2-10 single OFC neurons.

Neural activity was analyzed with respect to the button press. We first identified significant modulations in firing rate activity in any of five intervals defined with respect to the button press when compared to baseline. We found that two-thirds of the OFC neurons we encountered (394/585) were modulated in at least one interval in either the leisure or work period. Of these, approximately 40% were modulated in both periods, 40% were modulated in the work period exclusively, and 20% in the leisure period exclusively. The neurons that responded in only one period suggest that OFC contains at least two internal representations of distinct reward categories. The neurons that participated in both periods suggest that OFC also represents abstract commonalities between rewards of different kinds. These results are consistent with our

intuition that perceptual experience itself is rewarding, and indicate that the neural correlates overlap with that for appetitive rewards.

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**Funding:** JST.ERATO Shimojo Implicit Brain Functions Project, National Eye Institute to RAA.

**203. Direct instrumental conditioning of neural activity using fMRI derived reward feedback**

*Signe Bray[1], Shinsuke Shimojo, John P. O'Doherty[1,2]*

Successful learning is often contingent on feedback. In instrumental conditioning, an animal or human learns to perform specific responses in order to obtain reward. Instrumental conditioning is often used by behavioral psychologists in order to train an animal (or human) to produce a desired behavior. Shaping involves reinforcing those behaviors that in a step-wise fashion are successively closer to the desired behavior until the desired behavior is reached. Here, we aimed to extend this traditional approach in order to directly shape neural activity instead of overt behavior. To achieve this we scanned 22 human subjects with fMRI and performed image processing in parallel with acquisition. We delineated regions of interest (ROIs) in finger and toe motor/somatosensory regions, and used an instrumental shaping procedure to induce a regionally-specific increase in activity by providing an explicit monetary reward to reinforce neural activity in the target areas. After training, we found a significant and regionally-specific increase in activity in the ROI being rewarded (finger or toe) and a decrease in activity in the non-rewarded region. This demonstrates that instrumental conditioning procedures can be used to directly shape neural activity, even without the production of an overt behavioral response. This procedure offers an important alternative to traditional biofeedback-based approaches, and may be useful in the development of future therapies for stroke and other brain disorders.

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**Publication**

Bray, S., Shimojo, S. and O'Doherty, J.P. (2007) Direct instrumental conditioning of neural activity using functional magnetic resonance imaging-derived reward feedback. *J. Neurosci*. **27**:7498-7507.

**204. The neural mechanisms underlying the influence of Pavlovian cues on human decision making**

*Signe Bray[1], Antonio Rangel[1,2], Shinsuke Shimojo, Bernard Balleine[3], John P. O'Doherty[1,2]*

In outcome-specific transfer, Pavlovian cues that are predictive of specific outcomes bias action choice towards actions associated with those outcomes. This transfer occurs despite no explicit training of the instrumental actions in the presence of Pavlovian cues. The neural substrates of this effect in humans are unknown. To address this we scanned 23 human subjects with fMRI while they made choices between different liquid food rewards in the presence of Pavlovian cues previously associated with one of these outcomes. We found behavioral evidence of outcome-specific transfer effects in our subjects, as well as differential BOLD activity in a region of ventrolateral putamen when subjects chose, respectively, actions consistent and inconsistent with the Pavlovian-predicted outcome. Our results suggest that choosing an action incompatible with a Pavlovian-predicted outcome might require the inhibition of feasible but non-selected action-outcome associations. The results of this study are relevant for understanding how marketing actions can affect consumer choice behavior, as well as for how environmental cues can influence drug seeking behavior in addiction.

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**205. Distortions of subjective time perception within and across senses**

*Virginie van Wassenhove, Dean Buonomano[1,2], Shinsuke Shimojo, Ladan Shams[1]*

***Background***: The ability to estimate the passage of time is of fundamental importance for perceptual and cognitive processes. One experience of time is the perception of duration, which is not isomorphic to physical duration and can be distorted by a number of factors. Yet, the critical features generating these perceptual shifts in subjective duration are not understood.

***Methodology/Findings***: We used prospective duration judgments within and across sensory modalities to examine the effect of stimulus predictability and feature change on the perception of duration. First, we found robust distortions of perceived duration in auditory, visual and auditory-visual presentations despite the predictability of the feature changes in the stimuli. For example, a looming disc embedded in a series of steady discs led to time dilation, whereas a steady disc embedded in a series of looming discs led to time compression. Second, we addressed whether visual (auditory) inputs could alter the perception of duration of auditory (visual) inputs. When participants were presented with incongruent audio-visual stimuli, the perceived duration of auditory events could be shortened or lengthened by the presence of conflicting visual information; however, the perceived duration of visual events was seldom distorted by the presence of auditory information and was never perceived shorter than their actual durations.

***Conclusions/Significance***: These results support the existence of multisensory interactions in the perception of duration and, importantly, suggest that vision can modify auditory temporal perception in a pure timing task. Insofar as distortions in subjective duration can neither be accounted for by the unpredictability of an auditory, visual or auditory-visual event, we propose that it is the intrinsic

features of the stimulus that critically affect subjective time distortions.

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**Citation**

van Wassenhove, V., Buonomano, D., Shimojo, S. and Shams, L. (2008) Distortions of subjective time perception within and across senses. *PLoS One* **3**(1)e1437.

**206. Learning probability and reward through experience: Impact of value structure on reach planning**

*Erik Schlicht[1], Shinsuke Shimojo, Ken Nakayama[2]*

Throughout our everyday routine we must make actions in the face of uncertainty. From a decision theoretic standpoint, optimal actions are those that maximize the value associated with the task. However, in order for humans to act optimally, it necessitates the brain has an accurate representation of both the reward and probability associated with each outcome. Previous research investigating how humans use value structure to perform reaching movements has exclusively focused on asymptotic performance, ignoring how this structure is learned. Therefore, this project investigates how value is learned by requiring subjects to reach to targets that appear after completing a portion of their movement towards the possible target locations. Since subjects have no information about the target at the beginning of the reach, their initial trajectories provide a way to quantify reach plans. Value is manipulated by varying either the probability or reward associated with each target. Subjects are awarded points for correctly acquiring the target, no points for reaching to the incorrect target, and are penalized points for taking too much time. Subjects receive bonus money after the experiment that is based on their point total, assuring that value structure in this paradigm has actual utility. Furthermore, we developed a model that learns through the subject's experience what initial biases result in maximal points. We can use the model to make predictions about the biases people should use and what experience is important for forming value estimates. The results show that as the difference in value between the targets increases, subjects' biases also increase at a rate that closely matches the maximum-point predictions. Moreover, changes in biases across trials are better predicted by recent experience, rather than global experience. Together, this suggests that people learn value structure through recent experience, and this knowledge is used to guide reach planning.

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**Funding**: Shimojo Implicit Brain Function Grant (JST)

**207. Dynamic perceptual changes in audiovisual simultaneity**

*Ryota Kanai, Bhavin R Sheth[1,2], Frans A.J. Verstraten[3], Shinsuke Shimojo*

***Background:*** The timing at which sensory input reaches the level of conscious perception is an intriguing question still awaiting an answer. It is often assumed that both visual and auditory percepts have a modality-specific processing delay and their difference determines perceptual temporal offset. ***Methodology/Principal Findings***: Here, we show that the perception of audiovisual simultaneity can change flexibly and fluctuates over a short period of time while subjects observe a constant stimulus. We investigated the mechanisms underlying the spontaneous alternations in this audiovisual illusion and found that attention plays a crucial role. When attention was distracted from the stimulus, the perceptual transitions disappeared. When attention was directed to a visual event, the perceived timing of an auditory event was attracted towards that event. ***Conclusions/Significance***: This multistable display illustrates how flexible perceived timing can be, and at the same time offers a paradigm to dissociate perceptual from stimulus-driven factors in crossmodal feature binding. Our findings suggest that the perception of crossmodal synchrony depends on perceptual binding of audiovisual stimuli as a common event.

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**208. Perceiving the present and a systematization of illusions**

*Mark Changizi, Andrew Hsieh, Romi Nijhawan[*], Ryota Kanai, Shinsuke Shimojo*

Over the history of the study of visual perception there has been great success at discovering countless visual illusions. There has been less success in organizing the overwhelming variety of illusions into empirical generalizations (much less explaining them all via a unifying theory). Here, this article shows that it is possible to systematically organize more than 50 kinds of illusion into a 7 × 4 matrix of 28 classes. In particular, this article demonstrates that: (1) smaller sizes; (2) slower speeds; (3) greater luminance contrast; (4) farther distance; (5) lower eccentricity; (6) greater proximity to the vanishing point; and (7) greater proximity to the focus of expansion all tend to have similar perceptual effects, namely, to (a) increase perceived size; (b) increase perceived speed; (c) decrease perceived luminance contrast; and (d) decrease perceived distance. The detection of these empirical regularities was motivated by a hypothesis, called "perceiving the present," that the visual system possesses mechanisms for compensating neural delay during forward motion. This article shows how this hypothesis predicts the empirical regularity.

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**209. Distance in feature space determines exclusivity in visual rivalry**

*Tomas Knapen[*], Ryota Kanai, Jan Brascamp[*], Jeroen van Boxtel[*], Raymond van Ee[*]*

Visual rivalry is thought to be a distributed process that simultaneously takes place at multiple levels in the visual processing hierarchy. Also, the different types of rivalry, such as binocular and monocular rivalry, are thought to engage shared underlying mechanisms. We hypothesized that the amount of perceptual suppression during rivalry as measured by the total duration of fully exclusive perceptual dominance is determined by a distance in a neurally represented feature space. This hypothesis can be contrasted with the possibility that the brain constructs an internal model of the outside world using full-fledged object representations, and that perceptual suppression is due to an appraisal of the likelihood of the particular stimulus configuration at a high, object-based level. We applied color and stereo-depth differences between monocular rivalry stimulus gratings, and manipulated color and eye-of-origin information in binocular rivalry using the flicker and switch presentation paradigm. Our data show that exclusivity in visual rivalry increases with increased difference in feature space without regard for real-world constraints, and that eye-of-origin information may be regarded as a segregating feature that functions in a manner similar to color and stereo-depth information. Moreover, distances defined in multiple feature dimensions additively and independently increase the amount of perceptual exclusivity and coherence in both monocular and binocular rivalry. We conclude that exclusivity in visual rivalry is determined by a distance in feature space that is subtended by multiple stimulus features.

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**210. Disruption of implicit perceptual memory by intervening neutral stimuli**

*Ryota Kanai[1], Tomas H.J. Knapen[2], Raymond van Ee[2], Frans A.J. Verstraten[2]*

After viewing directional motion, one is likely to perceive a subsequently presented directionally ambiguous motion as being in the same direction as the prior motion. The perceptual bias towards the most recent percept gradually develops as the interval between the prior stimulus and a subsequent test becomes longer. This form of positive bias, or priming, is created in an automatic fashion. It remain unclear how such perceptual bias could be eliminated by a stimulus manipulation. Here we examine whether presentation of a stimulus, which was neutral as to the competing perceptual interpretations, during the interval between prior and test stimuli, disrupts the development of the priming effect. In experiments with ambiguous motion, we used stationary gratings as the neutral stimuli, and in an experiment with binocular rivalry between orthogonal gratings, we used a plaid pattern consisting of the two rival gratings. In both cases, presenting the neutral stimuli reduced the perceptual bias. These findings show that the visual system dynamically calibrates its internal bias using a recent percept and that this internal bias can be nullified by presenting neutral stimuli.

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**211. Multi-timescale perceptual history resolves visual ambiguity**

*Jan W. Brascamp[1], Tomas H.J. Knapen[2], Ryota Kanai, André J. Noest[1], Raymond van Ee[2], Albert V. van den Berg[1]*

When visual input is inconclusive, does previous experience aid the visual system in attaining an accurate perceptual interpretation? Prolonged viewing of a visually ambiguous stimulus causes perception to alternate between conflicting interpretations. When viewed intermittently, however, ambiguous stimuli tend to evoke the same percept on many consecutive presentations. This perceptual stabilization has been suggested to reflect persistence of the most recent percept throughout the blank that separates two presentations. Here we show that the memory trace that causes stabilization reflects not just the latest percept, but perception during a much longer period. That is, the choice between competing percepts at stimulus reappearance is determined by an elaborate history of prior perception. Specifically, we demonstrate a seconds-long influence of the latest percept, as well as a more persistent influence based on the relative proportion of dominance during a preceding period of at least one minute. In case short-term perceptual history and long-term perceptual history are opposed (because perception has recently switched after prolonged stabilization), the long-term influence recovers after the effect of the latest percept has worn off, indicating independence between time scales. We accommodate these results by adding two positive adaptation terms, one with a short time constant and one with a long time constant, to a standard model of perceptual switching.

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**212. Speech perception at the interface of neurobiology and linguistics**

*David Poeppel[1,2], William Idsardi[1], Virginie van Wassenhove*

Speech perception consists of a set of computations that take as input continuously varying acoustic waveforms (or visual kinematics), and generate as output discrete representations that make contact with the lexical representations stored in long-term memory.

Because the perceptual objects that are recognized by speech perception enter into subsequent linguistic computations, the format that is used for lexical representation and processing fundamentally constrains the (auditory and/or visual) speech perceptual processes. Consequently, theories of speech perception must, at some level, be tightly linked to theories of lexical representation. Minimally, speech perception must yield representations that smoothly and rapidly interface with stored lexical items. Adopting the perspective of David Marr (1982), we argue and provide neurobiological and psychophysical evidence for the following research program. First, at the implementation level, speech perception is a multi-time resolution process, with perceptual analyses occurring concurrently on at least two time scales commensurate with (sub)segmental (~20-80 ms) and syllabic analyses (~150-300 ms). Second, at the algorithmic level, we suggest that speech perception proceeds on the basis of internal forward models and uses an 'analysis-by-synthesis' approach (Halle and Stevens 1959, 1962). From a neurobiological point of view, the perception of speech would thus entail a predictive mechanism which affords the generation of discrete representations. Third, at the computational level (in the sense of Marr), the theory of lexical representation that we adopt here is principally informed by phonological research and assumes that words are represented in the mental lexicon in terms of sequences of discrete segments composed of distinctive features. One important goal of the research program is to develop linking hypotheses between the putative neurobiological primitives (for example, temporal primitives) and those primitives that are derived from linguistic inquiry, in order to ultimately reach a biologically sensible and theoretically satisfying model of representation and computation in speech.

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**Citation**

Poeppel, D., Idsardi, W. and van Wassenhove, V. (2007) Speech perception at the interface of neuroscience and linguistics. *Phil. Trans. R. Soc. London B*. In press.

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**213. The effects of temporal acceleration and deceleration on auditory-visual speech perception**

*Douglas S. Brungart*[*]*, Virginie van Wassenhove, Eugene Brandewie*[*]*, Griffin Romigh*[*]

The speaking rate can be a determining factor for speech comprehension in second language learners, hearing-impaired or elderly populations. Modern signal processing techniques make it possible to speed up or slow down the apparent speaking rate of a natural or synthetic talking face i.e., of an audio-visual speech event. However, little is known about the effect this processing might have on the intelligibility of auditory-visual (AV) speech signals. In these experiments, we used AV recordings of sentences from the Modified Rhyme Test (MRT). These sentences were accelerated or decelerated and participants were tested on their intelligibility in auditory, visual and auditory-visual conditions. In order to modify the speaking stimuli, we use the PSOLA speech processing algorithm (PRAAT) to change the duration of the audio signal. Subsequently, the frame rate of the AVI file was changed in order to maintain synchronization of the audio and visual stimuli. The original speech sentences were recorded at either a fast speaking rate (roughly five syllables per second (syl/s)), a normal conversational rate (3.3 syl/s), and a slow rate (1.7 syl/s). The results of a preliminary experiment showed that the conversational-rate AV recordings that were shifted in speed to match the slow or the fast recordings produced the same audio and audiovisual intelligibility levels as the original recordings. However, some degradation in performance occurred when the fast recordings were slowed down or the slow recordings were speeded up. In the main experiment, the sentences were processed to set their speaking rates to eight different fixed values ranging from 0.6 syl/s to 20 syl/s. The results show that AV advantages were preserved at speaking rates as fast as 12.5 syl/s, but that they disappeared when the rate was increased to 20 syl/s. Notably, the results also failed to show any improvement in AV performance for phrases presented slower than their original speaking rates.

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**Citation**

Brungart, D.S., van Wassenhove, V., Brandewie, Romigh, E., Griffin, R.E. (2007) The effects of temporal acceleration and deceleration on auditory-visual speech perception. *Proceedings of the International Conference on Auditory-Visual Speech Processing* (AVSP07), Kasteel Groenendael, Hilvarenbeek, The Netherlands.

## 214. Analysis-by-synthesis in auditory-visual speech perception: Multisensori-motor interfacing

*Virginie van Wassenhove*

In conversational settings, one sees as much as one hears the interlocutor. Watching the interlocutor's face not only provides information about the identity or the emotion of the interlocutor, but also about facial kinematics, a complex and dynamic structuring of visual information which can influence auditory speech processing. The natural co-occurrence of auditory-visual speech has been shown to affect both speech detection and intelligibility. Compelling demonstrations of auditory-visual (AV) integration in speech perception are the classic McGurk effects (McGurk and MacDonald, 1976) in the McGurk "fusion," an auditory [p] dubbed onto a face articulating [k] is perceived as a single fused percept [t]. However, in the McGurk "combination," an auditory [k] dubbed onto a visual [p] is heard as multiple combinations of [k] and [p]. The natural co-occurrence of AV speech signals is a likely feature used by the brain to integrate multisensory signals but, as illustrated by the diversity of McGurk effects, the speech informational content provided by each sensory modalities matters for the multisensory perceptual outcome. AV speech integration thus offers interesting challenges for neuroscience and speech sciences alike. How, when, where, and in what format do auditory and visual speech signals integrate? A set of empirical studies are herein described which include psychophysics, electroencephalography (EEG) and fMRI evidence for an 'analysis-by-synthesis' (or forward) model of AV speech perception. The findings support the view that visual speech plays a crucial role in shaping an internal prediction of the auditory speech signals that follow. It is proposed that the dynamics of the facial articulators perceived by the listener enable the brain to narrow down the set of potential auditory speech inputs. This visual-based prediction is shown to engage a large-scale (predictive) brain network that includes the motor system which is hypothesized to operate on the distinctive features of speech (i.e., abstract, hence, amodal speech representations).

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### Citation

van Wassenhove, V. (2007) Analysis-by-synthesis in auditory-visual speech perception: sensori-motor interfacing. *Proceedings of the 16th International Congress of Phonetic Sciences*, Säärbrucken, Germany.

## 215. Self and world: Large-scale installations at science museums

*Shinsuke Shimojo*

This paper describes three examples of illusion installation in a science museum environment from the author's collaboration with the artist and architect. The installations amplify the illusory effects, such as vection (visually-induced sensation of self motion) and motion-induced blindness, to emphasize that perception is not just to obtain structure and features of objects, rather than to grasp the dynamic relationship between the self and the world. Scaling up the size and utilizing the live human body turned out to be keys for installations with higher emotional impact.

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**Postdoctoral Scholar:** Evgueniy Lubenov
**Graduate Students:** Ming Gu, Casimir Wierzynski

**Support:** The work described in the following research report has been supported by:
The Bren Foundation
The Caltech Center for Biological Circuits Design
The James S. McDonnell Foundation
The McKnight Foundation
The Moore Foundation
The Hixon Foundation
The Gimbel Discovery Fund in Neuroscience
The Whitehall Foundation

**Summary:** Our research focuses on the study of learning and memory formation in freely behaving animals at the level of networks of neurons. Previous research has shown that the hippocampus is critical for the formation of long-term declarative memories, and that this hippocampal involvement is time-limited. The current predominant conjecture is that memories are gradually established across distributed neocortical networks through the interactions between cortical and hippocampal circuits.

However, the direct experimental investigation of these interactions has been difficult since, until recently, simultaneous chronic recordings from large numbers of well-isolated single neurons were not technically feasible. These experiments became possible with the advent of the technique of chronic multi-area tetrode recordings in freely behaving rodents. Using this technique we monitor the simultaneous activity of large numbers of cortical and hippocampal cells during the acquisition and performance of memory tasks, as well as during the sleep periods preceding and following experience.

Our research efforts focus on analyzing the structure of cortico-hippocampal interactions in the different brain states and on characterizing how this structure is modulated by behavior; how it evolves throughout the learning process; and what it reflects about the intrinsic organization of memory processing at the level of networks of neurons.

Our experimental work is complemented by theoretical studies of network models and the development of tools for the analysis of multi-neuronal data.

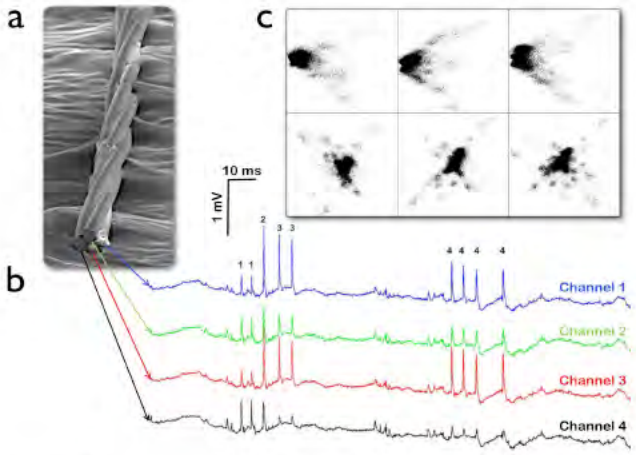


**Figure 1**: *Hippocampal Tetrode Recordings.* (a) Scanning EM image of a tetrode, which consists of four microwires twisted together to form a single recording probe. (b) Because of the spatial separation between the wire tips and the signal sources, each action potential is recorded simultaneously on all four wires, but with different amplitudes. This enables isolating the activity of multiple neurons in the vicinity of the tetrode tip, through a process analogous to triangulation. (c) The six 2-D projections of the Hadamard transformation of the 4-D space of amplitudes. Individual cells correspond to clusters in this space.

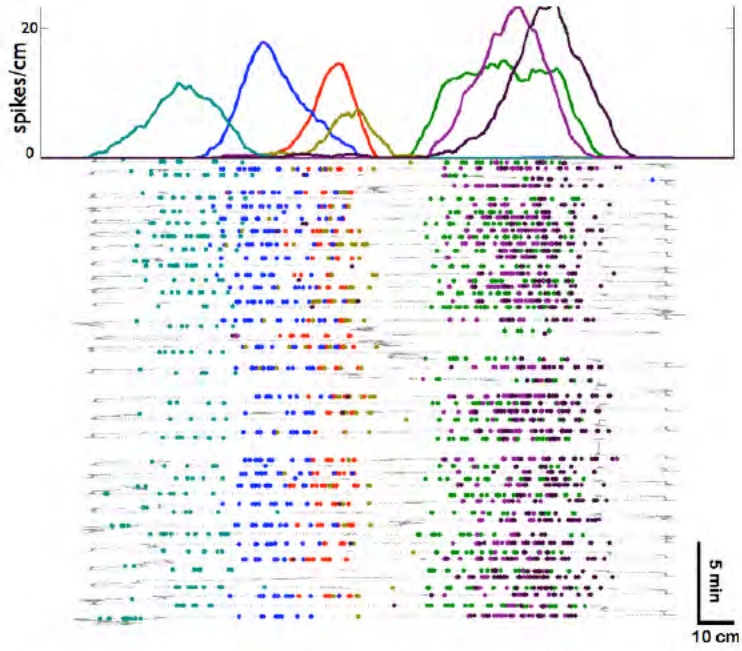


**Figure 2**: *Place fields.* The bottom panel shows that position of a rat in gray as a function of time (total time 28 minutes). The rat runs on a linear track back and forth and the positions and times of the spikes of seven simultaneously recorded place cells are shown in different colors. The top panel displays histograms of all spikes of each cell as a function of position along the track. Each of these cells ("place cells") fires in a specific location on the track ("place field"). The histograms are binned at 1cm and smoothed with a Gaussian with $\sigma$ =10 cm.

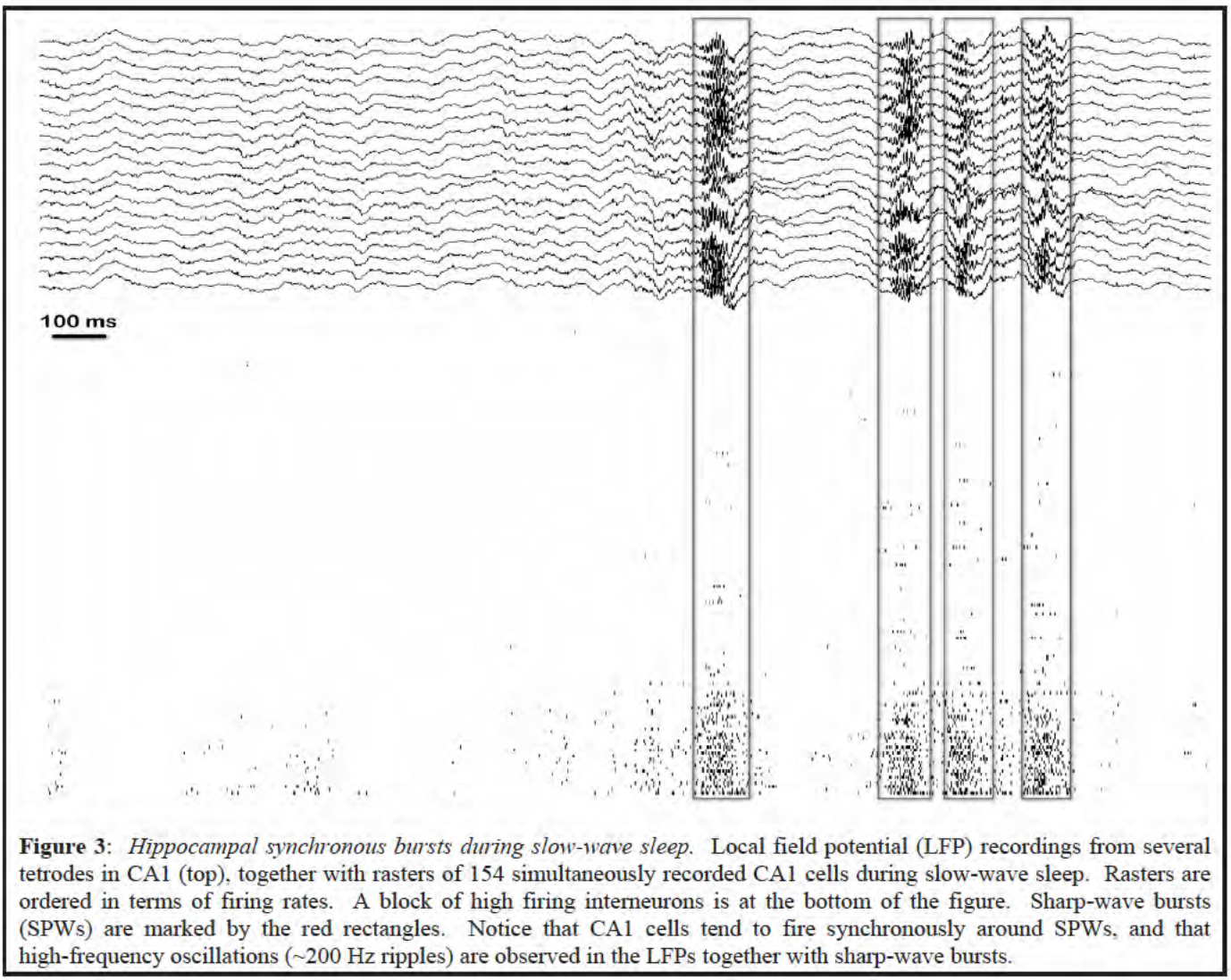


**Figure 3**: *Hippocampal synchronous bursts during slow-wave sleep.* Local field potential (LFP) recordings from several tetrodes in CA1 (top), together with rasters of 154 simultaneously recorded CA1 cells during slow-wave sleep. Rasters are ordered in terms of firing rates. A block of high firing interneurons is at the bottom of the figure. Sharp-wave bursts (SPWs) are marked by the red rectangles. Notice that CA1 cells tend to fire synchronously around SPWs, and that high-frequency oscillations (~200 Hz ripples) are observed in the LFPs together with sharp-wave bursts.

### 216. Decoupling through synchrony in recurrent networks

*Evgueniy Lubenov, Thanos Siapas*

Synchronization is a key property of many biological, physical, and engineering systems. The level of synchronization in distributed systems is often controlled by the strength of the interactions between individual elements. In brain circuits the connection strengths between neurons are constantly modified under the influence of spike-timing-dependent plasticity rules (STDP). However, the consequences of these local rules for the global dynamics of brain networks are not fully understood. We have shown that when recurrent networks exhibit population bursts STDP rules exert a strong decoupling force that desynchronizes activity. Conversely, when activity is random, the same plasticity rules can have a coupling and synchronizing influence. The presence of these opposing forces promotes the self-organization of spontaneously active recurrent networks to a state at the border between randomness and synchrony. These results may have implications for the transfer of information in cortico-hippocampal networks during memory formation, and for understanding the therapeutic effects of deep brain stimulation for Parkinson's disease and epilepsy.

### 217. Mechanisms and functional consequences of synchronous hippocampal bursts during slow-wave sleep

*Evgueniy Lubenov, Casimir Wierzynski, Ming Gu, Thanos Siapas*

Hippocampal activity during slow-wave sleep is characterized by the presence of highly synchronous bursts (sharp-wave bursts, **Figure 3**). Within each of these bursts about 40,000 CA1 cells (~10%) fire within a window of less than 100 ms. These massive population events are believed to be very effective in driving hippocampal postsynaptic targets and engaging plasticity mechanisms. We study the patterns of neuronal firing during these bursts, and analyze how these patterns evolve throughout sleep. These experimental efforts are complemented with the development of computational models of the mechanisms underlying the generation of synchronous bursts within recurrent networks.

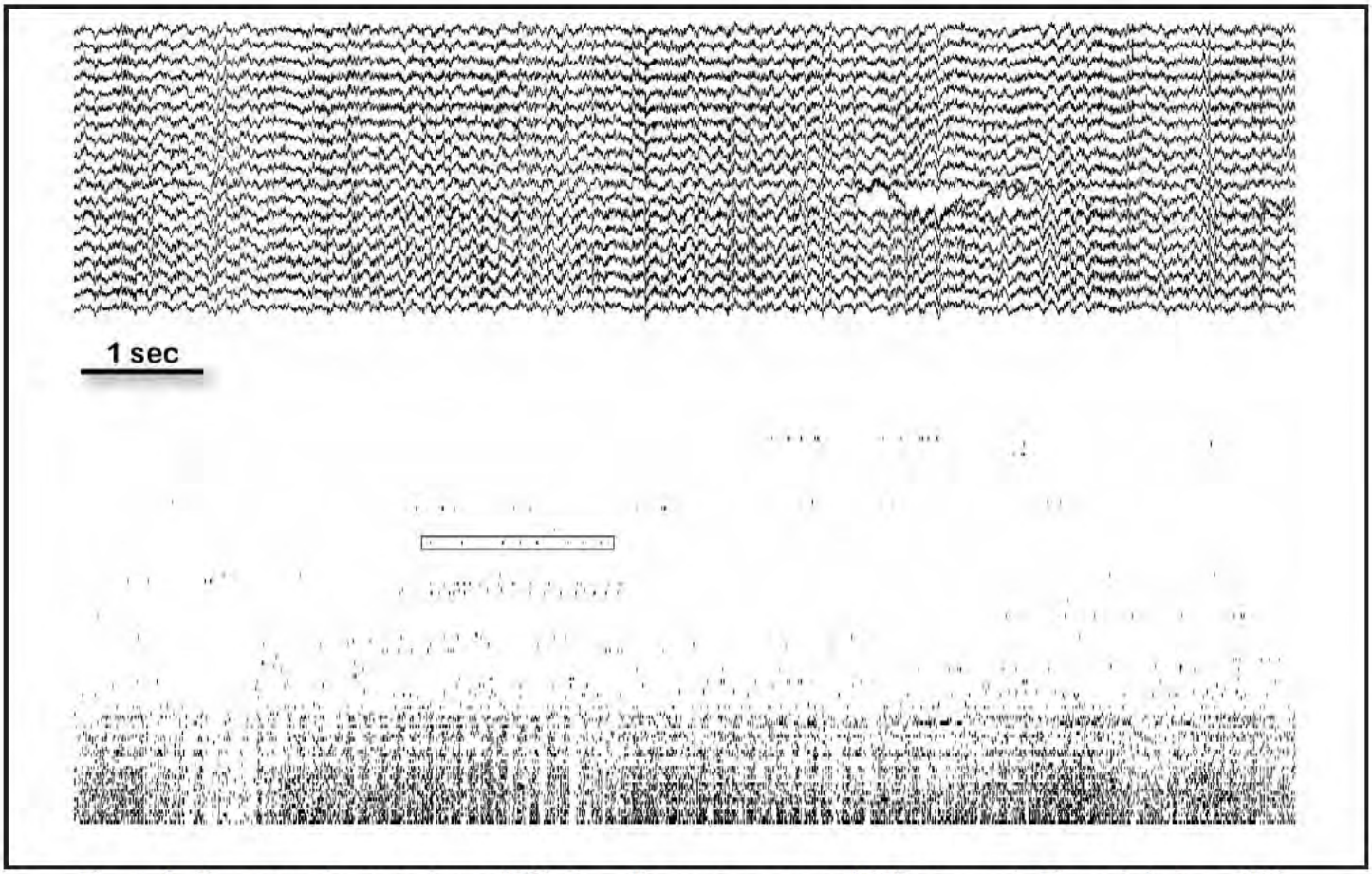


**Figure 4**: *Hippocampal activity during REM sleep.* The same neurons and LFP traces as in **Figure 1** during a REM episode (2 minutes earlier than in **Figure 3**). Notice the scale change from **Figure 1**. Theta oscillations (4-10 Hz) are clearly visible in the LFPs, and interneurons fire rhythmically phase-locked to the theta oscillations. Pyramidal cells are rhythmically activated over multiple theta cycles, similar to the activation during a pass through a place field (for example the segment marked by the red rectangle).

## 218. Hippocampal activity patterns during REM sleep

*Evgueniy Lubenov, Ming Gu, Casimir Wierzynski, Thanos Siapas*

REM sleep activity looks remarkably similar to the awake activity, hence the name, *paradoxical sleep*. As illustrated in **Figure 4**, hippocampal LFPs are characterized by theta oscillations, regular 4-10 Hz oscillations that also characterize hippocampal activity during the awake behavior. Hippocampal cells fire phase-locked to the theta rhythm both in awake behavior and REM sleep, but the preferred phases in these two brain states need not be the same [1]. The timing of spikes of hippocampal neurons with respect to the theta rhythm has strong effects on synaptic plasticity [2,3] hence quantifying changes in phase-locking may provide important insights into the functional role of sleep in memory formation. We study the evolution of phase locking to theta oscillations across multiple sleep-wake cycles, and characterize the effects of experience on phase locking in sleep. In addition, we develop tools for analyzing and identifying network activity motifs in REM sleep and study their experience specificity.

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## 219. Electrical stimulation of hippocampal circuits

*Evgueniy Lubenov, Thanos Siapas*

We chronically implant multiple tetrodes and stimulating electrodes in order to both observe and alter neuronal activity patterns. Our efforts focus on stimulating area CA3 and its afferent/efferent pathways. Low intensity pulses produce a field EPSP (fEPSP) whose slope reflects the mean synaptic coupling in the network. We track the evolution of fEPSP slopes during the awake experience and sleep, and relate systematic changes in these slopes to patterns of CA3 population activity.

**220. Prefrontal-hippocampal interactions in associative learning**

*Casimir Wierzynski, Thanos Siapas*

Eyeblink conditioning is a form of associative learning that has been shown to engage the hippocampus across a wide range of species and parameters [1]. Moreover, in its trace form, where the conditioned and unconditioned stimuli do not overlap in time, eyeblink conditioning has been shown to require an intact hippocampus for successful acquisition [2]. This hippocampal dependence falls off with time, implying that the long-term locus of the CS-US association is extra-hippocampal. Furthermore, lesions to the medial prefrontal cortex in rats have been shown to disrupt the long-term recall of the eyeblink response, but not its acquisition [3]. Using simultaneous chronic recordings from the hippocampus and medial prefrontal cortex, we are characterizing the relationships between the activity patterns in these brain areas during the acquisition of the CS-US association. These experiments enable us to study how the CS-US association is represented across prefrontal and hippocampal circuits, and how this representation evolves with learning.

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**221. Dynamics of phase locking across cortico-hippocampal networks**

*Evgueniy Lubenov, Casimir Wierzynski, Thanos Siapas*

During the awake behavior, hippocampal activity is marked by the presence of pronounced 4-10 Hz LFP oscillations known as theta oscillations [1]. We demonstrated that a significant proportion (about 40%) of the cells in the medial prefrontal cortex of the rat fire preferentially during particular phases of the hippocampal theta rhythm [2]. Furthermore, we showed that prefrontal neurons phase lock best to theta oscillations delayed by approximately 50 ms and confirmed this hippocampo-prefrontal directionality and timing at the level of correlations between single cells. Finally, phase locking of prefrontal cells is predicted by the presence of significant correlations with hippocampal cells at positive delays up to 150 ms. This indicates that direct hippocampal input is likely to have a considerable contribution to the observed prefrontal phase locking. We are studying how these phase-locking properties evolve over time and how they are modulated by behavior.

**References**

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[2] Siapas, A.G., Lubenov, E.V. and Wilson, M.A. (2005) *Neuron* **46**:141-151.

**222. Prefrontal-hippocampal interactions during slow-wave sleep**

*Casimir Wierzynski, Thanos Siapas*

Sleep is characterized by a structured combination of neuronal oscillations. In the hippocampus, slow-wave sleep (SWS) is marked by high-frequency network oscillations (200 Hz "ripples" - **Figure 3**), whereas neocortical SWS activity is organized into lo++w-frequency delta (1–4 Hz) and spindle (7–14 Hz) oscillations. We have previously shown that hippocampal ripples tend to occur together with neocortical spindles[*]. This coactivation of hippocampal and neocortical pathways may be important for the process of memory consolidation, during which memories are gradually translated from short-term hippocampal to longer-term neocortical stores. We are investigating the precise patterns of firing around ripple-spindle episodes and their relationship to awake patterns.

**Reference**

Siapas, A.G. and Wilson M.A. (1998) *Neuron* **21**:1123-1128.

**223. Comparison of CA3 and CA1 place fields**

*Ming Gu, Thanos Siapas*

Pyramidal cells ("place cells") in the hippocampal subfields CA3 and CA1 fire in specific regions of space ("place fields" – **Figure 2**). These subfields have very different intrinsic architecture: CA3 is characterized by extensive recurrent collaterals that are not prominent in CA1. Does the different architecture imply different place field properties in CA3 and CA1? We characterize the precise temporal structure of firing, and the relationships of place field firing across these subfields. We, furthermore, examine how place field representations evolve and mature when the animal is exposed to novel environments.

**224. Reward learning and VTA-hippocampal interactions**

*Thanos Siapas*

Many lines of evidence suggest that the ventral tegmental area (VTA) interacts with the hippocampus to modulate the entry of information into long-term memory[*]. The VTA-hippocampal loop has been hypothesized to be important for the detection of novelty and signaling of the behavioral relevance of stimuli in the environment. However, the organization of VTA-hippocampal interactions remains unknown, as simultaneous recordings from these areas have not been reported in the literature. Leveraging our experience with chronic multi-area tetrode recordings, we are examining the simultaneous response of multiple dopaminergic cells in the VTA and pyramidal cells in hippocampus during exposure to novel stimuli as well as during the acquisition of instrumental conditioning paradigms.

**Reference**

*Lisman, J.E. and Grace, A.A. (2005) *Neuron* **46**:703-713.

**225. Wireless recording of multi-neuronal spike trains in freely behaving animals**

In collaboration with Alan Litke of the University of California, Santa Cruz, and Markus Meister of Harvard University, we are developing a wireless system that would allow recording neuronal activity from freely behaving animals. The system will acquire signals from 64 channels, amplify and filter the waveform from each channel, and transmit the data with a broadband radio transmitter to a remote receiver. This technique will enable examining neuronal network activity while animals explore much larger spatial extends than previously possible, and may enable probing neural function in natural behaviors, such as flying, that were difficult to study in the past.

**Publications**

Lubenov E.V. and Siapas A.G. (2008) Decoupling through synchrony in neuronal circuits with propagation delays. *Neuron* **58**:118-131.

Tolias A.S., Ecker A.S., Siapas A.G., Hoenselaar A., Keliris G. and Logothetis N.K. (2007) Recording chronically from the same neurons in awake, behaving primates. *J. Neurophysiol.* **98**(6):3780-3790.

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**Staff:** Elena Armand, Lakshmi Bugga, Amy Cording, Kaushiki Menon, Violana Nesterova, Yelizaveta Nesterova, Viveca Sapin

**Support:** The work described in the research reports has been supported by:
Della Martin
Gates Grubstakes Funds
Hixon Fund
McKnight Foundation
National Institute on Aging
National Institute of Diabetes and Digestive and Kidney Diseases
National Institute of Neurological Disorders and Stroke
National Institutes of Health

**Summary of Zinn group research**

Our group studies the molecular mechanisms of axon guidance and synaptogenesis. The fruit fly *Drosophila melanogaster* is our primary experimental system. In the embryo, we examine signaling systems involved in motor and CNS axon guidance. In the larva, we study synaptogenesis and synaptic plasticity in the neuromuscular system. Our approaches combine genetics, molecular biology, electrophysiology, biochemistry, and cell biology. The current group members include three postdocs (Kaushiki Menon, Anna Salazar, Ed Silverman, and Peter (Hyung-Kook) Lee, a graduate student (Ashley Wright), a lab manager/technician (Violana Nesterova), a technician working on motor axon targeting (Amy Cording), and a technician participating in various projects by performing dissections and *Drosophila* crosses (Elena Armand). A postdoctoral scholar (Mili Jeon), and an Assistant Biologist (Lakshmi Bugga), recently left the group, but their work is described here.

**Motor axon guidance and muscle targeting**

The *Drosophila* motor axon network has provided one of the best systems in which to study axonal pathfinding mechanisms. The network is simple: 32 motoneurons innervate 30 body wall muscle fibers in each abdominal segment. Each motoneuron axon is targeted to a specific muscle fiber, and very few projection errors are made during normal development. Thus, the motor axon network is a genetically hard-wired map, and is an ideal system in which to study how genes control the formation of specific synaptic connections. In much of our work, we have focused on the roles of tyrosine phosphorylation in regulating motor axon guidance decisions. We are now conducting genetic screens to determine the mechanisms by which cell surface proteins label specific muscle fibers for recognition by motor axon growth cones.

**Targeting of motor axons to specific muscle fibers**

Despite the advances in characterizing molecules that regulate motor axon pathfinding, we still understand little about how specific muscle fibers are recognized as targets for synapse formation by these axons. Many mutations affect pathfinding decisions, leading to aberrant wiring of the neuromuscular system, but no single loss-of-function (LOF) mutations are known that block recognition of specific muscle targets. These results are most easily explained by invoking genetic redundancy in target labeling. If each muscle fiber were defined by a combination of several cell surface labels, removing one of the labels might not have a major effect on targeting of axons to that fiber. This would explain why targeting molecules have not been identified in conventional LOF genetic screens.

Studies of gain-of-function (GOF) phenotypes by other groups are consistent with the redundancy hypothesis. For example, the homophilic cell adhesion molecule Fasciclin III (Fas III) is expressed on only two muscle fibers, 6 and 7, and on the growth cone of the RP3 neuron that innervates these two fibers. Fas III appears to be a functional target label, because when it is ectopically expressed on other muscle fibers near 6 and 7, the RP3 neuron makes abnormal synapses on these Fas III-expressing fibers. However, when Fas III is removed by a LOF mutation, there is no effect on targeting of RP3 to 6 and 7. These results imply that Fas III can be used for muscle targeting, but that targeting of 6 and 7 can still proceed in its absence, presumably because these fibers are also labeled by other surface molecules that can be recognized by the RP3 growth cone when Fas III is not present.

These findings suggest that cell-surface proteins that label specific targets in the motor axon system might be identifiable by a GOF genetic screen in which candidate labels are ectopically expressed on all muscle fibers. If these proteins are functional labels, their misexpression might produce alterations in target recognition, as observed in the Fas III experiments described above. By identifying genes encoded in the *Drosophila* genome that can confer GOF phenotypes in which targeting of specific muscle fibers is altered, we will acquire the tools to understand the mechanisms involved in target recognition in this system. This type of screen should allow us to overcome the redundancy problem. For example, suppose one could identify three different cell-surface proteins that are normally expressed on a specific muscle fiber, but whose misexpression on other muscle fibers produces targeting errors. One might then predict that removing all three of these proteins by making a triple LOF mutant (through conventional or RNAi techniques) would now prevent targeting of this muscle fiber. Through these kinds of experiments, we could begin to understand the combinatorial code for muscle targeting. Insights into the motor axon targeting code would be likely to facilitate an understanding of targeting in other neuronal systems (*e.g.* the antennal lobe, optic lobe, and mushroom body),

since candidate target labels are usually expressed by a variety of neuronal and non-neuronal cell types.

To conduct this GOF screen, we first created a database of all cell-surface and secreted (CSS) proteins in *Drosophila* that are likely to be involved in specific cell-cell interactions. The database was generated by database mining and reiterative computational screening. We defined all fly genes encoding proteins that contain domains known to be present in CSS proteins in other eukaryotes (including all of the 240 domains in the 'extracellular' portion of the SMART database, http://smart.embl-heidelberg.de/browse.shtml, that are represented in flies). We then eliminated several hundred genes that we thought were unlikely to be important for cell recognition, and defined a CSS cell recognition candidate collection of 976 genes.

To drive expression of these genes in muscles, we used the 'EP' system, in which a P element containing a block of UAS sequences that are responsive to the yeast transcription factor GAL4 is jumped around the genome. Like other P elements, EPs usually land upstream of genes. If a line bearing an EP upstream of a gene is crossed to a 'driver' line expressing GAL4 in all muscle fibers, the gene will now be expressed at high levels in muscles in the resulting progeny embryos and larvae. To find EP-like elements upstream of the CSS genes, we searched through about 40,000 different insertions that have been maintained in collections of *Drosophila* lines. These include the original EP set generated by Pernille Rorth, the EY insertion lines generated in the Bellen lab, the GS lines developed in Japan, insertions generated by Exelixis, Inc. and maintained at Harvard, and the GE lines developed by GenExel, Inc. We were able to identify insertions that can confer expression of 410 of the 976 CSS genes in our database, representing about 40% of the repertoire and including members of all CSS protein families.

To screen for genes encoding potential targeting molecules, we crossed each of these insertions to a strong pan-muscle GAL4 driver and visualized motor axons and neuromuscular junction synapses in the resulting F1 progeny larvae by immunostaining. We have already identified 30 genes that cause synaptic mistargeting on muscles 12 and 13, and 55 genes that cause synaptic morphology phenotypes.

We have focused initially on the analysis of the mistargeting genes, as this is our primary interest. In a paper in press in *Neuron*, we show that one class of receptor, the leucine-rich repeat (LRR) family, is overrepresented within the mistargeting set. Using a combination of loss-of-function and gain-of-function analysis, we demonstrate that four of these LRR proteins participate in the decision of the RP5 motor axon to choose muscle 12 as its target (Kurusu *et al*., in press.). These proteins are: Tartan (Trn), Capricious (Caps), CG14351/Hattifattener (Haf), and CG8561/Als (Kurusu/Cording abstract).

**Neural receptor tyrosine phosphatases**

In the 1990s, we showed that receptor-linked protein tyrosine phosphatases (RPTPs) are selectively expressed on CNS axons and growth cones in the *Drosophila* embryo, and that these RPTPs regulate motor and CNS axon guidance during embryonic development. RPTPs directly couple cell recognition *via* their extracellular domains to control of tyrosine phosphorylation *via* their cytoplasmic enzymatic domains. The extracellular regions of the fly RPTPs all contain immunoglobulin-like (Ig) and/or fibronectin-type III (FN3) domains, which are usually involved in recognition of cell-surface or extracellular matrix ligands. Their cytoplasmic regions contain either one or two PTP enzymatic domains. The fly genome encodes six RPTPs (LAR, PTP10D, PTP69D, PTP99A, PTP52F, PTP4E), and we have generated or obtained mutations in all six of the genes encoding these proteins.

We have now performed a detailed characterization of the genetic interactions among all six RPTPs. We find that each growth cone guidance decision in the neuromuscular system has a requirement for a unique subset of RPTPs; thus, in a sense, there is an "RPTP code" for each decision. In some cases, the RPTPs work together, so that defects are only observed when two or more are removed. In other cases, however, phenotypes produced by removal of one RPTP are suppressed when a second RPTP is also absent. Our results provide evidence for three types of relationships among the RPTPs: partial redundancy; collaboration; and competition. The first Jeon abstract describes our most recent findings, on Ptp4E; these were recently published.

**A genetic approach to identification of RPTP ligands**

The ligands recognized by RPTPs *in vivo* have not been identified in any system. In order to understand how RPTPs regulate axon guidance, it is essential to know when and where they engage ligands, and how ligand binding affects enzymatic activity and/or localization.

One of our current approaches to identifying ligands is based on our observation that fusion proteins in which the extracellular domains of RPTPs are joined to human placental alkaline phosphatase (AP) can be used to stain live-dissected *Drosophila* embryos. Each of four fusion proteins (LAR-AP, PTP69D-AP, PTP10D-AP, PTP99A-AP) binds in a specific manner. Each fusion protein stains a subset of CNS axons and also binds to other cell types in the periphery. To identify the genes encoding the RPTP ligands, we are screening deficiency mutations that remove specific portions of the genome. We began by screening the Bloomington 'deficiency (Df) kit' of 266 fly lines. Each Df line was crossed to GFP balancers so that Df/Df embryos could be identified, and we then stained these embryos with each of four fusion proteins (LAR-HS2-AP, 69D-AP, 10D-AP, 99A-AP). Since each Df lacks a specific region of the genome, if homozygous Df embryos don't stain with a fusion protein, this indicates that this genomic region contains a gene required for ligand expression. Overlapping Dfs and point

mutants can then be screened in order to identify the relevant gene within the Df.

Using this screen, we found a Df that contains a gene encoding a ligand that binds to LAR-AP, and have identified this ligand as Syndecan (Sdc). This work has been published (Fox, A.N. and Zinn, K. (2005). The heparan sulfate proteoglycan Syndecan is an *in vivo* ligand for the *Drosophila* LAR receptor tyrosine phosphatase, *Curr. Biol.* **15**:1701-1711.) Sdc is a heparan sulfate proteoglycan (HSPG). Our results show that LAR binds to the glycosaminoglycan side chains of Sdc with nanomolar affinity, and that Sdc is required for DLAR-mediated axon guidance. We can generate motor axon guidance errors by overexpressing LAR on neurons, and find that the same errors are generated by ectopically expressing Sdc on muscles. This Sdc GOF phenotype is suppressed by LOF mutations in the *Lar* gene, indicating that LAR is epistatic to (downstream of) Sdc. This result shows that muscle Sdc can function in *trans* as a ligand for LAR on neuronal growth cones, and suggests that binding to Sdc increases LAR's signaling activity.

We have continued the Df screen, and have identified four regions required for 99A-AP staining. Ashley Wright is now screening overlapping Dfs and point mutations to find the responsible genes. Our results thus far already indicate that a novel glial-neuronal interaction is required to specify expression of the 99A ligand.

Our approach is general, and can be used to identify ligands for any 'orphan receptor' that has a *Drosophila* ortholog. We also used the method to define genomic regions required for expression of selected cell surface antigens, including those recognized by the 1D4 and BP102 monoclonal antibodies (mAbs). As part of the analysis, we have defined a new Df kit for embryonic screening, which uses alternative Bloomington Dfs to allow screening of regions of the genome whose removal in the normal Df kit causes early developmental failure. This new kit contains about 450 lines, and covers about 89% of polytene chromosome bands. It can be used to analyze any region of the genome for the desired embryonic phenotype. We have already analyzed about half of the genome for regions necessary for motor axon guidance by staining Df embryos with 1D4 (A. Wright *et al*., manuscript in preparation).

**A gain-of-function screen for RPTP ligands**

Despite the success of the Df screen (an LOF approach), it is clearly not capable of identification of all RPTP ligands, and may not even be capable of finding most of them. First, about 11% of the genome still cannot be screened, either because no Dfs exist there or because embryos homozygous for those regions do not develop. Second, and most important, the four RPTP-AP probes all stain subsets of CNS axons, in addition to other patterns outside the CNS. If multiple ligands for an RPTP were all expressed on CNS axons, removal of one ligand gene by a Df might not perturb staining enough to detect a difference from wild type. We already know that this is the case for LAR: Sdc is expressed both on CNS axons and in the periphery, but only peripheral staining is eliminated in an *Sdc* mutant. CNS axons in *Sdc* mutants continue to stain with LAR-AP, and are also stained by a mutant version of LAR-AP that cannot bind to Sdc[7]. These data show that there is at least one non-HSPG ligand for LAR that is expressed on CNS axons together with the HSPG ligand Sdc. Because of these limitations, we have developed a new GOF approach to ligand screening that allows direct identification of proteins that bind in embryos to an RPTP probe, regardless of whether such proteins are normally expressed in patterns that overlap with those of other ligands. This approach is also general and can be applied to any orphan receptor of interest that has *Drosophila* orthologs. It is based on observations made by Fox and Zinn (2005), who showed that when Sdc is ectopically expressed on muscle fibers, this produces ectopic muscle staining with LAR-AP, which normally does not bind to muscles. Thus, if one were able to express ligand genes in new patterns in the embryo, one would expect to be able to see additional staining with RPTP-AP probes and identify ligands in this manner.

Our approach is a directed EP screen. It uses the collection of EP element lines described above to ectopically express CSS proteins in new patterns in the embryo. To screen for new RPTP ligands, we are crossing each line in our CSS EP collection to GAL4 driver lines that confer ectopic gene expression in cells that normally do not stain with RPTP-AP fusion proteins. If I detect new staining patterns in embryos derived from such a cross, this may indicate that the gene driven by that EP-like element encodes a protein that can bind to the RPTP. Peter Lee has already found a number of such lines, and these define several potential new RPTP ligands.

**Searching for RPTP substrates**

It is difficult to identify PTP substrates biochemically because PTPs usually do not display strong specificity *in vitro*. To find substrate candidates, we performed yeast two-hybrid screens with 'substrate-trap' mutant versions of PTP10D, PTP69D, PTP52F, and PTP99A. These 'trap' proteins form stable complexes with tyrosine-phosphorylated substrates because they bind normally but cannot catalyze dephosphorylation. We introduced a constitutively activated chicken Src tyrosine kinase into yeast together with the PTP trap constructs and the cDNA library, in the hope that it would phosphorylate relevant substrate fusion proteins made from cDNA library plasmids. We identified several classes of clones whose interactions with the substrate-trap RPTPs are dependent on coexpression of the tyrosine kinase, suggesting that they may be substrates. One of these encodes Tartan (Trn), and we have submitted a paper showing that Trn is in fact a Ptp52F substrate and that Trn and Ptp52F participate in the same signaling pathway in motor neurons (Bugga abstract).

**Tracheal development: Regulation of EGFR tyrosine kinase activity by the Ptp10D and Ptp4E RPTPs regulates lumen formation**

In the process of examination of double mutants lacking expression of the closely related proteins Ptp4E and Ptp10D, we noticed that the tracheal network exhibits severe defects in these embryos. We have shown that these defects, which include formation of huge 'bubbles' along the tracheal tubes, arise from mislocalization of apical proteins. Localization of basolateral proteins is unaffected. These trafficking defects are associated with upregulation of EGFR activity, and Ptp10D and EGFR directly associate in cultured cells. We have submitted a paper describing this work (Jeon abstract #2).

**Genes controlling synaptogenesis in the larval neuromuscular system**

Motor growth cones reach their muscle targets during late embryogenesis and then mature into presynaptic terminals that are functional by the time of hatching. The pattern of Type I neuromuscular junction (NMJ) synapses in the larva is simple and highly stereotyped, with boutons restricted to specific locations on each muscle fiber. These synapses continue to expand and change as the larva grows, because their strengths must be matched to the sizes of the muscle fibers they drive. This growth represents a form of synaptic plasticity, because it is controlled by feedback from the muscle to the neuron. Studies of NMJ synapses in flies are relevant to an understanding of synaptic plasticity in the mammalian brain, because the fly NMJ is a glutamatergic synapse, organized into boutons, that uses ionotropic glutamate receptors homologous to vertebrate AMPA receptors.

**Control of synaptic local translation by Pumilio and Nanos**

Our recent work on synapses has focused on control of synaptic protein translation. Local translation at synapses has been studied in *Aplysia*, mammalian, and arthropod systems. It has attracted interest because it is a mechanism that allows neurons to separately adjust the strengths of individual synapses.

To identify genes involved in synaptogenesis in larvae, including those that regulate local translation, we devised and executed a GOF screen of live third instar larvae. In the screen, we identified *pumilio* (*pum*), which encodes an RNA-binding protein that shuts down translation of specific mRNAs by binding to their 3' untranslated regions. Translational repression by Pum controls posterior patterning during embryonic development. In a 2004 paper, we showed that Pum is an important mediator of synaptic growth and plasticity at the NMJ. Pum is localized to the postsynaptic side of the NMJ in third instar larvae, and is also expressed in larval neurons. Neuronal Pum regulates synaptic growth. In its absence, NMJ boutons are larger and fewer in number, while Pum overexpression increases bouton number and decreases bouton size. Postsynaptic Pum negatively regulates expression of the essential translation factor eIF-4E (the cap-binding protein) at the NMJ, and Pum binds selectively to the 3'UTR of *eIF-4E* mRNA. These data suggest that Pum is a direct regulator of local eIF-4E translation, and that eIF-4E (which is normally limiting for translation) in turn switches on translation of other synaptic mRNAs. Pum also directly regulates the GluRIIa glutamate receptor. These results, together with genetic epistasis studies, suggest that postsynaptic Pum modulates synaptic function *via* direct control of local synaptic translation.

In our current work, we have studied the Pum cofactor Nanos, which works together with Pum to repress translation in the early embryo, as a participant in Pum regulation of targets at the NMJ. In *nos* mutants (or transgenic *nos* RNAi larvae), GluRIIB is downregulated, while the alternative subunit GluRIIB is upregulated. Thus, the phenotypes of *nos* and *pum* are opposite in this system. We also show that Pum represses Nos expression. Regulation of GluRIIA and Nos by Pum involves direct binding of Pum to the 3' UTRs of their mRNAs. We have submitted a paper describing these results (Menon abstract).

**Assembly of Pumilio into ordered aggregates as a regulatory switching mechanism**

We are also studying Pum in another context: its potential role as switch that could control synaptic translation via regulated assembly into an ordered aggregate. This project emerged from a computational search we performed to identify switch proteins that might have the capacity to form ordered aggregates. This is relevant to human disease as well, since proteins involved in many human neurodegenerative diseases share a propensity to form amyloid aggregates. One class of sequences that can form amyloids are domains rich in glutamine (Q) and asparagine (N). These are present in many metazoan proteins, including ~450 in *Drosophila*. Q/N domains are found in all yeast prions, and these domains have been positively selected during evolution, perhaps in order to allow reversible switching of the functional domain of the prion into an inactive aggregated state. We wondered this type of selection might also maintain Q/N domains in metazoans. To examine this question, we devised a computational search strategy to identify candidates for nucleic-acid binding prion switches in metazoan proteomes.

One of the two strong *Drosophila* candidates identified in this search is Pum. As described above, work by our group had shown that Pum is localized to the postsynaptic side of the larval NMJ, where it acts as a regulator of local mRNA translation. We found that a Q/N-rich domain (denoted NQ1) from Pum exhibits prion-like behavior in budding yeast, including heritable phenotypic switching and reversibility by guanidine hydrochloride. NQ1 purified from *E. coli* forms amyloid fibrils *in vitro*. To test whether NQ1 aggregate formation can perturb Pum's function in the nervous system, we created transgenic fly lines in which NQ1 expression is driven by GAL4. Our results show that postsynaptic NQ1

expression generates alterations in the NMJ that phenocopy the *pum* loss-of-function phenotype and interact genetically with *pum* mutations. Postsynaptic Pum overexpression is lethal, but co-overexpression of NQ1 rescues this lethality, suggesting that NQ1 can inactivate endogenous Pum. We have submitted a paper describing these findings (Salazar/Silverman abstract).

**226. The cell surface receptor Tartan is a potential *in vivo* substrate for the receptor tyrosine phosphatase Ptp52F**

*Lakshmi Bugga[1], Anuradha Ratnaparkhi[2]*

Receptor tyrosine phosphatases (RPTPs) are essential regulators of motor axon guidance in *Drosophila*. To search for substrates for these RPTPs, we conducted a modified two-hybrid screen in yeast using substrate-trapping mutants as "bait." We identified the cell-surface receptor Tartan (Trn) as a candidate substrate for the Ptp52F RPTP. We examined whether Trn can interact with the Ptp52F substrate-trapping mutant in transfected *Drosophila* Schneider 2 (S2) cells. Trn-GFP fusion proteins were tyrosine-phosphorylated in S2 cells treated with pervanadate or transfected with v-Src expression plasmids. Trn and Trn-GFP bound to the Myc-tagged Ptp52F substrate-trapping mutant, but only if the v-Src kinase was also expressed. Coexpression of wild-type Ptp52F caused dephosphorylation of v-Src-phosphorylated Trn-GFP. To examine the specificity of the Trn-Ptp52F interaction *in vitro*, we incubated purified wild-type and substrate-trap Ptp52F-GST fusion proteins with pervanadate-treated S2 cell lysates. Wild-type Ptp52F dephosphorylated Trn-GFP, but could also dephosphorylate most other bands in the lysate. The substrate-trapping mutant, however, exhibited high specificity, because it bound to only one prominent phosphoprotein band in pervanadate-treated S2 cell lysates, and we identified this band as endogenous Trn. To evaluate whether Trn and Ptp52F are part of the same pathway *in vivo*, we examined motor axon guidance in mutant embryos. We found that *trn* and *Ptp52F* mutations produce identical phenotypes affecting the SNa motor nerve. The genes display dosage-dependent interactions, so that *Ptp52F/+*, *trn/+* embryos have the same phenotypes as *Ptp52F/Ptp52F* or *trn/trn* embryos. These results suggest that Ptp52F regulates Trn signaling in SNa motor neurons.

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**227. Redundancy and compensation in axon guidance: Genetic analysis of the *Drosophila* Ptp10D/Ptp4E receptor tyrosine phosphatase subfamily**

*Mili Jeon, Kai Zinn*

*Drosophila* has six receptor protein tyrosine phosphatases (RPTPs), five of which are expressed primarily in neurons. Mutations in all five affect axon guidance, either alone or in combination. Highly penetrant CNS and motor axon guidance alterations are usually observed only when specific combinations of two or more RPTPs are removed. Here, we examine the sixth RPTP, Ptp4E, which is broadly expressed. Ptp4E and Ptp10D are closely related Type III RPTPs. Non-drosophilid insect species have only one Type III RPTP, which is closest to Ptp10D. We found that *Ptp4E* mutants are viable and fertile. We then examined *Ptp4E Ptp10D* double mutants. These die before the larval stage, and have a mild CNS phenotype in which the outer longitudinal 1D4 bundle is frayed. *Ptp10D Ptp69D* double mutants have a strong CNS phenotype in which 1D4 axons abnormally cross the midline and the outer and middle longitudinal bundles are fused to the inner bundle. To examine if *Ptp4E* also exhibits synthetic phenotypes in combination with *Ptp69D*, we made *Ptp4E Ptp69D* double mutants and *Ptp4E Ptp10D Ptp69D* triple mutants. No phenotype was observed in the double mutant. The triple mutant phenotype differs from the *Ptp10D Ptp69D* phenotype in two ways. First, the longitudinal tracts appear more normal than in the double mutant; two or three bundles are observed, although they are disorganized and fused. Second, axons labeled by the SemaIIB-τMyc, marker often cross in the wrong commissure. We also examined motor axon guidance, and found that no phenotypes are observed in any *Ptp4E* double mutant combination. However, triple mutants in that *Ptp4E Ptp10D* was combined with *Ptp69D* or *Ptp52F* exhibited stronger phenotypes than the corresponding *Ptp10D* double mutants. Type III RPTPs are required for viability in *Drosophila*, since *Ptp4E Ptp10D* double mutants die before the larval stage. Unlike Ptp10D, Ptp4E appears to be a relatively minor player in the control of axon guidance. Strong phenotypes are only observed in triple mutants in that both Type III RPTPs are eliminated together with Ptp69D or Ptp52F. Our results allow us to construct a complete genetic interaction matrix for all six of the RPTPs.

**228. Type III receptor tyrosine phosphatases regulate apical protein trafficking and lumen formation in the *Drosophila* tracheal system**

*Mili Jeon, Kai Zinn*

Receptor tyrosine phosphatases (RPTPs) are transmembrane proteins that regulate tyrosine kinase signaling pathways. We have defined a new role for RPTPs in epithelial tube development. Loss of both Ptp4E and Ptp10D, the two *Drosophila* Type III RPTPs, produces a unique phenotype in which large intracellular vacuoles appear along specific tracheal branches. The vacuoles contain apical but not basolateral membrane proteins.

They appear to be generated by endocytosis from the apical membrane *via* a process that requires dynamin and Rab5. Lumen formation is blocked in vacuole-containing branches. The phenotype involves upregulation of EGFR tyrosine kinase signaling and activation of Rho family GTPases. Our data are consistent with a model in which alterations in phosphotyrosine signaling in the double *Rptp* mutant tip the balance between exo- and endocytosis or induce a new endocytic process. This leads to massive internalization of apical membrane in tracheal cells that are undergoing rearrangement and generating new lumens.

**229. A screen of cell-surface molecules identifies leucine-rich repeat proteins as key mediators of synaptic target selection**

*Mitsuhiko Kurusu[*], Amy Cording, Misako Taniguchi[*], Kaushiki Menon, Emiko Suzuki[*], Kai Zinn*

In *Drosophila* embryos and larvae, a small number of identified motor neurons innervate body wall muscles in a highly stereotyped pattern. Although genetic screens have identified many proteins that are required for axon guidance and synaptogenesis in this system, little is known about the mechanisms by which muscle fibers are defined as targets for specific motor axons. To identify potential target labels, we screened 410 genes encoding cell-surface and secreted proteins, searching for those whose overexpression on all muscle fibers causes motor axons to make targeting errors. Thirty such genes were identified, and a number of these were members of a large gene family encoding proteins whose extracellular domains contain leucine-rich repeat (LRR) sequences, which are protein interaction modules. By manipulating gene expression in muscle 12, we showed that four LRR proteins participate in the selection of this muscle as the appropriate synaptic target for the RP5 motor neuron.

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**230. The translational repressors Nanos and Pumilio have divergent effects on presynaptic terminal growth and postsynaptic glutamate receptor subunit composition**

*Kaushiki Menon, Shane Andrews[*], Mala Murthy, Elizabeth R. Gavis[*], Kai Zinn*

Pumilio (Pum) is a translational repressor that binds selectively to target mRNAs and recruits Nanos (Nos) as a corepressor. Pum and Nos are best known for their roles in embryonic pattern formation, but they also regulate dendritic branching in the peripheral nervous system and $Na^+$ channel expression in motor neurons. Pum represses expression of the translation factor eIF-4E and the glutamate receptor subunit GluRIIA in the larval neuromuscular system. Nos, like Pum, is expressed at the neuromuscular junction (NMJ) and in neuronal cell bodies. Surprisingly, however, Nos and Pum have divergent functions on both the pre- and postsynaptic sides of the NMJ. In *nos* mutant and neuronal *nos* RNAi larvae, the number of NMJ boutons is increased, while loss of Pum reduces bouton number. On the postsynaptic side, Nos acts in opposition to Pum in regulating the subunit composition of the glutamate receptor. NMJ active zones are associated with GluRIIA- and GluRIIB-containing receptor clusters. Loss of Nos causes downregulation of GluRIIA and increases the levels of GluRIIB. Consistent with this finding, the electrophysiological properties of NMJs lacking postsynaptic Nos suggest that they employ primarily GluRIIB-containing receptors. Nos is a target for Pum repression, and Pum binds selectively to the 3' UTRs of the *nos* and *GluRIIA* mRNAs. Our findings suggest a model in which the regulatory interplay among Pum, Nos, GluRIIA, and GluRIIB could cause a small change in Pum activity to be amplified into a large shift in the balance between GluRIIA and GluRIIB synapses.

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**231. Regulation of synaptic Pumilio function by an aggregation-prone domain**

*Anna M. Salazar, Edward J. Silverman, Kaushiki Menon, Kai Zinn*

We identified Pumilio (Pum), a *Drosophila* translational repressor, in a computational search for metazoan regulatory proteins whose activities might be controlled by assembly into ordered aggregates. The search algorithm was based on evolutionary sequence conservation patterns observed for yeast prion proteins, which contain aggregation-prone glutamine/asparagine (Q/N)-rich domains attached to functional domains of normal amino acid composition. Pum controls pattern formation in the early embryo, and also regulates postsynaptic translation at larval neuromuscular junctions (NMJ). To assess how NQ1, the Pum domain identified in our search, might affect Pum's translational repression activity, we expressed it in *Drosophila* muscles, and found that it interacts selectively with *pum* mutations. NQ1 negatively regulates endogenous Pum, producing gene dosage-dependent *pum* loss-of-function NMJ phenotypes. Deletion of the Q/N sequences from NQ1 eliminates this genetic interaction. The phenotypes were assessed by counting NMJ boutons and by examining expression of the GluRIIA glutamate receptor subunit, which is a target for Pum repression. NQ1 coexpression also rescues animals from lethality caused by Pum overexpression in muscles. We have not been able to observe NQ1 aggregates in transgenic *Drosophila*. Thus, to evaluate whether NQ1's effects might be mediated through aggregation, we studied its behavior in yeast and *in vitro*. NQ1 and the nematode Pum ortholog PUF-9 localize to macroscopic aggregates (foci) in yeast, and NQ1 assembles into amyloid fibrils *in vitro*. NQ1 and PUF-9 can also generate the yeast *Pin+* prion trait, which is transmitted by a heritable aggregate.

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# Developmental and Regulatory Biology

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**Support:** The work described in the following research reports has been supported by:
CIRM
National Institutes of Health (NINDS, NIDCR, NHGRI)

**Summary:** This laboratory's research centers on the early formation of the nervous system in vertebrate embryos. The peripheral nervous system forms from two cell types that are unique to vertebrates: neural crest cells and ectodermal placodes. We study the cellular and molecular events underlying the formation, cell lineage decisions and migration of these two cells types. The neural crest is comprised of multipotent stem-cell-like precursor cells that migrate extensively and give rise to an amazingly diverse set of derivatives. In addition to their specific neuronal and glial derivatives, neural crest cells can also form melanocytes, craniofacial bone and cartilage and smooth muscle. Placodes are discrete regions of thickened epithelium that give rise to portions of the cranial sensory ganglia, as well as form the paired sense organs (lens, nose, ears). Placodes and neural crest cells share several properties including the ability to migrate and to undergo an epithelial to mesenchymal transition. Their progeny are also similar: sensory neurons, glia, neuroendocrine cells, and cells that can secrete special extracellular matrices.

Our laboratory concentrates on studying the cellular and molecular mechanisms underlying the induction, early development and evolution of the neural crest and placodes. This research addresses fundamental questions concerning cell commitment, migration and differentiation using a combination of techniques ranging from experimental embryology to genomic approaches to novel gene discovery and identification of gene regulatory regions. These studies shed important light on the mechanisms of neural crest and placode formation, migration and differentiation. In addition, the neural crest and placodes are unique to vertebrates. In studying the evolution of these traits, we hope to better understand the origin of vertebrates.

Because these cell types are involved in a variety of birth defects and cancers such as neurofibromatosis, melanoma, neuroblastoma, our results on the normal mechanisms of neural crest development provide important clues regarding the mistakes that may lead to abnormal development or loss of the differentiated state.

**232. Robo2/Slit1-dependent cell-cell interactions mediate assembly of the trigeminal ganglion**

*Celia Shiau, Peter Lwigale, Marianne Bronner-Fraser*

Vertebrate cranial sensory ganglia, responsible for sensation of touch, taste and pain in the face and viscera, are comprised of both ectodermal placode and neural crest cells. The cellular and molecular interactions allowing generation of complex ganglia remain unknown. We have found that proper formation of the trigeminal ganglion, the largest of the cranial ganglia, relies on reciprocal interactions between placode and neural crest cells in chick, since removal of either population results in severe defects. We demonstrate that ingressing placode cells express the Robo2 receptor, whilst early migrating cranial neural crest cells express its cognate ligand Slit1. Perturbation of this receptor-ligand interaction by blocking Robo2 function or depleting either Robo2 or Slit1 using RNAi disrupts proper ganglion formation. The resultant disorganization mimics the effects of neural crest ablation. Thus, our data reveal a novel and essential role for Robo2/Slit1 signaling in mediating neural crest-placode interactions during trigeminal gangliogenesis.

**233. Essential role for PDGF signaling in trigeminal placode induction**

*Katherine McCabe, Marianne Bronner-Fraser*

Much of the peripheral nervous system of the head is derived from ectodermal thickenings, called placodes, which delaminate or invaginate to form cranial ganglia and sense organs. The trigeminal ganglion, arising lateral to the midbrain, forms via interactions between the neural tube and adjacent ectoderm. This induction triggers expression of Pax3, ingression of placode cells and their differentiation into neurons. However, the molecular nature of the underlying signals remains unknown. We investigate the role of PDGF signaling in ophthalmic trigeminal placode induction. By *in situ* hybridization, PDGF receptor b is expressed in the cranial ectoderm at the time of trigeminal placode formation, with the ligand PDGFD expressed in the midbrain neural folds. Blocking PDGF signaling *in vitro* results in a dose-dependent abrogation of Pax3 expression in recombinants of quail ectoderm with chick neural tube that recapitulate placode induction. *In ovo* microinjection of PDGF inhibitor causes a similar loss of Pax3, as well as the later placodal marker, CD151, and failure of neuronal differentiation. Conversely, microinjection of exogenous PDGFD increases the number of Pax3+ cells in the trigeminal placode and neurons in the condensing ganglia. Our results provide the first evidence for a signaling pathway involved in the ophthalmic (opV) trigeminal placode induction.

**234. EWS-Fli1 causes neuroepithelial defects and abrogates emigration of neural crest stem cells**

*Ed Coles, Elizabeth Lawlor, Marianne Bronner-Fraser*

The most frequently occurring chromosomal translocation that gives rise to the Ewing's sarcoma family of tumors (ESFT) is the chimeric fusion gene EWS-FLI1 that encodes an oncogenic protein comprised of the N terminus of EWS and the C terminus of FLI1. Although the genetic basis of ESFT is fairly well understood, the putative cellular origin of ESFT remains to be determined. Previous work has proposed that neural crest stem cells may be the causative cell type responsible for ESFT. However, surprisingly little is known about the expression pattern or role of either wild-type EWS or FLI1 in this stem cell population during early embryonic development. Using the developing chick embryo as a model system, we identified EWS expression in emigrating and migratory neural crest stem cells, whereas FLI1 transcripts were found to be absent in these populations and were restricted to developing endothelial cells. By ectopically expressing EWS-FLI1 or wild-type FLI1 in the developing embryo, we have been able to study the cellular transformations that ensue in the context of an *in vivo* model system. Our results reveal that mis-expression of the chimeric EWS-FLI1 fusion gene, or wild-type FLI1, in the developing neural crest stem cell population leads to significant aberrations in neural crest development. The disruption of the neural crest stem cell population, and subsequent loss of proper neural crest formation, that occurs upon mis-expression of the EWS-FLI1 oncogene may be an initiating event in ESFT genesis.

**235. A critical role for Cadherin6B in regulating avian neural crest emigration**

*Ed Coles, Lisa Taneyhill, Marianne Bronner-Fraser*

Neural crest cells originate in the dorsal neural tube but subsequently undergo an epithelial-to-mesenchymal transition (EMT), delaminate and migrate to diverse locations in the embryo where they contribute to a variety of derivatives. Cadherins are a family of cell-cell adhesion molecules expressed in a broad range of embryonic tissues, including the neural tube. In particular, cadherin6B (Cad6B) is expressed in the dorsal neural tube prior to neural crest emigration but is then repressed by the transcription factor Snail2, expressed by premigratory and early migrating cranial neural crest cells. To examine the role of Cad6B during neural crest migration, we have perturbed Cad6B protein levels in the cranial neural crest-forming region and have examined subsequent effects on emigration and migration. The results show that knock down of Cad6B leads to premature neural crest cell emigration, whereas Cad6B overexpression disrupts migration. Our data reveal a novel role for Cad6B in controlling the proper timing of neural crest emigration and delamination from the neural tube of the avian embryo.

**236. Insights from the amphioxus genome on origin of neural crest in the "new" vertebrate head**

*Jr-Kai Yu, Daniel Meulemans, Marianne Bronner-Fraser*

The evolution of the neural crest has been proposed to play a key role in vertebrate origins by remodeling the chordate head into a "new head" that enabled early vertebrates to shift from filter feeding to active predation. In the embryo, neural crest cells arise at the neural plate border, undergo an epithelial-mesenchymal transition, and become a migratory cell population that forms defining features of vertebrates, including craniofacial skeleton, peripheral nervous system, and pigment cells. We show that the genome of a basal chordate, amphioxus, contains homologues of nearly all vertebrate genes implicated in a putative gene regulatory network for neural crest development. Our survey of gene expression shows that early inducing signals, neural plate border patterning genes, and genes involved in pigment cell differentiation appear conserved between amphioxus and vertebrates. Furthermore, exogenous BMP affects expression of amphioxus neural plate border genes as in vertebrates, suggesting that conserved signaling and regulatory circuits are responsible for specifying the neural plate border throughout chordates. In contrast to this core conservation, homologues of many neural crest specifier genes are not expressed at the amphioxus neural plate border or neural tube. These results raise the intriguing possibility that this level of the network was co-opted for evolution of the vertebrate neural crest in order to confer migratory ability to these precursor cells.

**237. Discovery of transcription factors and other candidate regulators of neural crest development**

*Meghan Adams, Laura Gammill, Marianne Bronner-Fraser*

After induction at the neural plate border, neural crest cells leave the neural tube by undergoing an epithelial to mesenchymal transition in order to migrate long distances and form divergent derivatives. Despite several previous efforts to identify genes upregulated in neural crest populations, transcription factors have proved to be elusive due to their relatively low expression levels and their sometimes, transient expression. We have screened newly induced neural crest cells for early target genes, with the aim of identifying transcriptional regulators, as well as other developmentally important genes. This has yielded numerous candidate regulators, including more than a dozen transcription factors, many of which were not previously associated with neural crest development. The level of upregulation of several transcription factors was confirmed using quantitative real-time PCR in newly induced neural crest populations *in vitro*. We then performed a secondary screen by *in situ* hybridization to verify the expression of >100 genes in the neural crest. In addition to the neural crest, we note that several of the transcription factors and other genes are expressed in other

migratory cell populations and have been implicated in diverse forms of cancer.

**238. The amphioxus SoxB family: Implications for the evolution of vertebrate placodes**

*Daniel Meulemans, Marianne Bronner-Fraser*

Cranial placodes are regions of thickened ectoderm that give rise to sense organs and ganglia in the vertebrate head. Similar structures exist in urochordates, but not cephalochordates, suggesting the first chordates lacked placodes. It is unclear how placodes arose and diversified in the chordate lineage. While cephalochordates possess epidermal sensory cells and regions of invaginating ectoderm, these properties are not juxtaposed as placodal tissue. To elucidate the origins of placodes, we isolated the complete set of SoxB genes from amphioxus and analyzed their expression in embryos and larvae. We find that while amphioxus possesses a single SoxB2 gene, it has three SoxB1 paralogs. Like vertebrate SoxB1 genes, one of these paralogs is expressed in epidermal ectoderm destined to give rise to sensory cells. When considered in the context of amphioxus placode marker homolog expression, amphioxus SoxB1 expression suggests a surprising diversity of sensory cell types was present in the first chordates. We propose a model for placode evolution and diversification whereby the full complement of vertebrate placodes evolved by serial recruitment of distinct sensory cell specification programs to anterior pre-placodal ectoderm.

**239. A genetic model for the evolution of the vertebrate pharynx**

*Daniel Meulemans, Marianne Bronner-Fraser*

Central to understanding vertebrate evolution is uncovering the origin of the vertebrate head, a problem difficult to approach using paleontology and comparative morphology due to a lack of clear intermediate forms. Recent work in protochordates has shed light on the developmental bases of vertebrate head evolution, though outstanding questions remain regarding the origin of cranial cartilage. We present evidence from the cephalochordate amphioxus that supports a novel premise for the evolution of vertebrate head cartilage. Our data suggest the genetic network controlling vertebrate head skeletogenesis was assembled largely from pre-existing gene networks operating in the neural tube and pharyngeal mesoderm of a protochordate ancestor. This model explains how neural crest-derived cartilages could have evolved relatively quickly from migratory neural tube cells and provides a conceptual bridge between the protochordate and vertebrate body plans.

**240. Ancient evolutionary origin of the neural crest gene regulatory network**

*Tatjana Sauka-Spengler, Daniel Meulemans, Matt Jones, Marianne Bronner-Fraser*

The neural crest, a uniquely vertebrate cell type, migrates from its origin, the neural plate border, to form diverse derivatives. We previously hypothesized that a neural crest gene regulatory network (NC-GRN) guides neural crest formation. We investigate when during evolution this hypothetical network emerged by experimentally analyzing neural crest formation in lamprey, a basal extant vertebrate. We identify 50 NC-GRN homologs and use morpholinos to demonstrate a critical role for eight transcriptional regulators. The results reveal conservation in deployment of upstream factors, suggesting that proximal portions of the network arose early in vertebrate evolution and have been conserved for >500 million years. We further demonstrate biphasic expression of neural crest specifiers and differences in deployment of some specifiers and effectors expected to confer species-specific properties. By testing the collective expression and function of neural crest genes in a single basal vertebrate, we reveal the ground state of the NC-GRN and resolve ambiguities between model organisms.

**241. Spalt4 mediates morphogenesis of ectodermal placodes**

*Meyer Barembaum, Marianne Bronner-Fraser*

Vertebrate placodes are regions of thickened head ectoderm that contribute to paired sensory organs and cranial ganglia. We demonstrate that the transcription factor spalt4 is broadly expressed in chick preplacodal epiblast and later resolves to otic and olfactory placodes. Ectopic expression is sufficient to induce invagination of non-placodal ectoderm along the entire body axis and prevents neurogenic placodes from contributing to cranial ganglia. Conversely, loss of spalt4 function in the otic placode results in abnormal otocyst development. Intriguingly, spalt4 appears to initiate the placode program appropriate for the axial level but is not involved in later development of specific placode fates. The results suggest that spalt4 is involved in early stages of placode development, initiating ectodermal invagination and region-specific gene regulatory networks.

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**Support:** The work described in the following research reports has been supported by:
Arrowhead Research
Beckman Institute
Camilla Chandler Frost Fellowship
Lucille P. Markey Charitable Trust
National Institutes of Health, USPHS
National Science Foundation
Norman Chandler Professorship in Cell Biology

**Summary:** The major focus of research in our laboratory is on gene regulatory networks (GRNs) that control development, and the evolution of these networks. Most of our research is done on sea urchin embryos, which provide key experimental advantages. Among these are: an easy gene transfer technology, which makes this a system of choice for studying the genomic regulatory code; advanced technologies for high throughput perturbation of gene expression in the embryo; multiple means of visualizing and measuring gene expression; availability of embryonic material at all seasons of the year; an optically clear, easily handled embryo that is remarkably able to withstand micromanipulations, injections and blastomere recombination and disaggregation procedures; a very well understood and relatively simple embryonic process; and in-house egg-to-egg culture of the species we work with, *Strongylocentrotus purpuratus* (in a special culture system we have developed, located at Caltech's Kerckhoff Marine Laboratory). There is also a rich collection of arrayed cDNA and BAC libraries for many other species of sea urchins, at various degrees of relatedness to this one. The genome of *S. purpuratus* has been sequenced at HGSC (Baylor) and annotated. We utilize additional experimental echinoderm models for evolutionary GRN comparisons, viz. the starfish *Asterina miniata* also of local provenance, and the primitive "pencil urchin" *Eucidaris tribuloides*. The embryos of both these animals prove to be as excellent subjects for gene regulation molecular biology as is that of the sea urchin.

We pursue an integrated, "vertical" mode of experimental analysis, in that our experiments are directed at all levels of biological organization, extending from the transcription factor-DNA interactions that control spatial and temporal expression of specific genes to the system-level analysis of large regulatory networks. It has become apparent that the only level of analysis from which causal explanations of major developmental phenomena directly emerge, is the GRN system level.

The main research initiatives in our laboratories at the present time are as follows: ***i. Analysis of the gene regulatory network underlying endomesoderm specification in* S. purpuratus *embryos*:** At present about 50 genes have been linked into this GRN. The architecture of the network is emerging from an interdisciplinary approach in which computational analysis is applied to perturbation data obtained by gene expression knockouts and other methods, combined with experimental embryology. A predictive model of the GRN has emerged which indicates the inputs and outputs of the *cis*-regulatory elements at its key nodes. This model essentially provides the genomic regulatory code for specification of the endomesodermal territories of the embryo, up to gastrula stage. Most of the individual projects reported below are contributing to understanding of this network. ***ii. Testing the* cis*-regulatory predictions of the GRN:*** The GRN was constructed essentially by integrating the results of a massive perturbation analysis of expression of individual genes with spatial and temporal expression data for these genes. It predicts the required specific regulatory inputs and outputs linking the genes within the GRN. These predictions are subject to direct experimental *cis*-regulatory test, and correction. We have now authenticated the predicted *cis*-regulatory inputs into genes at a majority of the key nodes of the current GRN. At these nodes are regulatory genes into which there are multiple regulatory inputs from genes elsewhere in the GRN, and multiple outputs to other genes in the GRN. For some regions of the GRN the analysis is approaching

maturity, in that it extends convincingly from maternal inputs to cell-type differentiation. The best example is the GRN subregion determining skeletogenic micromere specification. Overall, the results of these experiments are converting the GRN from a model proposition into a hard-wired map of the genomic control logic for this portion of development. At present among *cis*-regulatory systems that are the subject of experimental analysis are those of the following genes: *alx1*, *tgif*, *hex*, *foxa*, *brachyury*, *rel*. ***iii. Completion of the repertoire of regulatory genes engaged in the endomesoderm GRN:*** We used the data emerging from the genome sequence project to identify and assemble computationally all gene sequences that encode transcription factors. The temporal patterns of expression of these genes were determined, and for those genes sufficiently expressed in the embryo, the spatial patterns as well. Regulatory genes were identified in this manner that evidently play a role in endomesoderm specification, because they are expressed specifically in the endomesodermal territories at the relevant times, but that had not yet been incorporated into the GRN. All of these genes are now being linked into the GRN by perturbation and *cis*-regulatory analysis; this project has been completed for the skeletogenic micromere lineage and is in process for the non-skeletogenic mesoderm and the endoderm. ***iv. Evolution, viewed as a process of change in GRN architecture:*** Starfish and sea urchins shared a last common ancestor about 500 million years ago. Thus, analysis of the GRN controlling endomesoderm specification events in the starfish embryo will reveal both the nature of functional change in the GRN, and conservation of features that are so essential that they have resisted alteration for half a billion years. Examples of both have now been documented. The underlying processes are of course change, or alternatively, conservation, of functional *cis*-regulatory features. To study this we are examining starfish/sea urchin GRN differences at the *cis*-regulatory level. A second ongoing project is an attempt to reprogram the development of the skeletogenic cell lineage in a primitive sea urchin, *Eucidaris tribuloides*, by inserting regulatory apparatus from *S. purpuratus*. These echinoids diverged from a common ancestor in the Middle Triassic and generate their embryonic skeleton in different ways. We term this Synthetic Experimental Evolution. ***v.* cis*-regulatory evolution and interspecific recognition of* cis*-regulatory modules:*** We are carrying out a sequence level evolutionary analysis of experimentally authenticated *cis*-regulatory modules from nine different genes in four species of sea urchin. At a 50 my divergence distance (*S. purpuratus* and *Lytechinus variegatus*) *cis*-regulatory modules and exons are the only conserved sequence elements. Surprisingly, of authenticated transcription factor target sites, about a third are either novel additional occurrences of given sites in one or the other species, or are sites that have changed position within the conserved module. However, some proximal site pairs recur repeatedly. At the closer distance represented by two congeners for which genomic sequence exists (*Allocentrotus fragilis* and *Strongylocentrotus franciscanus*), *cis*-regulatory modules are marked by sharply decreased frequencies of large indels. These are otherwise a major mechanism of divergence in unselected sequence. A library of >100 sequence patches conserved between *L. variegatus* and *S. purpuratus* is under study for indel frequencies as a function of indel size in all four species. ***vi. Oral and aboral ectoderm GRNs:*** We have recently attained draft GRNs for oral and aboral ectoderm specification including about 30 more regulatory genes. This is part of an effort to extend the same kind of causal, system level GRN analysis to the whole embryo, and represents a major step toward that goal. There is only one additional early embryonic territory, the apical neurogenic region, which is being studied in other sea urchin laboratories. The aboral ectoderm generates a single cell type, but the oral ectoderm gives rise to several distinctly functioning domains: mouth, columnar "facial" epithelium, neurogenic ciliary band, and the ectodermal signaling stripes which determine the location of the skeletal rods. The approach is to obtain all the regulatory players expressed in oral and aboral ectoderm from the analysis of all genes encoding transcription factors predicted in the genomic sequence, and engage them in a provisional network by carrying out a matrix of perturbation experiments. The network is anchored at the onset of the ectodermal specification process, of which the initial gene zygotically expressed on the oral side is *nodal*. The *cis*-regulatory module controlling early oral ectoderm expression of *nodal* has been thoroughly characterized: its target sites provide the direct links between the initial cytoplasmic anisotropy by which the future oral and aboral sides of the embryo are distinguished, i.e., differences in redox potential, and differential zygotic gene expression. ***vii. Various explorations by new methods and approaches:*** As always, we are trying to expand knowledge by use of novel technologies for analysis of the GRN and the genome. Current applications of new technology include increasingly widespread use of *in vitro* reengineered BAC recombinants, which we are supplying to the whole sea urchin field; use of these in first attempts to "redesign" the process of embryonic development, by introduction of altered regulatory subcircuits in novel spatial domains; and extensive application of two-color gene transfer experiments in which the control version of a *cis*-regulatory construct drives expression of a reporter detectable in one color and a mutated version injected with it and incorporated in the same cells drives expression of a reporter detected in a different color. We have also developed a completely novel method for blocking expression of any gene whenever and wherever desired, though this has so far been tested only in sea urchin embryo skeletogenic cells. This method allows us to determine the function of regulatory genes that have multiple activity phases in one of the later phases, in embryos that develop normally up to that point. Another new technology that has had a major impact is utilization of NanoString technology, a new instrument that permits direct automated counting of transcripts of one-several

hundred genes in control and experimentally perturbed lysates of small numbers of embryos with high accuracy efficiency. ***viii. Revolutionizing* cis*-regulatory analysis:*** We are seeking to improve the efficiency of *cis*-regulatory analysis by large factors. First, we have shown that high coverage Solexa reads of *Lytechinus* BACs containing given genes can be used to identify conserved *cis*-regulatory modules by mapping them on the *S. purpuratus* genome sequence, using an elegant display apparatus, "Cis-Browser," developed by our colleague Sorin Istrail (Brown University). Second, we have found that FACS sorted, disaggregated embryos expressing regulatory constructs producing GFP provide populations of expressing cells that can be analysed by QPCR to determine spatial and temporal domain of *cis*-regulatory expression. This obviates time consuming microscopic screening and can be done on, many samples at once. Third, we have developed multiple tags for use as reporters so that many samples can be injected into embryos at once and analysed together by NanoString or QPCR. ***ix. Computational approaches to regulatory gene network analysis:*** The GRN visualization software BioTapestry, developed by our collaborators Hamid Bolouri and Wm. Longabaugh at ISB, is now in wide use, and we are further expanding its capacities so that it will automatically generate allowed network architectures from machine readable time and space of expression data plus results of perturbation analysis. A second-generation version with much enhanced capacities has been published. Many additional computational genomics and other projects are summarized below.

**The Center for Computational Regulatory Genomics CCRG**

*R. Andrew Cameron, Director*
*Eric H. Davison, Principal Investigator*

The Center for Computational Regulatory Biology and its subsidiary, the Genomics Technology Facility, is an integrated unit whose goal is to develop, refine and test computational approaches in genomics broadly and *cis*-regulatory analysis specifically. It consists of three overlapping areas of activity. The Genomics Facility is a high-throughput library arraying and printing operation that supplies macroarray libraries and clones to the community. The molecular biology branch tests and refines techniques associated with molecular developmental biology, gene transfer, and the routine aspects of *cis*-regulatory analysis developed in the Davidson laboratory. The computational branch is an enterprise that supplies software to the sea urchin developmental biologist and maintains databases fundamental to the Sea Urchin Genome Project, an initiative that began in the Davidson laboratory and at the Genomics Technology Facility.

**Genomics Technology Facility**

The operation of the Facility centers on the Genetix Arraying Robot, a large flatbed robotic arm with video camera used to produce bacterial macro-array libraries and filters. We currently maintain in -80°C freezers 27 different echinoderm libraries comprising a total of approximately three million arrayed clones. In addition to providing these materials to academic research groups, we also offer the opportunity for outside groups from Caltech and elsewhere to array and spot their own libraries. The existing genomic DNA and cDNA libraries that were so extensively employed for the annotation of the sea urchin genome are stably maintained in our freezers. We print new filters for these as needed. In addition, we have made and arrayed an Asterina miniata mixed-stage larval cDNA library in the pSport plasmid. During the past year we have arrayed a total of 120,000 colonies and printed a total of 317 macroarray filters. Research materials have been provided to research laboratories ranging from University of Washington to Stazione Zoologica Anton Dohrn (Italy).

Our collaboration with the Baylor College of Medicine, Human Genome Sequencing Center continues on several fronts. We provided material for, and later computational analysis of a skim-sequencing project in two related species, *Strongylocentrotus franciscanus* and the deepwater species, *Allocentrotus fragilis*. The 1X coverage provided only a glimpse into the genomic parameters between these and our reference species, *Strongylocentrotus purpuratus*. Baylor has completed another round of 1X coverage for both related species and we expect to have it available for analysis in the next few weeks. These sequences will be used to confirm our model that predicts a constraint for large insertions and deletions in *cis*-regulatory modules.

**Research Center**

The goal of the Center for Computational Regulatory Biology is to develop, refine and test computational approaches in genomics broadly and *cis*-regulatory analysis specifically. The primary focus for the latter is the elucidation of gene regulatory networks in development. The Center interacts with the wider research community in several ways: it provides open source software for use by academic research groups; it provides web-based servers for genomic analysis using software developed locally; and it maintains databases fundamental to the Sea Urchin Genome Project, an initiative that began in the Davidson laboratory and at the Genomics Technology Facility. The Facility provides to the Caltech and external scientific community upon request services and materials stemming from the macroarray libraries and arraying equipment that we maintain.

One aspect of the Center is the Sea Urchin Genome Resource that maintains information resources that are used widely in the sea urchin research community. We provide sequence information through the Sea Urchin Genome Project web site (http://spbase.org). With the advent of the web resources for annotation established at the Human Genome Sequencing Center, Baylor College of Medicine and the Sea Urchin Genome Resources at NCBI, we have not seen the need to expand our local databases. However, we have refined the cross-index between our library clones and sequences stored in public databases at

NCBI. Since so many of our libraries were used for the sequencing project, and the library location for the clones was preserved in the sequence information, we can provide a searchable sequence database from which the user can obtain clone information and order the clone. This "clone by computer" method renders our arrayed libraries extremely useful and readily accessible for the working molecular biologist.

**Beowulf cluster hardware and configuration**

Our 40-unit Beowulf cluster continues to operate with a minimum of downtime. The Rocks software stack of software components has indeed proven to be an efficient system with which to build, operate and maintain the cluster. We are processing the entire genome sequence on the cluster in various ways as part of the genome database. We further expect to queue search jobs from the genome database through this cluster.

**242. Experimental and mathematical analysis of *foxa cis*-regulation in the sea urchin embryo**

*Smadar Ben-Tabou de-Leon, Eric H. Davidson*

We combine mathematical modeling and experimental analysis to study the *cis*-regulation of the *foxa* gene. The *foxa* transcription factor is one of the key regulatory genes in the gene regulatory network governing endomesoderm development in the sea urchin embryo. Initially expressed in the endomesoderm progenitor field, *foxa* is necessary for endoderm specification and for exclusion of mesodermal fate in endoderm cells. As the segregation of the two domains occurs, *foxa* leaves the mesoderm territory and expresses only in the endoderm cells. FoxA protein exerts negative feedback on its own expression and its levels oscillate with time. In order to understand *foxa* regulation we perform a *cis*-regulatory analysis of the *foxa* gene. Interspecies sequence comparison and reporter construct assays were used to identify a 2.5 kb sequence that drives the correct expression pattern up to 72 h after fertilization. The sequence includes three distinct modules located upstream of the transcription start site: a proximal module responds to ubiquitous activation, a middle module responds to endodermal activators and a distal module restricts the expression to the correct spatial pattern. We tested putative consensus sites of possible inputs by mutating the sites in GFP reporter constructs and monitoring the GFP expression. The distal module contains a TCF site that represses expression in the ectoderm and permits expression only in the endomesoderm territory. The middle module contains a Brachyury site that contributes positively to the endoderm expression of *foxa*. The three modules contain Otx sites that provide ubiquitous positive activation. The distal module contains five putative binding sites for FoxA itself; though these have not been tested yet, they might mediate the observed auto-repression. We combined in a mathematical model the *cis*-regulatory analysis results and the dynamic expression levels of the input genes measured by quantitative real-time PCR. The mathematical model consists of a set of differential equations for the mRNA and protein levels of the input genes and *foxa*. We included the positive inputs of Brachyury and Otx and also *foxa* auto-repression. We were able to simulate the experimentally observed time course of *foxa* expression up to 27 h after fertilization. Our study elucidates the mechanism by which a static genomic code controls a dynamic expression pattern.

**243. The Sea Urchin Genome Database - SpBase**

*R. Andrew Cameron, Qiu Autumn Yuan, Wick Gankanda, David Felt*

The Sea Urchin Model Organism Database (http://spbase.org) was made publicly available on March 14, 2008. The web site and the database software that supports it have been in development during this past year. We have been tailoring our datasets to use the open source GMOD programs (http://gmod.org). These provide a consistent way to display, update and maintain the annotation data that emerged from the draft genome sequence done by BCM-HGSC. A considerable amount of our effort was directed toward formatting the annotations from the draft genome project into a suitable structure for loading into a very complete data schema, dubbed Chado by the software developers. The basic system includes the database itself, a graphical sequence browser, a sequence search function and a gene search function to contain the information from manual annotation. We are adding features to this basic structure as rapidly as possible. A graphical sequence curation tool has been tied into the system as well. It is the familiar Apollo package developed at Berkeley. We have retained most of the additional information accumulated by BCM_HGSC during the curation process. Our last task before going public was to map all of the original features to the newest version of the genome assembly (version 2.1).

The Sea Urchin Genome Resource that provided a searchable sequence database (http://sugp.caltech.edu/) from which the user can obtain clone information and order clones is now obsolete. It will remain publicly available until we are assured that all of the information it contains has been successfully moved to SpBase.

**244. The sea urchin repeat sequence complement**

*Susan Ernst, Emmanuelle Morin, Manoj Samanta, R. Andrew Cameron*

Although repeat sequences make up 35% of the genome in sea urchins, they are difficult to analyze thoroughly in draft genome sequence assemblies. Almost invariably, gaps in sequence lie at repeat margins and unassembled repeat sequences cannot be placed or precisely counted. Nevertheless, we have collected a dataset of repeats from the version 2.1 assembly of the sea urchin. The basic strategy was to iterate through an all-by-all blast analysis followed by repeat clustering and genome sequence masking on ever increasing portions of the genome. Less simple sequence repeats, this collection yields 2500 consensus repeat sequences of which the most abundant was estimated to be present 17,000 times. To

complement this approach, another method, k-mer analysis, is being used. We expect these will complement each other and give us a better estimate of the number and variety of the repeats. We have also begun to annotate the repeat families in the consensus set: Piggybac and Mariner DNA transposons are two distinctive families that are quite abundant in sea urchins. A measure of the divergence among the members of these two classes will allow an estimate of how recent these may have been active in the genome. Not unexpectedly, we are finding good representatives of both LTR and non-LTR retrotransposons and a rich complement of short interspersed nuclear elements or SINES is also detected.

**245. Network gene annotation project**

*Qiu Autumn Yuan, R. Andrew Cameron*

One of the strengths of the sea urchin embryo research model is the ease with which one can describe gene regulatory networks. As this experimental approach matures it is becoming clear that a gene annotation knowledge base is indispensible for designing experiments. Gene sequences, expression patterns and responses to perturbation are the central classes of information to be used here. Our previously built and currently maintained database of sequence and expression data for sea urchin regulatory genes fills that role. The data housed in this suite of databases continues to grow as additional genes, expression patterns, and network linkages are added to the existing information. The new ectoderm regulatory network studies from the Davidson laboratory have added a unique chunk of data to this effort. These unpublished data are available to a registered user group through a private Caltech web site. Our client base continues to grow as new users add more biological data. The latest addition is a function that graphically displays gene expression time-courses during embryogenesis.

**246. The evolution of *cis*-regulatory module sequence in lower deuterostomes**

*R. Andrew Cameron, Eve Helguero, Qiu Autumn Yuan, Ping Dong, Julie Hahn*

The echinoderm taxa make an unusually good set of species in which to examine the evolution of *cis*-regulatory modules (CRM). We have used genomic sequence comparisons covering a range of divergences from 20-540 million years (MY) in order to address a variety of questions about CRMs. Conserved sequence patches across 50 MY between *Strongylocentrous purpuratus* (Sp, our reference species) and *Lytechinus variegatus* (Lv) reveal candidate CRM sequences for gene regulatory network analysis. Interestingly, even though many of these conserved patches contain CRMs as shown by reporter analysis in the reference species, the exact position and order of the transcription factor binding sites are seldom conserved between the two species. However the number of sites is very similar if not identical. This observation suggests that simple sequence similarity will not distinguish CRMs.

Comparisons of functionally characterized *cis*-regulatory modules from the Sp genome, and the orthologous regulatory and flanking sequences from the genome of a congener, *S. franciscanus* (Sf), cover a 20 MY divergence and reveal that single nucleotide substitutions and small indels occur freely at many positions within the regulatory modules of these two species, as they do without. However, large indels (>20 bp) are statistically almost absent within, though they are common in flanking intergenic or intronic sequence. We continue to expand the panel of comparisons to be made in this analysis. We now have available sequence from *Allocentrotus fragilis* (Af, also 20 MY diverged) provided by the Baylor College of Medicine Human Genome Sequencing Center to add to the analysis discussed above. The amount of genome coverage is expected to reach 2X this year and we expect more thorough four-species analyses to quickly emerge from these data.

A number of across species tests are being conducted to test the sequence analyses. We use recombinant BACs in which GFP or RFP is substituted for the gene-coding region while the surrounding genomic sequence is preserved. First, we are examining the significance of the small differences between Sp and Lv discussed above by injecting Sp constructs into Lv embryos. We are modifying the Sp sequences to resemble the Lv ones and test their transcriptional activity in Sp as well. From these tests we will be able to assess the significance of these small sequence differences that evolved in the CRMs.

**247. Transcriptional control of the sea urchin Brachyury gene**

*Elly Chow, R. Andrew Cameron*

Previously, we have identified a minimal enhancer for brachyury, a gene intermediate in the endomesoderm specification gene regulatory network. Up to the peak of embryonic expression at 24 hrs, it recapitulates, in time and space, the expression of the endogenous gene. This enhancer contains binding sites for Tcf, a transcription factor that is activated by the wnt pathway and Gatae, the output of the endomesoderm kernel subcircuit. An Otx binding site in the larger intronic sequence that contains the minimal enhancer also plays a role in activating this gene. To determine more precisely the expression pattern of our recombinant BAC GFP reporter and smaller constructs derived from it, we have built a red fluorescent protein (RFP) containing recombinant BAC. Co-injection of this control red marker with various reporter constructs precisely demonstrates the spatial extent of the constructs. Using our GFP-BAC and mutated blimp constructs developed by Joel Smith in the Davidson laboratory, we have demonstrated that the expression of brachyury is most likely kept out of the mesoderm by TCF-groucho repression. Our current hypothesis for brachyury early transcriptional control is: a wnt activity in veg2 endoderm precursors leads to the nuclearization of beta-catenin and the activation of TCF in those cells. Thus, brachyury transcription elevates

beginning at about 10 hours pf. Later as Gatae and Otx begin to be expressed in these cells the expression is pushed to the maximum at 24 hours.

The next phase of brachyury expression is especially interesting. The zone of expression is stable in the blastopore lip even though the endoderm cells are moving through the zone of expression in order to form the archenteron. Thus, these cells must first turn on brachyury as they enter the zone and then turn it off as they exit. We will use our previous results as a base to explore the molecular basis of this dynamic expression pattern.

**248. Re-engineering embryonic skeletogenesis at the gene-regulatory network level in the sea urchin, *Strongylocentrotus purpuratus***

*Sagar Damle, Eric Davidson*

Early development of skeletogenic and non-skeletogenic mesenchyme cells in *S. purpuratus* embryos are becoming well understood at level of expression and regulation of transcription factors and signaling molecules. However an ultimate demonstration of our understanding of these systems will be to rewire those regulatory relationships in novel ways to produce predictable developmental outcomes. We have used BAC recombinant constructs to reprogram development of skeletogenic precursors, known as primary mesenchyme cells (PMCs), at the gene regulatory level by driving the expression of the non-skeletogenic mesenchyme cell (NSM)-specific transcription factor, SpGcm, using the regulatory system controlling the PMC-specific T-box factor SpTbr. This BAC recombinant causes exogenous gcm expression in PMCs in a manner that precisely mirrors the early expression pattern of SpTbrain. PMCs expressing Gcm remain in the vegetal plate when non-expressing PMCs ingress and fail to participate in PMC-syncitium, leading to a significant delay in skeletogenesis. Whole-mount *in situ* of blastula-stage embryos show PMCs expressing exogenous Gcm also show a loss of several transcription factors necessary for PMC-specification or skeletogenesis including SpAlx, Spfoxb and SpJun as well as a differentiation gene SpMsp130. At the same time these PMCs express markers of pigment cells, a subtype of NSMs. We have shown that expression of SpGcm in the context of another mesodermal cell type is sufficient to repress expression of genes that control that cell€™'s expected fate and redirect it towards a pigment cell fate.

Current work involves the *cis*-regulatory analysis of SpAlx, a factor expressed early during PMC specification. SpAlx is expressed at early blastula stage as a direct consequence of a double-repression gate involving the homeobox factor SpPmar1 and the hairy-enhancer-of-split ortholog, SpHes-C. Later in development Alx expression is maintained in part by Ets1. Hes-C binding sites have been identified in sequences conserved with the sea urchin *Lytechinus variegatus* and their function confirmed by mutational analysis with GFP reporters. Additionally, putative conserved Ets1 sites have been identified 3kb upstream of the basal promoter. Finally, it has been shown that Gcm overexpression is capable of repressing expression of a BAC-GFP reporter containing Alx regulatory DNA. At present, the binding sites mediating this repression are being investigated.

**249. Evolution of developmental gene regulatory networks GRN)**

*Feng Gao*

The developmental gene regulatory networks (GRNs) of sea urchin and sea star are providing almost the only direct, head-to-head comparative study so far available on gene regulatory network evolution. Comparative GRN analysis has revealed the diverse subcircuits of the orthologous GRNs of these animals evolve in dramatically different ways: some network subcircuits are astoundingly conserved in their linkages, which are termed the kernels of the GRN; others are changed, which are particularly evidenced as the same genes doing very different things (1, 2).

Our study is to exemplify and test these discoveries, and try to produce a causal explanation of specific evolutionary differences in gene regulatory network architecture between sea urchins and sea stars, which had a common ancestor half a billion years ago.

GRN mapping indicated the skeletogenic regulatory apparatus may have existed as a modular GRN unit since early in echinoderm history, and it was this same unit that was inserted into the sea urchin embryological developmental address defining the embryonic skeleton (3). There is presumptive evidence for a highly conserved GRN kernel. But this proposition could only be substantiated only by determining whether the actual *cis*-regulatory linkages are as pleisiomorphic as is the skeletogenic gene expression complex. This could be tested by reintroduction into sea urchin eggs the *cis*-regulatory modules of the sea star skeletogenic regulatory genes, together with computational genomics. If there is indeed a pan-echinoderm skeletogenic kernel, then the sea star regulatory modules thereof should display most of the same functional linkages and require most of the same inputs when introduced in reporter constructs into sea urchin eggs as are portrayed in the sea urchin GRN.

The GRN analysis predicts that the changes in GRN architecture are due to differently designed *cis*-regulatory modules of orthologous genes, which receive different inputs, and function differently to produce their specific modes of expression. This could be tested by isolation and direct functional comparison of the respective *cis*-regulatory modules of orthologous genes. Three candidate genes to be studied are Tbr, Delta and GataC, which are known to play different roles in the GRNs of sea star and sea urchin.

**References**

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**250. The subnetwork underlying specification of mesoderm in sea urchins**

*Stefan C. Materna, Eric Davidson*

We have identified a multitude of transcription factors that are specifically expressed in the early mesoderm of the sea urchin. Currently, we are probing the regulatory interactions of these with other known mesodermal genes to better understand the regulatory network that regulates the specification of mesodermal cells.

Initially, nuclearization of beta-catenin – as part of a re-enforcing, regulatory circuit that involves the *blimp1/krox* and the *wnt8* gene – activates endomesodermal genes. This subcircuit is first active in the cells of the skeletogenic lineage and then moves in a torus-like motion across the vegetal half of the embryo. Downstream of this 'wave' are some transcription factors that have an important function in pigment cell formation, as the *z13* gene. Other genes include the previously known *hox11/13/b, ets1/2,* and *eve* genes as well as the newly identified *z48*. Following the subdivision of endomesoderm into endodermal and mesodermal cells, a Delta signal is expressed by the skeletogenic cells and received by the mesodermal cells via the Notch receptor. Downstream of this signal are exclusive mesodermal genes such as *glial cells missing*. It is essential for the specification of pigment cells and activates markers, as the *polyketide synthase* gene that, are specific to pigment cells. At early gastrulation *gcm* expression recedes from the oral quadrant of the mesodermal territory; simultaneously genes like *gata-c* are activated specifically in these cells. The oral quadrant will produce primarily blastocoelar cells that are thought to fulfill immune functions. Interestingly, a number of bHLH transcription factors whose vertebrate orthologs are involved in specifying immune cells are expressed in the same cells, among them *scl* and the ubiquitously expressed *e2a* and *id* genes. Other newly identified genes include the orally expressed *prox* gene, and the aboral *six1/2* gene. While it is not clear which signaling system is involved in the subdivision of the mesodermal cells, many of the mesodermal genes on the oral and aboral side are activated by Notch signaling. Current perturbation experiments are aimed at linking all newly identified genes to the beta-catenin/blimp/wnt8, and Delta/Notch systems as well as establishing the regulatory linkages among them.

**251. New experimental approaches for system-wide *cis*-regulatory analyses**

*Jongmin Nam, Eric H. Davidson*

Deciphering detailed features of *trans*- and *cis*-regulatory gene network codes is a key step in understanding how life forms develop and evolve. Building a system-wide *trans*-regulatory gene network is becoming a common procedure with the sea urchin *S. purpuratus* and some pioneering studies successfully demonstrated its power. However, system-wide *cis*-regulatory network analysis is still practically impossible in multicellular systems. Our goal in this project is to develop a set of system-wide and general experimental approaches for building *cis*-regulatory gene networks and to better relate *cis*- and *trans*-regulatory gene networks using the sea urchin as a model system. The proposed experimental approaches are as follows: Aim 1) Active *cis*-regulatory modules will be identified by a heuristic version of phylogenetic footprinting followed by simultaneous Q-PCR detection of 13 unique DNA Tags driven by 13 different candidate modules in one experiment. Aim 2) Spatial activity of each of the active modules will be examined by fluorescence-activated cell sorting followed by quantitative expression analysis of a large set of endogenous genes. By comparing the known spatial expression pattern of each gene to its enrichment or depletion in GFP positive cells it is possible to infer the spatial activity of a module in a quantitative manner. This method also gives an averaged regulatory state in cells where the module is active. Aim 3) Potential transcriptional inputs will be tested by simultaneously examining the effect of gene perturbation on a large set of active *cis*-regulatory modules (~100 modules) discovered from Aim 1 and on endogenous genes. Either Q-PCR or Nanostring will be used for the detection of expression. Predicted inputs will further be tested by mutating putative binding sites.

The method of Aim 1 has been successfully applied to a set of about 25 genes, and more genes will be analyzed by using this method in the near future. A proof of principle experiment for the method stated in Aim 2 was conducted with a partial success. For Aim 3 a set of over 100 unique DNA Tags have been developed and are being tested for ≤10 embryos with promising results. Once established, the set of quantitative and large-scale methods proposed here are easily applicable to many other systems including mammalian and plant systems. Therefore, these methods will have a significant impact on the mechanistic understanding of how life forms develop and evolve, and of abnormalities in disease conditions.

**Publication**

Nam, J., Su., Y.H., Lee, P.Y., Robertson, A.J., Coffman, J.A. and Davidson, E.H. (2007) *cis*-Regulatory control of the nodal gene, initiator of the sea urchin oral ectoderm gene network. *Dev. Biol*. **306**(2):860-869.

**252. Regulatory logic of endoderm development in sea urchin embryos**

*Isabelle Peter, Yi Fan, Jina Yun, Eric H. Davidson*

Embryonic endoderm development relies on the transition of a common endomesoderm regulatory state to the distinct endoderm regulatory states that specify different domains of the tripartite gut. The logical sequence in this process is determined by the regulatory wiring among endoderm transcription factors and signals acting on presumptive endoderm cells. We aim at solving the architecture of the complete endoderm gene regulatory network (GRN) in pre-gastrula stage sea urchin embryos. An earlier model of the endoderm GRN included 12

regulatory genes and genome-wide characterizations of transcription factor gene expression patterns have revealed an additional set of 11 candidate genes. We have further analyzed their spatial expression pattern, confirming the expression of seven of the candidate genes in endoderm precursor cells. To identify the regulatory linkages between all endoderm regulatory genes, we have perturbed the expression of each transcription factor by morpholino injection and detected the resulting changes in expression levels of all endoderm network genes.

Our results indicate that common endomesoderm transcription factors, regulated by nuclear β-catenin, control the expression of early endoderm transcription factors, which are at first expressed in a broad endomesoderm area but become restricted to endoderm precursor cells soon thereafter. Among these early endoderm factors are FoxA and Brachyury (Bra). Our analyses has identified several transcription factor genes as regulatory targets of Bra: *foxA* (later expressed in foregut and hindgut), *foxP* (foregut), *hnf1* and *tgif* (both mid- and hindgut). Bra therefore constitutes an important node in the early endoderm GRN, controlling the expression of genes later involved in the formation of all three-gut compartments. In addition to Bra, *Hnf1* responds also to an activating input from GataE, which represents a conserved regulatory linkage.

We have almost completed the perturbation analysis for all known early endoderm transcription factors and we are currently attempting to fill the remaining gaps in the endoderm GRN model by analyzing perturbation-induced changes in the spatial organization of gene expression.

**253. *cis*-Regulatory linkages coordinating the sequential expression of Wnt and Delta ligands during early sea urchin embryogenesis**

*Joel Smith, Eric H. Davidson*

We dissect the transcriptional regulatory relationships that align and coordinate the dynamic expression patterns of two signaling genes, *wnt8* and *delta*, central to sea urchin endomesoderm specification. By *cis*-regulatory analysis we find *delta* transcription, while being broadly activated by the widely-expressed Runx transcription factor, is restricted by HesC-mediated repression through a site in the *delta* 5´UTR. Further *cis*-regulatory analysis reveals transcription of the *hesC* gene is itself shut down by Blimp1 repression. Blimp1 therefore represses the repressor of *delta*, thereby permitting its transcription. As Blimp1 autorepression is the mechanism responsible, indirectly, for extinguishing *wnt8* expression in the center of an expanding torus pattern of expression, Blimp1 repression of *hesC* couples *delta* expression with the extinction of *wnt8* transcription, resulting in side-by-side alignment of *wnt8* and *delta* expression domains and their coordinated movement. The specific *cis*-regulatory linkages of the gene regulatory network thus, guarantee a reproducible developmental sequence.

**254. A spatially dynamic cohort of regulatory genes in the endomesodermal gene network of the sea urchin embryo**

*Joel Smith, Ebba Kraemer*[*]*, Hongdau Liu, Christina Theodoris, Eric H. Davidson*

A gene regulatory network subcircuit comprising the *otx, wnt8* and *blimp1* genes accounts for a moving torus of gene expression that sweeps concentrically across the vegetal domain of the sea urchin embryo. Here we confirm by mutation the inputs into the *blimp1 cis*-regulatory module predicted by network analysis. Its essential design feature is that it includes both activation and autorepression sites. The *wnt8* gene is functionally linked into the subcircuit in that cells receiving this ligand generate a β-catenin/Tcf input required for *blimp1* expression, while the *wnt8* gene in turn requires a Blimp1 input. Their torus-like spatial expression patterns and gene regulatory analysis indicate that the genes *even-skipped* and *hox11/13b* are also entrained by this subcircuit. We verify the *cis*-regulatory inputs of *even-skipped* predicted by network analysis. These include activation by β-catenin/Tcf and Blimp1, repression within the torus by Hox11/13b, and repression outside the torus by Tcf in the absence of Wnt8 signal input. Thus, *even-skipped* and *hox11/13b*, along with *blimp1* and *wnt8*, are members of a cohort of torus genes with similar regulatory inputs and similar, though slightly out-of-phase, expression patterns, which reflect differences in *cis*-regulatory design.

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**255. Dissecting a three-gene positive feedback subcircuit**

*Qiang Tu*

A regulatory state stabilization subcircuit is commonly used in gene regulatory networks (GRNs) for developmental specification. In sea urchin skeletogenic micromere specification, a 3-gene positive feedback loop, which is composed of *erg*, *hex* and *tgif*, is identified to be the stabilization device by large-scale perturbation analysis. Perturbation of any of the three genes will affect the other two, thereby also affecting the downstream differentiation gene battery. The *cis*-regulatory analysis of the genes in this loop is particularly interesting due to the complicated interactions among them. New tools are used to elucidate the subcircuit and investigate the *cis*-regulatory modules.

One tool, 'qPCRplot,' is a computational program we developed for qPCR data processing and visualization. In the course of perturbation experiments, large amounts of quantitative measurements of gene expression are generated. The program is able to process the raw data, run all calculations, and visualize the result. The inputs are simply the files generated from qPCR experiments, and the output graphs all measurements of gene expressions in experimental/control perturbations at different time points, with indicators of significant changes, experimental variations, and rough expression levels. This program is

currently being used to elucidate the sea urchin embryonic skeletogenesis GRN and other GRNs.

Another tool is the Solexa high-throughput sequencing platform, which facilitates discovery of *cis*-regulatory modules. Comparative sequence analyses have been shown to be reliable techniques to find *cis*-regulatory modules. Solexa technology is able to provide large amount of sequence data sufficient for this purpose. Here we apply the technology in the dissection of the *cis*-regulatory modules of the three genes in this subcircuit. Several modules have been identified which drive the expression of reporter genes in the sea urchin embryo, including two *tgif* modules driving the expression in the primary mesenchyme cells and the endoderm at mesenchyme blastula stage, respectively. These two modules fully recapitulate the endogenous expression pattern of *tgif* at this stage.

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**Support:** The work described in the following research reports has been approved by:
Air Force Office of Scientific Research
BAE Systems/ARL
Edelman Trust (Endowment)
MicroPropulsion Corp./DARPA
National Institutes of Health
National Science Foundation
UCSB/ARO
University of Washington/ONR

**Summary**: Complex and intellectually challenging problems can be so commonplace that they escape our attention. The research in my lab focuses on one such everyday phenomenon - the motion of a fly through the air. While the buzz of fly wings is more likely to elicit a sense of annoyance than wonder, insect flight behavior links a series of fundamental processes within both the physical and biological sciences: neuronal signaling within brains, the dynamics of unsteady fluid flow, the structural mechanics of composite materials, and the behavior of complex nonlinear systems. The aim research in my lab is to elucidate the means by which flies accomplish their aerodynamic feats. A rigorous mechanistic description of flight requires an integration of biology, engineering, fluid mechanics, and control theory. The long-term goal, however, is not simply to understand the material basis of insect flight, but to develop its study into a model that can provide insight to the behavior and robustness of complex systems in general. The following projects in my lab, some well underway, others nascent and moving in the direction of a deeper understanding of the genetic control and brain of this insect, are helping move us in the direction of reverse engineering a fly.

## 256. Ocelli-mediated flight control

*Matthias Wittlinger, William Dickson*

Insects use several sensory systems to control flight attitude. Visual and mechanosensory contributions from the compound eyes and the gyroscope-like halteres play important roles in maintaining flight equilibrium but have different temporal response properties. The fruit fly, as well as many other flying insects, has a second visual system that is likely to be involved in the control of flight stability – the ocelli - a triplet of simple eyes on the vertex of its head. This system is thought to operate faster than the compound eyes and cover a greater dynamic range of light intensities but is presumably be slower than the halteres. A combination of these three sensory systems – fast to slow – may allow the fly to optimize the bandwidth without sacrificing sensitivity. We have developed an apparatus to study these simple eyes in the context of pitch and roll response in flight within the laboratory's well-established virtual tethered-flight arena. Using optic fibers to transfer light stimuli to the simple eyes, we are capable of stimulating the three ocelli with light stimuli that mimic pitch and roll of the fly's body posture while the fly is tethered to a thungsten pin inside the virtual flight arena. Three blue 470 nm wavelength LED's are optically coupled into three optic fibers with an outer diameter of 60 μm each. At the output end the fibers are attached to each other in such a way as to fit them perfectly onto the fruit fly's simple eyes. The LED's are controlled with a programmable micro controller that allows us to perform a variety of stimuli. In addition to the tethered-flight experiments we are conducting 3D tracking and high-speed videography of freely flying flies that have undergone diverse manual and genetic manipulations to their visual systems. Whereas natural flies start smoothly when they perform a voluntary take off, flies with occluded ocelli have problems achieving flight equilibrium.

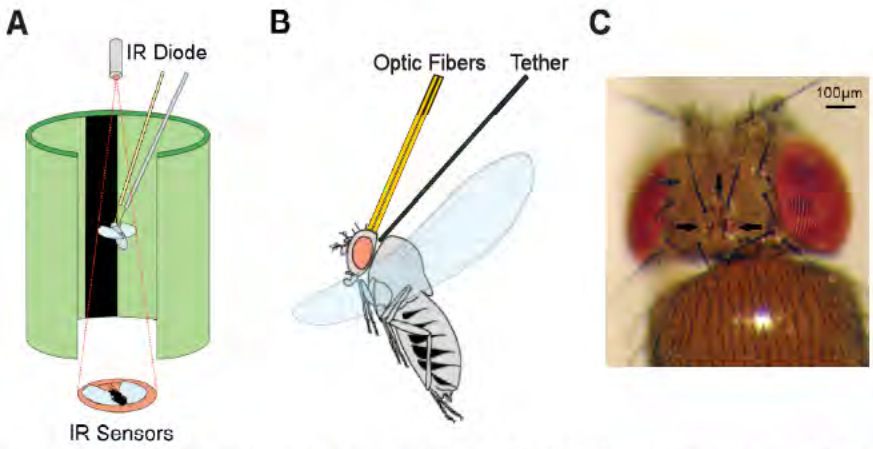


**Figure 1.** Tethered virtual flight arena to study two visual systems of fruit flies. Schematics of a fly in the virtual flight arena and with optic fibers positioned on top of its head (A, B). C. Image of a *Drosophila* head with arrows indicating the three simple eyes (ocelli).

**257. Visual feedback based control during *Drosophila* landing**

*Andrew D. Straw*

The fly brain guides flight using many sensors to perform a variety of goals, including maintaining flight stability, avoiding obstacles, localizing odor sources, navigating through three-dimensional structure, and landing. The compound eyes are prominent sensors (Figure 1E) that, with the nervous system, provide estimates of motion across the visual world (Figure 1F). A number of visual motion based algorithms for governing various behaviors have been proposed on the basis of many experiments in insects. One point emerges as the overarching principal behind visually guided behavior — feedback-based control is essential. At the receptor level, absolute quantities such as retinal velocity are usually not available, but the underlying control algorithms, when operating based on the sensory signals generated by the animal's own behavior, guide flight with performance that usually exceeds engineered controller designs. Such performance is all the more remarkable when considering the noisy, often sparse, and sometimes conflicting sensory data available. Our present efforts are focused on two lines of questions: first, what visual feedback based controllers mediate landing in freely flying flies, and how do other cues such as hunger and odor modify this control? Second, how do the suite of identified visual control algorithms interact within naturalistic contexts to produce flight behavior? To answer these questions, we have constructed a free-flight tracking arena that is large enough (2 m diameter, 80 cm high) on the scale of the organism (3 mm length) to allow unimpeded movement at ecologically relevant spatial scales (Figure 1C). Through the use of 11 digital video cameras, wide-angle lenses, and custom built software called 'flydra,' the trajectories of individual flies are automatically tracked with sub-millimeter accuracy over this volume (Figure 1A and B). Furthermore, because the tracking is performed online, we can trigger acquisition of high-speed, high-resolution video based on particular flight maneuvers such as landing.

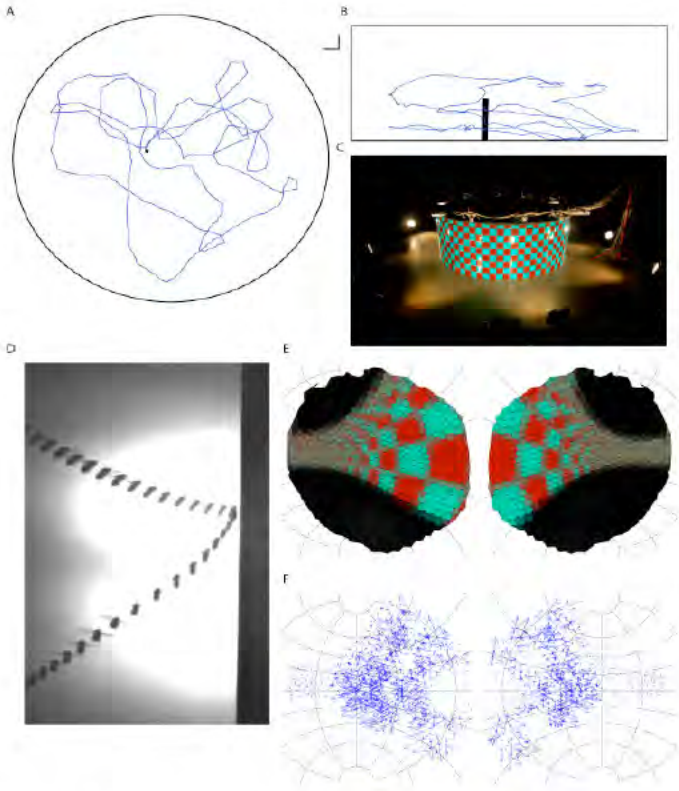


**Figure 2.** Flies use vision to navigate through the environment, gather information about features of interest, and land. (A) Top view of single trajectory in 2m diameter free-flight arena containing a vertical post. Scale bars are 10 cm. (B) Side view of trajectory in A. (C) Photograph of flight arena showing checkerboard pattern used to provide visual contrast to the fly and 11 cameras tracking fly movement. (D) Montage of high-speed video frames showing approach and retreat from a vertical post. (E) Simulated fly-eye view from inside arena shown in stereographic projection. (F) Simulated output of 4000 elementary motion detectors during trajectory shown in A. Arrow length represents magnitude of response.

**258. Walking straight – Freely walking flies in rotating visual environments**

*Peter Wier*

Fruit flies rely on walking in pursuing two of the most basic life goals: courtship and food discovery. In order to examine the principles of fly locomotion control we built an arena (Fig. 3) in which a fly can walk freely on a flat circular temperature-controlled surface (radius 9.1 cm). The organism is kept in the arena by heating the perimeter and clipping its wings. We illuminate the surface from above with infrared lights (invisible to the fly) and image it at 24 frames per second. Real-time tracking of the fly's location and orientation is accomplished via custom software (http://code.astraw.com/). Surrounding this walking platform is a cylindrical display of 192 by 24 LEDs, allowing us to present arbitrary visual surrounds to the fly. An important first step in this line of inquiry is in understanding how flies walk straight, that is, what sensory clues allow a fly to navigate in an intended direction. Optomotor balance – the tendency of an organism to minimize retinal motion by active turning responses – has been extensively studied. When we displayed a pattern of rotating vertical stripes, a fly displays a robust optomotor response – is turns in the direction of the stripe's rotation. Restricting our attention to times when a fly is moving, it rotates at the same angular velocity as the pattern. Although the fly's mechanoreceptors, olfactory system, etc. were intact, the visual information dominated the fly's locomotion. When a fly is stopped there is a bifurcation in behavior. Sometimes it is completely stationary, but at other times it turns in place, following the pattern motion. We examined responses to other patterns of visual motion (expanding/contracting patterns, single stripes, and others) and are able to elicit a rich diversity of behaviors. It has become clear that flies respond to visual information coming from different positions in different ways. In order to more closely investigate the transformation from visual information to motor output we have begun improving our tracking system to be able to resolve individual leg movements. When combined with the existing arena this set up will allow us to present visual stimuli in different positions and monitor how flies react.

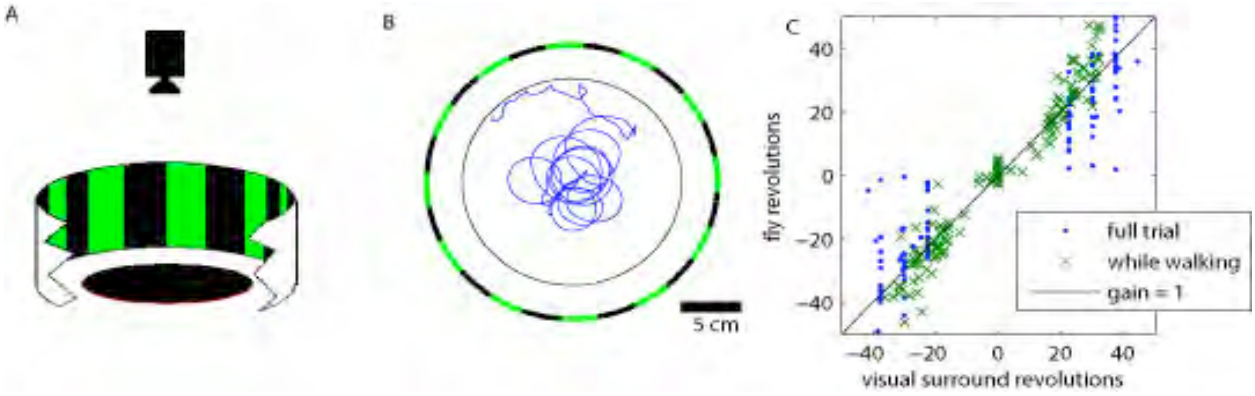


**Figure 3.** Analysis of optomotor responses in walking flies. (A) Experimental arena surrounded by cylindrical LED array. (B) Flies walk in circles if presented with rotatory visual motion. (C) Fly turning response as a function of the velocity of the rotatory visual stimulus.

**259. Hunger-driven dispersal: Individual fly behavior**

*Japer Simon*

Overriding the visual and olfactory cues from food, hunger can drive the dispersal of a fruit fly from patches of food. To describe this movement in terms of behavioral algorithms, we mounted single cameras above experimental chambers used to characterize this hunger-driven dispersal and developed a simple machine vision strategy to reconstruct the 3D trajectory of individual flies moving within these chambers. To observe the change in behavior underlying the dispersal of flies as they become hungry, we introduced single flies fed *ad labitum* to the first of two connected chambers and filmed their movement at a rate of 15 $s^{-1}$ for 6 hours (Fig. 4A). To reconstruct the 3D positions of a fly, we calculated the total pixel area representing a fly from a thresholded image (Fig. 4D) for each cropped image and used this information together with 2D coordinates to deduce the location of the fly throughout the length of a video. If the pixel area representing a fly was greater than a specific computed amount we would assume that the fly at this time point was closer to the camera and therefore on the underside of the chamber lid; conversely, if the pixel area was less than this amount we would assume that the fly was farther from the camera, either on the wall or floor of the chamber (Fig. 4E). Using this strategy, we could build up a fly's trajectory frame-by-frame over the 6 hours. We calibrated the projection between the 2D coordinates of a fly and its 3D positions using known anchor points assigned within an image of the experimental chamber corresponding with known positions within the chamber, using a direct linear transformation (standard DLT). Finally, we estimated the most likely sequence of positions (lid vs. wall or floor) for the fly between each video image, and used this estimate to reconstruct the trajectory for a fly

(Viterbi optimization). For example, the probability of a fly transitioning between a location on the lid to a location on the wall is quite low if the fly is in the center of the chamber; this transition is more probable if the fly is on the lid near the wall. We wrote custom code in Matlab for transforming, optimizing, and analyzing all data. Here we show a sample trajectory for a fly during a single 6 hours trial. The highlighted trajectory represents 10s segments of trajectory preceding all exits. To help illustrate the trajectory of a fly, we unwrapped its 3D positions within the experimental chambers and report its movement in a flattened representation (Fig. 4F).

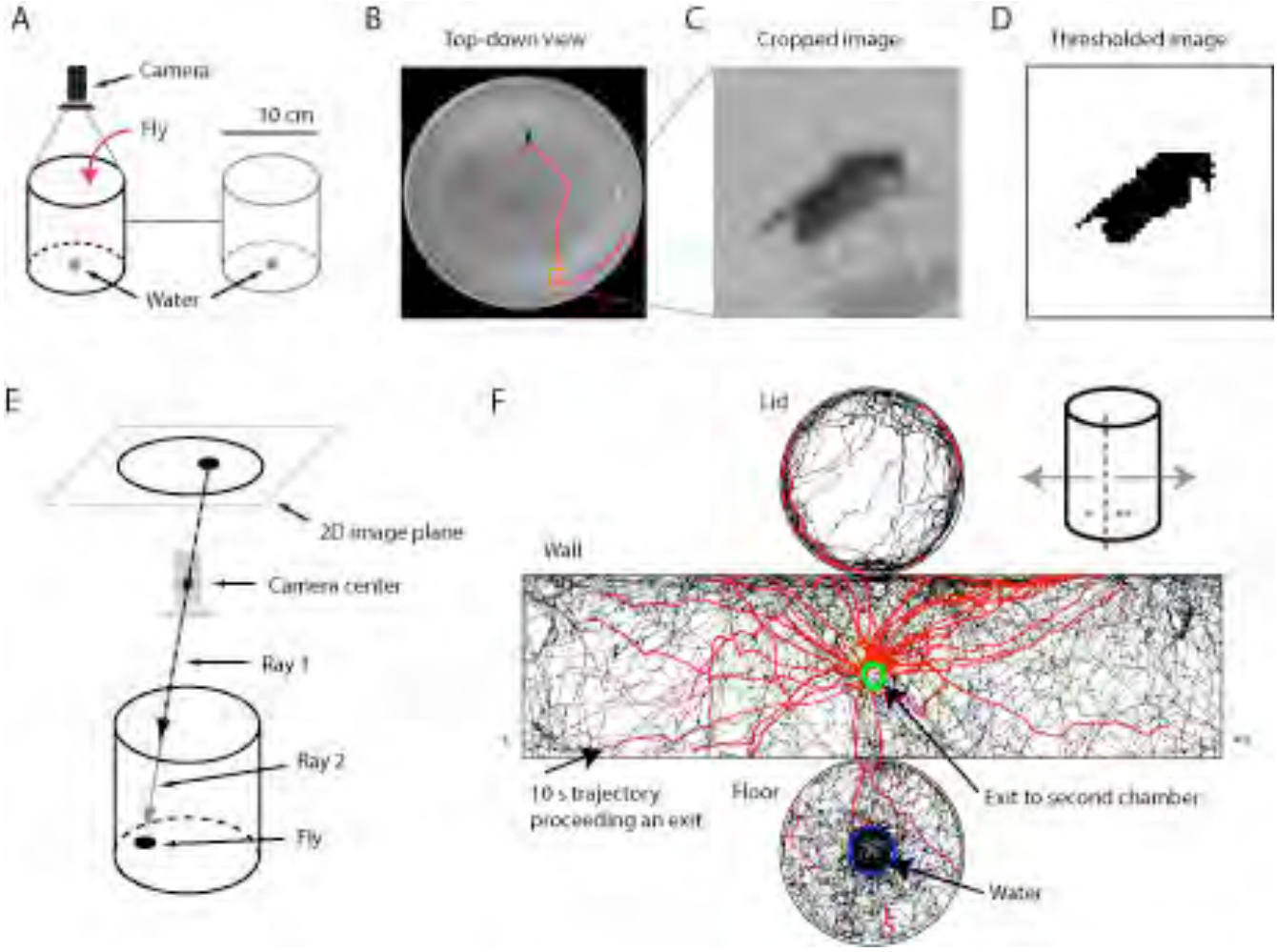


**Figure 4.** (A) Technology devised to study the movement of *Drosophila* within a controlled sensory environment. An individual fly is introduced to the first of two connected chambers, both containing only water. (B) An example of the 2D position of a fly obtained from video using custom software from the laboratory. (C) Cropped image of the fly from this video. (D) Thresholded image of the fly in C. (E) Cartoon illustrating the two possible locations for a fly from the perspective of the camera. Ray 1 represents the possible location of a fly on the underside of the chamber lid (DASHED; BLACK arrow); Ray 2 indicates the true location of the fly in this illustration (SOLID; GRAY arrow), which sits on the chamber floor. (F) Reconstructed trajectory of a fly filmed for 6 h as it became hungry (BLACK). We highlighted 10 s segments for all trajectories proceeding exits (RED) into the second chamber (GREEN circle).

**260. A search for a robust naturalistic behavior to study sensory motor transformation in flies**

*Akira Mamiya*

Producing context appropriate motor patterns is the most important function of the brain. Although many studies have focused on how the brain represents sensory information, few have investigated how these sensory representations in the brain influence the production of motor patterns. The overall goal of this project is to elucidate basic principles underlying this sensory-motor transformation using *Drosophila* as a model organism. We aim to achieve this by combining precise presentation of stimulus and accurate measurements of behavior with physiological recordings from multiple neurons involved in a particular sensory motor transformation. As an initial step of the project, we are currently searching for a naturalistic behavior that flies can perform even when their heads are held stationary under various recording instruments. One of the candidates for this type of robust behavior is an escape flight initiation by looming visual stimulus. To test whether flies can initiate escape flight even when their heads are immobilized, we presented a looming visual stimulus to flies whose heads were fixed to tungsten rods. Flies were placed on top of a floating styrofoam ball that allowed them to walk, or push down the ball with its legs and initiate flight. We found that even when their heads are immobilized, flies can initiate escape flight in response to a looming visual stimulus (Figure 5). Interestingly, stimuli coming from the back of the flies evoked flight responses at a higher rate than stimuli coming from the front of the flies. We also observed that flies move their legs in preparation of the flight initiation, confirming the previous finding in the lab by a graduate student Gwyneth Card in freely behaving flies. These results suggest that an escape flight initiation in *Drosophila* is not a simple reflex, but composed of multiple processing stages that involve sensing of stimulus direction and planned leg movements. We are currently extending the study to combine this behavioral paradigm with functional calcium imaging using 2-photon microscopy.

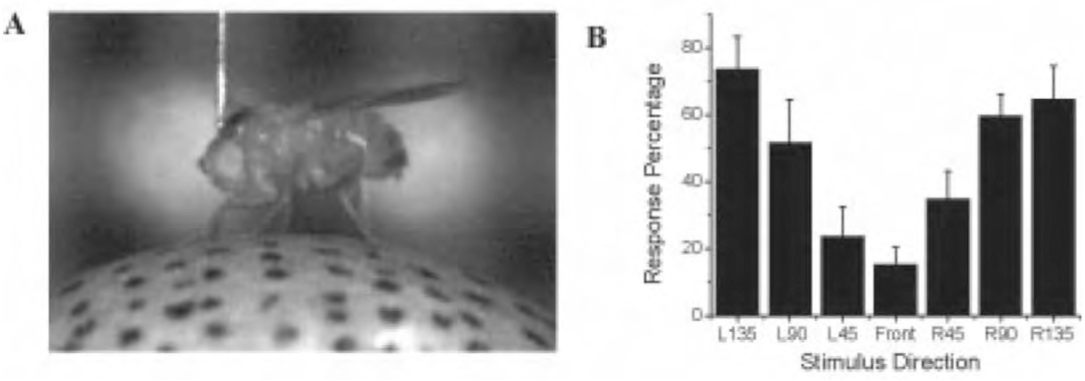


**Figure 5.** Flies can initiate escape flight in response to a looming visual stimulus even when their heads are fixed to tungsten rods. (A) An example of a fly standing on top of a Styrofoam ball with its head fixed to a tungsten rod. (B) Percentage of visual stimuli that evoked escape flight in flies that had their heads fixed to tungsten rods (mean±s.e.m., n = 8 flies). "L" and "R" indicates stimuli coming from the left and the right of the fly, and numbers indicate the angle of the stimulus direction relative to the head orientation. Response percentages for stimulus from different directions are significantly different (one-way ANOVA, $p < 0.001$).

**261. Method for simultaneous behavioral and electrophysiological recording in *Drosophila***

*Gaby Maimon*

Flies are highly visual creatures and, deservedly, much attention has been focused on trying to understand how their visual system operates. Our lab has contributed to this research program by characterizing a variety of visually mediated behaviors the fruit fly, *Drosophila melanogaster*. Importantly, many of these newly described behaviors are robustly observed even while the fly is rigidly tethered, which opens up the possibility of making concurrent physiological measurements as the animals perform these behaviors. Researchers in Michael Bate's and Gilles Laurent's laboratories have recently shown that it is possible to perform whole-cell, patch-clamp recordings from single neurons in the *Drosophila* nervous system, albeit in highly constrained preparations. We have been developing a new recording apparatus, in which whole-cell patch clamp measurements from single cells can be obtained simultaneously with behavioral measurements in the same fly. Using a high-precision, CNC mill we have manufactured a *Drosophila* 'physio-tether-plate' (Figure 6). Ultraviolet-activated glue is applied to the head and very front of the thorax of an anesthetized fly, and the animal is carefully attached to a small opening at the bottom of this plate. A tiny opening is made in the head cuticle, overlying the fly's brain, allowing one to approach neurons of interest with a microelectrode. The plate is designed such that the exposed brain is bathed in a perfused saline solution for

patch clamp recordings, while the rest of the animal's body is dry, and free to produce behaviors such as tethered walking or tethered flight. Genetic tools available in *Drosophila* allow one to expressed green fluorescent protein a subset of neurons, which allows one to target the same cells across preparations. To date, we have successfully recorded from identified, visually responsive cells, as the animals performed walking behaviors on a low-friction ball, acting as a treadmill below them. We have also recently recorded from other neurons during tethered flight. It will be necessary to spend some time maximizing the health of the animals in the preparation, at which point we will begin examining the electrophysiological basis for a variety of visually guided behaviors in the fly.

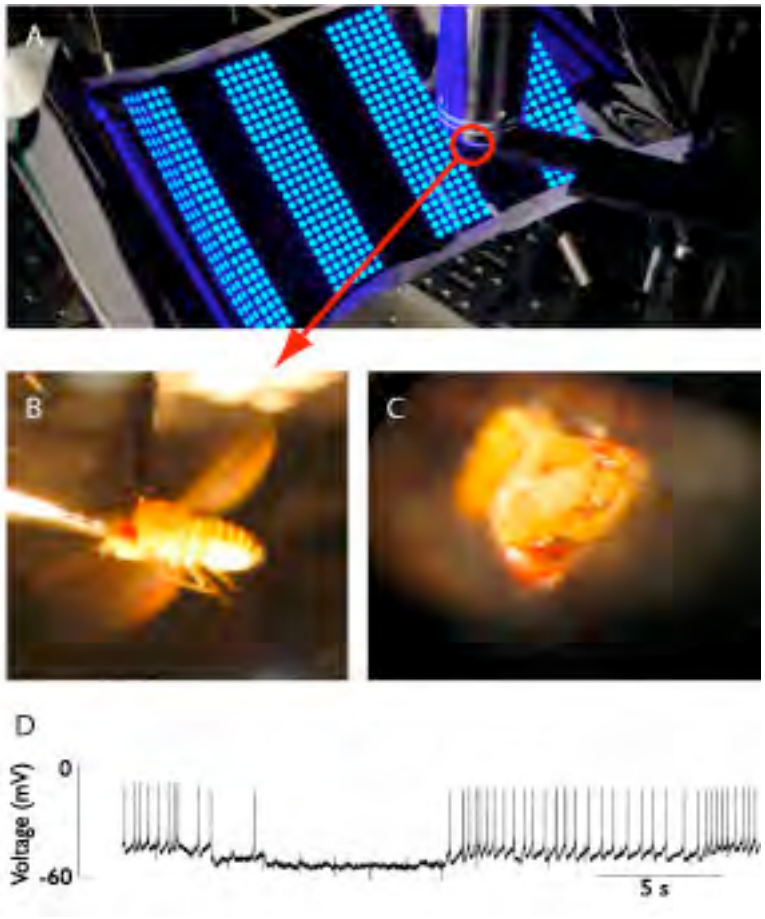


**Figure 6.** Electrophysiology in behaving flies. (A) The physio-tether-plate beneath a microscope objective, in front of an LED visual display showing a blue grating pattern. (B) Close-up of a fly glued to the plate, in tethered-flight. (C) Top view of the fly's head in the saline bath, with a small hole cut in the cuticle. (D) An example of an intracellular voltage trace made from a single neuron in the fly's brain (whole-cell current-clamp mode).

**262. Motor planning in the escape responses of flies**
*Gwyneth Card*

A key feature of reactive behaviors is the ability to spatially localize a salient stimulus and act accordingly. Such sensory-motor transformations must be particularly fast and well tuned in escape behaviors, in which both the speed and accuracy of the evasive response determine whether an animal successfully avoids predation. We have studied the escape behavior of the fruit fly, *Drosophila*, and found that flies can use visual information to plan a jump directly away from a looming threat. This is surprising, given the architecture of the hard-wired pathway thought to mediate escape jumps. Using high-speed videography, we found that approximately 200ms before take-off flies begin a series of postural adjustments that determine the direction of their escape (Fig. 7). These movements position their center of mass (COM) relative to the jumping legs so that leg extension will push them away from the expanding visual stimulus. These pre-flight movements are not the result of a simple feed-forward motor program, because their magnitude and direction depend on the flies' initial postural state. Furthermore, flies plan a take-off direction even in instances when they choose not to jump. This

sophisticated motor program is evidence for a form of rapid, visually-mediated motor planning in a genetically-accessible model organism.

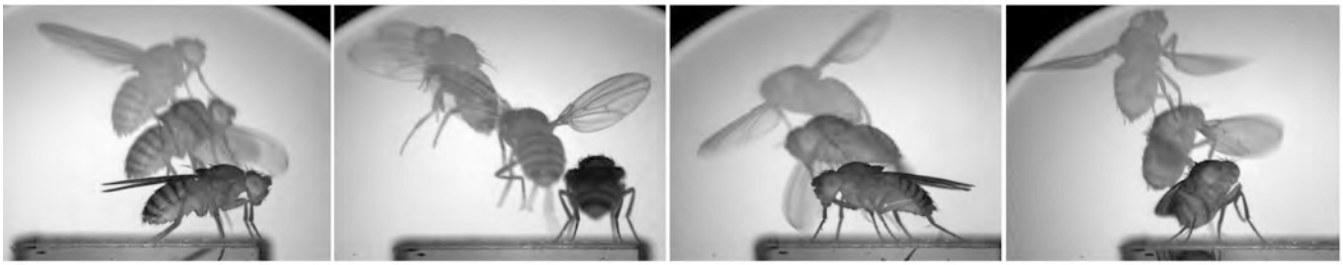

**Figure 7.** Still image composites of high-speed sequences showing flies jumping away from a looming stimulus. In each case the looming stimulus was approaching from the right; the flies jump to the left regardless of their initial posture.

**Publications**


Budick, S.A., Reiser, M.B. and Dickinson, M.H. (2007) The role of visual and mechanosensory cues in structuring forward flight in *Drosophila melanogaster*. *J. Exp. Biol.* **210**(23):4092-4103.

Card, G. and Dickinson, M. (2008) Performance trade-offs in the flight initiation of *Drosophila.* *J. Exp. Biol.* **211**(3):341-353.

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**Graduate Students**: Chiraj K. Dalal, Amit Lakhanpal, Joseph Levine, Shaunak Sen, Frederick E. Tan, John Yong, Jonathan Young
**Undergraduate Students**: Vishu Asthana, Atanu Paul, Kasra Rahbar
**Research and Laboratory Staff**: Melinda A. Kirk, Leah Santat, Michelle E. Shah

**Support**: The work described in the following research reports has been supported by:
Arnold and Mabel Beckman Fellowship Program
Betty and Gordon Moore Fellowship Program
Bren Foundation
Burroughs Wellcome Fund
European Molecular Biology Organization (EMBO)
Howard Hughes Medical Institute
Human Frontier Science Program (HFSP)
National Institutes of Health (NIH)
National Science Foundation (NSF)
Packard Foundation
Searle Scholars Program
Weston Havens Foundation

**Summary**: Cells process information, interact with one another, and exhibit patterned development using circuits composed of interacting genes and proteins. Although many of these components and their interactions are now known, it remains unclear how the circuits they compose function reliably within cells. In order to understand how genetic circuits operate at the single-cell level, we are applying experimental and theoretical techniques to key model systems:

First, we construct synthetic genetic circuits and study their behavior in individual cells. These synthetic circuits are simpler counterparts to the complex circuits one finds in nature. This approach, often called "synthetic biology," allows one to analyze how various circuit designs might work, and begin to understand what is special about the specific circuit architectures observed in organisms. We have constructed circuits that exhibit oscillations and other dynamic phenomena, (e.g., Elowitz and Leibler, 2000). We have used synthetic circuits to analyze the dynamics and variability of gene regulation at the single-cell level (e.g., Elowitz *et al.*, 2002 and Rosenfeld *et al.*, 2005). We also make use of 're-wiring' perturbations to alter the architecture of natural genetic circuits. Current synthetic biology projects focus on regulation of two-component systems in bacteria, and higher-level developmental pattern formation in mammalian cell culture.

Second, we analyze the dynamics of specific natural genetic circuits in order to understand basic principles of their operation. We have developed the ability to acquire and quantitatively analyze large time-lapse movie datasets. These movies allow tracking of circuit dynamics in individual cells as they grow and develop. By incorporating several distinguishable fluorescent protein reporter genes in these organisms, we can track multiple circuit components simultaneously. The results constrain models of the corresponding circuits and provide insight into basic principles of their operation. A recent example of this approach is our work on regulation of genetic competence in *Bacillus subtilis* (see Süel *et al.*, 2006 and Süel *et al.*, 2007).

Third, we are analyzing the generation of variability within cell populations. Genetically identical cells appear to actively generate variability, even in homogeneous environmental conditions. We focus specifically on two complementary questions: How do cells use intrinsic "noise" (stochasticity) in their own components to make effectively random cell fate decisions? And how do they suppress noise in order to operate reliably despite of variability? Current projects are examining these issues in *Bacillus subtilis,* a very simple prokaryote that exhibits both differentiation and development, as well as in more complicated mammalian cell culture systems.

Projects in the lab make extensive use of relatively simple mathematical models of genetic circuits. We are also developing software and tools to improve gene circuit construction and quantitative analysis of movie data.

**263. A quantitative approach to Notch-Delta interactions at the single-cell level**

*David Sprinzak, Lauren LeBon, Amit Lakhanpal, Michael B. Elowitz*

Understanding how genetic circuits that control the differentiation of cells into well-defined patterns in developmental systems has been a major goal of modern biology. Basic principles of developmental circuit design, as well as specific molecular components, appear to be shared by many developmental systems.

In this work we study, both theoretically and experimentally, the Notch-Delta signaling system, which has been implicated in fine-grained (e.g., checkerboard) developmental patterning processes such as the hair patterning in the inner ear or the differentiation pattern of neurons in the developing embryo. We employ an alternative approach to standard genetics whereby we construct synthetic expression systems in mammalian cells and analyze the interactions using quantitative time-lapse microscopy and image analysis tools to quantitatively analyze the dynamics of the canonical Notch-Delta signaling system, typically used in the natural lateral inhibition processes. We combine these experimental results with a theoretical model for lateral inhibition patterning. Such a synthetic approach developed in mammalian cells can serve as a general platform for

studying many other developmental processes in the future and may have application in tissue engineering and applied synthetic biology.

**264. Global regulatory dynamics of *B. subtilis* stress response**

*James Locke, Jon Young, Michael B. Elowitz*

Clonal populations of cells contain the same genome, and may be grown in the same environment. However individual cells are frequently found to exhibit a heterogeneous set of cellular states. A fundamental question in biology is how this heterogeneity is generated. Recent work suggests that stochasticity, or noise, in underlying reactions is used by cells for probabilistic differentiation. Thus, a major problem is to understand, first, how such noise enables heterogeneous decision-making in cell populations and, second, how genetic circuits within the cell constrain variability to generate specific cellular states.

We are using *B. subtilis* as a model system to study this problem. This bacterium exhibits a broad range of differentiated states even in a single environment, including terminal and transient differentiation states (sporulation and genetic competence, respectively) as well as an array of metabolic and regulatory states that are activated in a subset of cells and may persist for more than a cell generation. These states are often controlled by master transcription factors, facilitating analysis with fluorescent protein reporter genes.

We are combining experiments at the single-cell level and theoretical modeling to identify the set of possible states in *B. subtilis*, and investigate their interactions. We have constructed a set of strains with multiple fluorescent reporter genes and are currently analyzing the modularity and compatibility of specific differentiated states, and the dynamic transitions among them.

**265. The dynamics of a phosphorelay-mediated signal transduction system**

*Shaunak Sen, Michael B. Elowitz*

Allosteric modification of a protein in response to stimulus is a ubiquitous signaling methodology in cells. Often, sensing and response are functionally encoded on two separate, but coupled, proteins. In a two-component system, the sensor and regulator are phosphorylable proteins coupled via a phosphotransfer interaction. Cells of the bacterium *Bacillus subtilis* use a cascade of two two-component systems (kinase, Spo0F and Spo0B, Spo0A), called a phosphorelay, to initiate sporulation. It is unclear why *B. subtilis* uses a phosphorelay and not a two-component system. We used mathematical models as guidelines for how a phosphorelay responded to increasing stimulus levels and if this differed from a two-component system. We noticed, for example, that the second two-component system in the phosphorelay could keep the first two-component system free of phosphates and unsaturated. This increased the range of stimulus levels that could be sensed in a phosphorelay relative to a two-component system. In *B. subtilis*, the interactions of the phosphorelay with the genetic circuit that it is embedded in may modify its response. We observed a characteristic increase in Spo0A activity just at the initiation of sporulation. This increase and sporulation itself was almost completely eliminated by multiple copies of *spo0F*. Deletion of a Spo0F phosphatase in the presence of multiple copies of *spo0F* significantly alleviated sporulation inhibition. In contrast, multiple copies of *spo0B*, which does not have a phosphatase, did not eliminate the characteristic increase in Spo0A activity at the initiation of sporulation. Paradoxically, we observed, in our attempts to transplant the phosphorelay into *E. coli*, that Spo0B appears to have a larger inhibitory effect on Spo0A activity than Spo0F. Using these results, we are analyzing the effects of the phosphorelay architecture on its dynamics as a way to understand why *B. subtilis* uses a phosphorelay and not a two-component system during sporulation.

**266. Variability in sporulation initiation in *B. subtilis***

*Joe Levine, Rajan Kulkarni, Michael Elowitz*

Under stressful conditions, *B. subtilis* cells respond by deciding whether, and when, to form robust spores that can survive harsh conditions and re-germinate later. Interestingly, even under conditions that favor this sporulation process, individual cells exhibit a large variability in the time it takes for them to initiate sporulation. This sporulation delay may function as a form of "bet-hedging" given the uncertainty of future environmental conditions that may improve (favoring vegetative growth) or may continue to deteriorate (favoring sporulation). The sporulation initiation circuitry has been well characterized using genetic and biochemical techniques, but it remains unclear how it functions to create long and variable delays in individual cells. In order to analyze the behavior of this circuitry at the single-cell level, we have constructed a variety of strains incorporating several different fluorescent reporter genes. Examining these strains with fluorescent time-lapse microscopy, we have observed variability in the expression of sporulation-specific genes that correlates with variability in the decision-making process. We are currently exploring how various dynamic mechanisms might generate this variability and looking for signatures of these mechanisms in gene expression patterns.

**267. Gene regulation dynamics underlying embryonic stem cell differentiation**

*Fred Tan, Julia Tischler, Michael Elowitz*

Mouse embryonic stem cells (mESCs) can differentiate into trophectoderm, primitive endoderm and embryonic germ layers to generate any cell type found in the mouse. Within mESCs, a unique transcriptional network exists to maintain an undifferentiated and self-renewing phenotype, anchored by the activity of three transcription factors (Oct4, Nanog and Sox2) and the supportive role of intercellular signaling. The interplay between pluripotency regulators and signaling pathway

second messengers define the dynamics of cell maintenance and cell specification in culture. We analyze the decision to remain pluripotent or commit to differentiation from the perspective of gene regulatory dynamics. We perturb signaling pathways and the expression of developmental cues to validate previously characterized or proposed regulatory interactions and to better understand the underlying transcriptional states. By tracking the expression of developmentally important genes, we hope to capture the initial moments that lead to lineage commitment. We believe such an analysis will yield further insights into processes occurring naturally in the mouse.

**268. Frequency-modulated nuclear localization bursts coordinate gene regulation**

*Long Cai, Chiraj Dalal, Michael Elowitz*

Regulation of transcription factor localization allows cells to respond rapidly to extracellular signals. Although the molecular mechanisms of nuclear import and export have been examined, it remains unclear how localization varies among individual cells, and how dynamic changes in localization affect expression of downstream genes. In the presence of extracellular calcium, Crz1, the calcineurin responsive zinc finger transcription factor of *Saccharomyces cerevisiae*, is dephosphorylated and translocates into the nucleus. By observing the localization of Crz1-GFP fusion proteins using time-lapse microscopy, we found that Crz1 exhibited bursts of nuclear localization with a characteristic nuclear residence time of ~2 minutes. These bursts occurred in a stochastic fashion in individual cells and propagated to the expression of downstream genes, contributing significantly to fluctuations in gene expression. Strikingly, calcium concentration controlled the frequency, but not duration, of nuclear localization bursts. Using an analytic model, we find that the observed stochastic frequency modulation (FM) of localization bursts can enable cells to proportionally coordinate expression levels of multiple target genes by regulating the fraction of time a promoter is active, rather than tuning the level of activity itself. We experimentally confirmed this theory by showing that both natural and synthetic Crz1 target promoters are expressed proportionally (in fixed ratios) across a wide range of calcium concentrations. Furthermore, we observe that another stress response transcription factor, Msn2, exhibits similar localization bursts under calcium stress, but its bursts are largely uncorrelated with Crz1 bursts. These results suggest that FM localization bursts may be a general control strategy utilized by the cell to coordinately regulate multi-gene responses to external signals. We are currently investigating the generality of this strategy in yeast and other species.

**Reference**

Cai, L., Dalal, C.K. and Elowitz, M.B. (2008) *Nature* **455**(7212):485-490.

**269. Regulatory activity revealed by dynamic correlations in gene expression noise**

*Mary J. Dunlop, Robert Sidney Cox III, Joseph H. Levine, Richard M. Murray, Michael B. Elowitz*

Gene regulatory interactions are context-dependent, active in some cellular states but not others. Stochastic fluctuations, or 'noise,' in gene expression propagate through active, but not inactive, regulatory links. Thus, correlations in gene expression noise could provide a non-invasive means to probe the activity states of regulatory links. However, global, 'extrinsic,' noise sources generate correlations even without direct regulatory links. Here we show that single-cell time-lapse microscopy, by revealing time lags due to regulation, can discriminate between active regulatory connections and extrinsic noise. We demonstrate this principle mathematically, using stochastic modeling, and experimentally, using simple synthetic gene circuits. We then use this approach to analyze dynamic noise correlations in the galactose metabolism genes of *E. coli.* We find that the CRP-GalS-GalE feed-forward loop is inactive in standard conditions, but can become active in a GalR mutant. These results show how noise can help analyze the context-dependence of regulatory interactions in endogenous gene circuits.

**270. Noise exposes alternative morphological cell fates in bacterial development**

*Avigdor Eldar, Vasant Chary, Panagiotis Xenopoulos, Michelle E. Fontes, Oliver C. Loson, Jonathan Dworkin, Patrick Piggot, Michael B. Elowitz*

Development normally proceeds through a consistent sequence of events that occurs similarly in all wild-type individuals. However, developmental mutations often exhibit partial penetrance, affecting the fate of individual organisms differently, even within an isogenic population in a homogeneous environment. Partial penetrance can originate from fluctuations, or noise, within developmental gene circuits, whose effects are buffered in the wild type. But it remains unclear how genetic circuit architecture determines the set of phenotypes a specific developmental mutation will generate, and how the relative frequencies of these fates evolve. Here, using *Bacillus subtilis* sporulation as a model developmental system, we identified a stochastic cell fate determination network that operates when inter-compartmental signaling components are mutated. Fate determination is largely independent of noise in the expression of the perturbed gene, but rather results from 'cryptic' noise sources whose effects are normally suppressed in wild-type organisms. Competition between three molecular processes—inter-compartmental signaling, septation, and chromosome replication—results in a set of discrete alternative fates not found in the wild-type strain, including the ability to form two "twin" spores, rather than one, in a single mother cell. The system enables rational tuning of the penetrance of alternative fates, and the construction of strains approaching complete penetrance of twin sporulation.

Intriguingly, twins resemble sporulation patterns observed in other microbial species. Thus, noise may facilitate evolution by enabling the expression of discrete new morphological traits at low penetrance that can be genetically assimilated by subsequent mutations that increase penetrance and reduce dependence on noise.

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**Undergraduates**: Rebecca Barter, Vamsidhar Chavakula, Csilla Felsen*
**Graduated or have left Caltech*

**Support:** The work described in the following research reports has been supported by:
American Heart Association
Anna L. Rosen Professorship
Atazoa Ltd.
Beckman Institute
Carl Zeiss Jena
Defense Advanced Research Projects Agency
House Ear Institute
Human Frontier Science Program
Jet Propulsion Laboratory
Moore Foundation
National Aeronautics and Space Administration
National Heart Lung Blood Institute
National Human Genome Research Institute
National Institute for Biomedical Imaging and Bioengineering
National Institute of Child Health & Human Development
National Institute of Neurological Disease and Stroke
National Science Foundation
That Man May See, Inc.

**Summary**: Our laboratory has dedicated itself to an integrative approach to defining the cell and molecular basis of embryonic patterning, in which *in vivo* imaging tools play a central role. Our approach has been to develop imaging tools that are sufficiently robust so that the movements, lineages and gene expression in living cells can be analyzed as an adjunct to the techniques more typically employed by systems biology. Systems biology provides a means to organize the explosion of data from molecular approaches and the dramatic progress from *in vitro* culture assays, but it requires some means to test the proposed linkages. Our goal is to test these proposed mechanisms in the intact embryo, with the hope of moving forward to an understanding of which of the potential mechanisms operate in the natural biological context. There are many challenges to such tests, including the tagging of cells or molecules so that they can be followed in the intact system, the visualization of the tagged structures, and the interpretation of the time-varying events these images represent. Solutions to these challenges require the coordinated efforts of researchers spanning the life and physical sciences.

In the past year we have made significant advances in imaging fast events in the developing embryo, ranging from the movements of cilia in the developing ear and left-right organizer and the flows these cilia generate, to the workings of the beating heart as it takes shape. This has required the development of new image acquisition and processing tools that offer quantitative analyses of the key events. These tools are proving to be generalizable to other systems such as the *Drosophila* embryo. As a result, we have great hope for exploring events that have previously been too difficult to address.

In parallel with the refinement of new imaging tools, we have been creating new and more efficient means for creating embryos with genetically-encoded fluorescent tags. Through random insertion into the genome, this approach permits the creation of functional fusions between a host protein and a fluorescent protein, permitting cells and developmental questions to be posed in normally developing embryos. The creation of these new lines is dramatically more efficient than previous approaches, making it possible for even modest-sized facilities to embark on large-scale screens. This increased efficiency of creating marked strains requires a parallel increase in imaging technologies, and the refinement of *in*

*toto* image acquisition and analysis tools answers this challenge.

There have been dramatic advances in other areas, as well ranging from the refinement of new sensor technologies with researchers in the Kavli Nanoscience Institute, to improved tools for acquiring information from MRI images. Our combination of modern and classical technologies continues to offer surprises, including the discovery of a novel sensory pathway in the mammalian olfactory system and the development of a molecular sensor with unprecedented sensitivity.

**271. Junctional dynamics during myocardial epithelia morphogenesis in heart tube formation**

*Le A. Trinh, Scott E. Fraser*

A fundamental challenge in epithelial biology is to understand how epithelial cells undergo collective cell migration as they participate in organ formation. To this end, we are using the migration of myocardial precursors of the embryonic heart tube as a model to study the dynamic molecular and cellular interactions of epithelial cells as they undergo morphogenesis. The myocardial precursors form bilateral epithelial tubes that move coordinately toward the embryonic midline to form the linear heart tube. Previous studies using static imaging of the migration process have shown that these cells form a maturing epithelia as they migrate. Additionally, the cadherin-catenin complexes have been shown to restrict to the basolateral domain of the myocardial epithelia. To study the dynamics of the junctional maturation process, we are performing 4-dimensional confocal imaging of a citrine (yellow fluorescent protein, YFP) fusion of the α-catenin gene product. α-catenin is a component of the cadherin-catenin complex, co-localizing with E-cadherin and β-catenin. The α-catenin-citrine fusion was generated using a transposon-mediated gene trapping strategy that we previously developed, in which we fluorescently tag zebrafish proteins at their endogenous loci with an internal exon encoding citrine. Using this gene trap line, we are able to detect the epithelial maturation process in real-time. We find that α-catenin localization changes dramatically during the migration process, starting in the cytoplasm at the 10-somite stage and localizing to the cell-cell boundary by the 16-somite stage. The localization at the cell-cell boundary is also marked by gaps between neighboring cells as they migrate, indicating the adhesion between the migrating cells are loose and not static. We are currently using this data in combination with analysis of α-catenin dynamics in mutant embryos that affect epithelial formation to provide a genetic framework into which these dynamic junctional behaviors can be placed. The analyses of α-catenin localization in wild-type and mutant cells during heart tube formation should further facilitate our understanding of the complex molecular and cellular interactions during cardiac development, as well as the mechanisms underlying epithelial morphogenesis.

**272. Endocrine disrupter exposures on cytochrome P450 aromatase activities: New lessons on endocrine and neuroanatomical development from teleost model systems**

*Rasheeda M. Hawk, Eduardo Rosa-Molinar, Le Trinh, Scott E. Fraser*

Exposures to pesticides, plasticizers, heavy metals, and other chemicals that accumulate in our environment have the potential to disrupt hormone-related processes of the reproductive system, neuronal development, and metabolism. Endocrine disrupting chemicals, in particular, act via estrogen receptors and aryl hydrocarbon receptors. We will study the effects of environmental estrogen homologues on the expression of *cyp19a* and *cyp19b*, genes that encode for aromatase P450 in zebrafish and mosquito fish. Zebrafish will be used for molecular studies because of the availability of genetic manipulations. Mosquitofish will be used because they have distinct sexual dimorphic characteristics, making them an ideal model for neuranatomical analysis of high aromatase activity. Thus, the aims of my project will be to: (1) study the effects of estrogen and aryl carbon activators on *cyp19a* and *cyp19b* gene expression found in the gonads and brains of zebrafish and mosquitofish; (2) study the physiological impacts of *cyp19b* activation and/or inhibition in the hypothalamus of mosquitofish; and (3) to analyze the role of aromatases located in glial cells on neuronal development and sexual dimorphism in mosquitofish.

**273. *In toto* single-cell imaging and cellular variations during somitogenesis in zebrafish**

*Frederique Ruf, Sean Megason, Scott E. Fraser*

Somitogenesis is a well-organized process that results in segmentation of the embryonic presomitic mesoderm into discrete structures, the somites, along the notochord. Somites are important conserved vertebrate structures that give rise to skeletal formations and muscles. The goal of my project is to continue developing *in toto* non-invasive 4D (3D and time) imaging with single-cell resolution to understand how individual cells organize to form each somite/myotome in zebrafish. Although somitogenesis has been investigated for years, this mechanism has not been looked at with single-cell resolution in living embryos. Since many zebrafish genes have human orthologs, the model will be useful to understand disease-related mutations affecting humans.

Each embryo is injected at the one-cell stage with RNA encoding for membrane-cherry and H2B-GFP/cerulean to label all individual cells. At 10 hpf, fluorescent embryos are sorted, dechorionated and placed in specifically designed "embryo arrays" for imaging. Time-lapses and digital z-sectioning are then performed on live embryos using confocal imaging (see Figs. 1 and 2). For analysis, digital movies will be segmented and individual cells tracked using cell identification and tracking softwares. Tracking single-cell movements will allow us to observe how cell-to-cell variations might be controlled in developing organisms, as well as giving us a

better understanding on how the process of cell rearrangement happens in wild-type versus mutant embryos. For that purpose we will image with high-resolution and analyze mutants, as well as new zebrafish lines with traps involved in somite/muscle formation from a flip-trap protein screen. We will make complete digital reconstructions of somite formation in each case.

Preliminary results show (1) that *in toto* imaging is feasible (Figure 1); and (2) that we are able to label and position the embryos to observe somite formation at the single-cell level, with staining intensities sufficient for cell tracking (Figure 2).

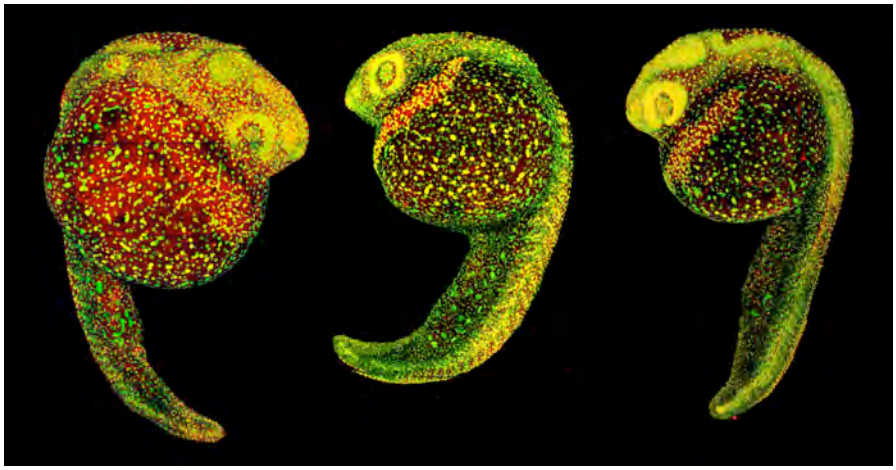

**Figure 1:** 3D reconstruction of three 24 hpf embryos following *in toto* imaging trials. Embryos were labeled with RNA encoding for membrane cherry and H2B-GFP. Imaging was performed in lateral mold on a 510 LSM Pascal, 40X 1.1NA objective, 2µm z-step.

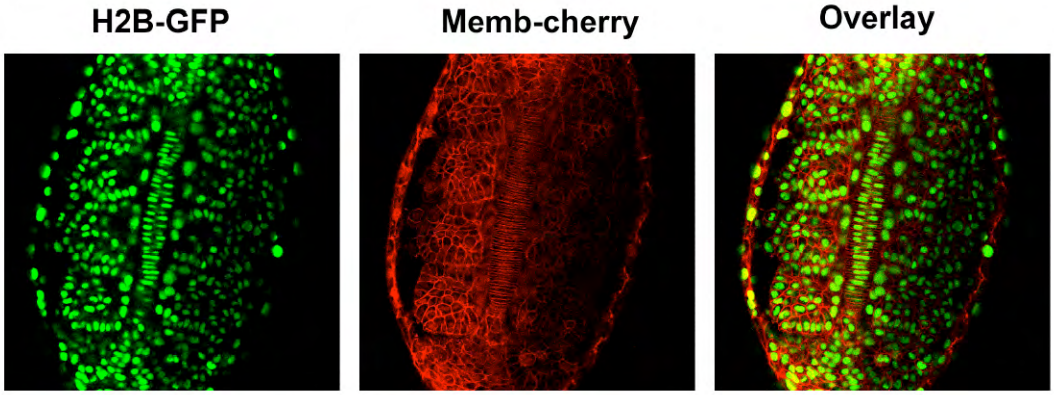


**Figure 2:** Somite single-cell resolution imaging. Embryos were labeled with RNA encoding for membrane cherry and H2B-GFP. Imaging was performed in dorsal mold on a 510 LSM Pascal, 40X 1.1NA objective.

**274. Forming the zebrafish spinal interneuron system: From neuronal progenitor domains onward**

*Alana Dixson, Sean Megason, Scott E. Fraser*

Earlier research on spinal cord (SC) development and circuitry formation focused on motoneurons and Rohon Beard cells with less attention given to interneurons (INs). Basic questions to answer include how many spinal INs exist and how they are further specified from their dorsal and ventral progenitors. Additionally, how do the anatomical and functional organization and reorganization of these cells change throughout developmental time? Understanding how the SC is built, repaired, and maintained over a lifetime will provide insights in to the biology, genetics and physiology of spinal neurons, and eventually, lead to corrective therapies for SC anomalies and injuries.

The organism, *Danio rerio* (zebrafish) is a strong model for vertebrate SC development. Its simplistic central nervous system (CNS) and suitability for genetic manipulation and optical imaging with confocal laser scanning microscopy have been exploited in neurodevelopmental research for several years. My thesis project relies on each of these strengths to meet two initial objectives: 1) To identify and track in three dimensions plus time (3D + t) the neuronal progenitor domains (NPDs) in the embryonic SC of transgenic and wild-type zebrafish (*in toto* imaging); and 2) To track each cell in the developing SC from neural induction to formation of the larval-stage cord at 72 hours post-fertilization (hpf).

To accomplish the first objective, NPDs in paraformaldehyde fixed embryos collected from 12-48 hpf are immunostained, using antibodies against transcription factors identified previously in chick and mouse. These molecular markers are then used to generate transgenic animals in which we locate and time the appearance of individual zebrafish NPDs. To meet our second objective, we inject one-cell stage zebrafish embryos with mRNA to create transient transgenic animals with fluorescently and ubiquitously labeled cell membranes and nuclei. These fish are then time-lapse imaged *in vivo* from 12-18 hpf and 18-24 hpf. The software programs GoFigure and Imaris will then track and segment individual SC cells in 3D + t. Eventually the transgenic fish developed in objective one will be used to follow cells arising from the NPDs, specifically, those of the spinal IN system in living zebrafish embryos and larvae.

**275. Cell dynamics and cell division during zebrafish gastrulation**

*Luca Caneparo, Periklis Pantazis, Scott E. Fraser*

During animal development a single cell is the origin of different cell types and through orchestrated cell movements generates several different tissues. The mechanisms through which cells generate such diversity strongly rely on organized and controlled cell divisions, and cell migration. During zebrafish development the anlage of the future central nervous system (the neural plate) undergoes a series of oriented cell divisions and directional cell migrations. Both events are at least partially regulated by Wnt family members. Wnt is one of the main pathways involved in cell migration and cell division during vertebrate gastrulation. The correct orientation along the antero-posterior axis of the embryo is randomized when Dsh, a cytoplasmic element of the Wnt pathway, is perturbed (Gong *et al.*, 2004). Similarly, lack of Wnt11 disrupted the directional cell migration (Heisenberg *et al.*, 2000). How the single cell perceives its position along the antero-posterior axis and coordinates its migration and division along this embryonic axis is not yet well understood in the zebrafish embryo, mainly due to the lack of molecular markers that can be followed *in vivo* in the gastrula embryos.

To try to elucidate this process we use Dendra protein linked to a membrane localized signal. The advantage of using Dendra instead of other fluorescence proteins to label the cell membrane is given by the intrinsic property of this protein: when exposed to UV light Dendra undergoes green to red photoconversion. This allows us to highlight single cells and appreciate the variability in a cell's shape and its relationship to the surrounding neural plate cells during gastrulation. Using image analysis software we can segment the cells and plan to correlate the great dynamic variability visible at the cell membrane level with the migratory directions of the neural plate cells. By photoconverting only a subregion of the cell we hope to gather information about the mechanism through which oriented cell divisions occur in the neural plate, and compare the wild type situation to embryos where signaling required for the proper alignment with the antero-posterior embryonic axis is perturbed.

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**276. Collective cell movements during embryo morphogenesis: From disorder to order**

*Willy Supatto, Thai Truong, Julien Vermot, Scott E. Fraser*

An embryo is shaped by a fascinating and complex choreography of coordinated cell movements that are highly regulated in both time and space. The recent development of imaging and image processing techniques, such as 4D microscopy and 3D cell tracking, gives access to the movement quantification of large cell populations within a developing embryo (Figure 1). These imaging approaches provide a unique opportunity to study embryonic morphogenesis from the level of cellular processes to the scale of an entire organism. In collaboration with several groups at Caltech (Amy McMahon and Angelike Stathopoulos, Biology Department) and at Harvard Medical School (Eric Brouzes and Norbert Perrimon, Genetic Department), we are developing the following research program: (i) 4D imaging by using optimized nonlinear microscopy that is adapted for the 4D, long-term and deep tissue imaging of *Drosophila* and zebrafish embryos without compromising viability; (ii) Systematic quantification of morphogenetic events by using advanced image analysis techniques; and (iii) Genetics and physical disruption to elucidate the mechanical and biochemical relationships involved in the coordination of movements.

This approach has been successfully applied to the study of mesoderm cell spreading during gastrulation in *Drosophila* embryos [1]. Despite the apparent disorganization of morphogenetic events when looking at the imaging data, the quantitative decomposition we developed reveals a high level of organization, involving a symmetrical and regular spatial pattern of collective spreading, spatial waves of cell divisions, and synchronized cell intercalation events leading to the formation of a cell monolayer. This study revealed surprising insights into the role of FGF signaling in concerted cell movements.

By using quantitative description inspired by condensed matter physics, we further developed cell movement analysis to investigate the cell kinetic ordering within a large population of cells. An order parameter is defined, which allows us to follow the correlations of cell movements and to describe the spatial and temporal propagation of these correlations. It allows us to identify specific cell collective behaviors leading to tissue and organ morphogenesis and to address questions, such as the spatio-temporal scale and the nature of cell-cell

interactions involved in morphogenesis [2]. These approaches are currently applied to analyze *Drosophila* and zebrafish embryo gastrulation.

**Publications**

1. McMahon, A., Supatto, W., Fraser, S.E. and Stathopoulos, A. (2008) Dynamic analyses of *Drosophila* gastrulation reveal spatial organization of cell behaviors. Submitted to *Science*. (*equal contribution.)
2. Supatto, W., Brouzes, E., Perrimon, N. and Fraser, S.E. (2008) Statistical analysis of cell movements during *Drosophila* embryo morphogenesis. Meeting abstract, *Biophysical J.* **94**:3194.

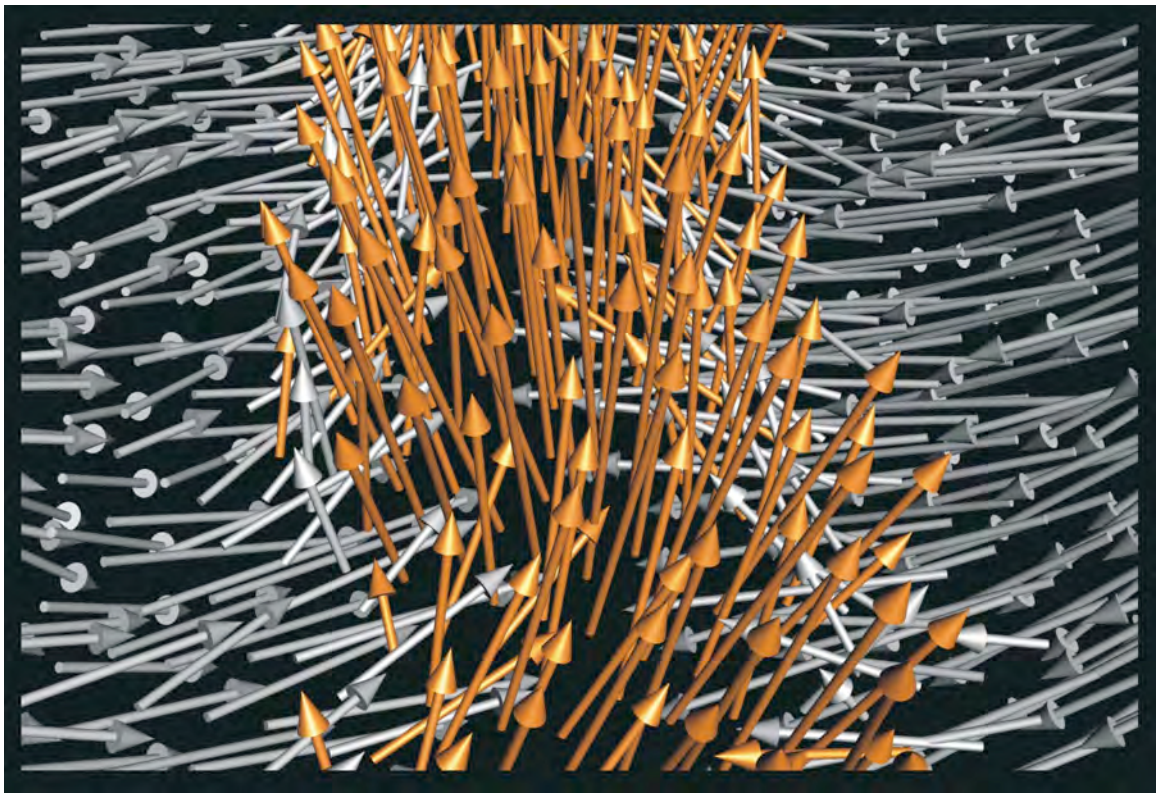


**Figure 1**: Quantification of cell movements during *Drosophila* gastrulation. The mesoderm cells (yellow arrows) are invaginating to shape the ventral furrow. This furrow formation is followed by the convergence-extension movement of the ectoderm cells (gray arrows).

**277. An all-optical approach for probing microscopic flows in living embryos**

*Willy Supatto, Scott E. Fraser, Julien Vermot*

Living systems rely on fluid dynamics from embryonic development to adulthood. To visualize biological fluid flow, devising the proper labeling method compatible with both normal biology and *in vivo* imaging remains a major experimental challenge. We developed a simple strategy for probing microscopic fluid flows *in vivo* that meets this challenge. An all-optical procedure combining femtosecond laser ablation, fast confocal microscopy and 3D-particle tracking was devised to label, image and quantify the flow [1]. This approach is illustrated by studying the flow generated within a micrometer-scale ciliated vesicle located deep inside the zebrafish embryo and involved in breaking left-right embryonic symmetry. By mapping the velocity field within the vesicle and surrounding a single beating cilium (Figure 1), we show this method can address the dynamics of cilia-driven flows at multiple-length scales, and can validate the flow features as predicted from previous simulations. This approach provides new experimental access to questions of microscopic fluid dynamics *in vivo*.

**Publication**

[1] Supatto, W., Fraser, S.E. and Vermot, J. (2008) An all-optical approach for probing microscopic flows in living embryos. *Biophysical J.* In press.

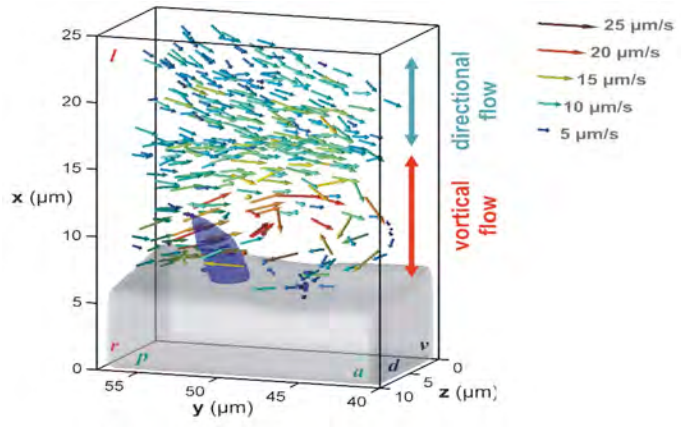



Figure 1: Velocity vector map around a single beating cilium extracted from 3D particle tracking *in vivo*. Dorsal view of the velocity field surrounding a beating cilium on the right side of the Kuppfer's vesicle and showing a transition between directional and vortical flow close to the cell surface. The gray volume represents the cell surface and the blue volume the cilium position. The vector map corresponds to the accumulation of instantaneous particle velocities recorded at different time points. The color of the arrows encodes for the norm of the velocity vectors. d: dorsal, v: ventral, a: anterior, p: posterior, l: left, r: right.

**278. Developing methods for imaging microscopic flow**

*Julien Vermot, Willy Supatto, Scott E. Fraser*

Techniques that permit us to directly address the interaction between beating cilia and their fluidic environment remain scarce and are often not well adapted to living samples. Many studies explored left-right axis specification in vertebrates, where the beating cilia are generating a directional flow necessary for breaking the embryonic symmetry in the so-called left-right organizer. So far, the obvious technical challenges of labeling microscopic flow in living tissue suitable for fast *in vivo* imaging have been a major barrier for understanding the cilia contribution to this process. To overcome these limitations, we have developed imaging techniques that allow us to follow cilia-driven flow with unprecedented temporal resolution. By combining highly localized femtosecond laser ablation, fast confocal imaging and 3D-particle tracking, we were able to label and quantify biological flows driven by cilia *in vivo*. Our results suggest that cilia generate a laminar flow in its far field and a vortical flow near its beating envelope, allowing the formation of a directional flow at the scale of the left-right organizer. Together, these observations suggest that breaking the initial embryonic symmetry relies on cilia positioning within the left-right organizer in order to generate a unidirectional flow.

**279. Imaging cilia-mediated flow in the developing inner ear**

*Julien Vermot, David Wu, Jessica Colantonio, Kent Hill, Scott E. Fraser*

In humans, auditory and balance defects are common among the elderly and deafness is one of the most common inherited diseases. In zebrafish, as in humans and other vertebrates, hearing and balance are mediated by mechanical sensors in the inner ear. These sensors consist of biomineralized composite crystals, called otoliths ("ear-stones"), situated atop ciliary bundles at the surface of epithelial cells. Otoliths are analogous to otoconia in human ears. They provide an inertial mass that facilitates deflection of ciliary bundles in response to vibration, gravity and linear acceleration, thereby initiating signaling pathways that underlie hearing and balance. The inner ear has multiple otoliths, whose number, position and morphology are under strict developmental control. Each otolith is assembled from a common pool of precursor particles, but the mechanisms that ensure particle seeding on specific cells of the sensory epithelium are unclear. It has been proposed that beating cilia distributed throughout the inner ear participate in otolith biogenesis, but direct evidence for this is lacking.

Based on direct imaging of cilia motility and quantitative analysis of precursor particle movements, we propose a new model for otolith biogenesis. In this model, motility of specialized tether cilia establishes a fluid vortex that draws in precursor particles, thereby biasing an otherwise random distribution of precursors and concentrating them at the poles of the otic vesicle. This process facilitates preferential seeding on developing hair cells of the sensory maculae. Tether cilia motility further promotes uniform otolith growth by maximizing effective contact area with precursor particles. Together, these findings provide a surprisingly simple model for explaining the biogenesis of biominerals *in vivo* and will help to clarify the ontogenesis of many inner ear defects that remained unexplained so far. Our findings also provide new insights for understanding the epigenetic influence of motile cilia and fluid dynamics on embryonic developmental programs.

**280. Blood flow and heart valve formation**

*Julien Vermot, Arian S. Forouhar, Michael Liebling, David Wu, Diane Plummer, Morteza Gharib, Scott E. Fraser*

The growth and development of vertebrates are critically dependent on efficient cardiac function to drive blood circulation. As the heart matures beyond the earliest heart tube stages, efficient cardiac function can only be maintained with properly working heart valves. Congenital heart valve defects remain one of the most prevalent manifestations of cardiac dysgenesis. Mitral valve defects occur in almost 1% of all live human births, emphasizing the importance of identifying the genetic and epigenetic factors that foster normal valve formation. Of the several genes that regulate valvulogenesis, many are responsive to fluid dynamic stimuli. Along with previous

observations demonstrating the importance of intracardiac fluid flow for cardiogenesis, this offers the exciting possibility that the genetic programs that govern valve formation *in vivo* depend on intracardiac hemodynamics. To address this question, we have developed imaging techniques that allow us to follow heart development with unprecedented temporal resolution. We took advantage of these technical advances to study the nature of intracardiac flow during the stages of valve specification. The experimental evidence argues that valve development is critically dependent on flow and the reversing nature of the flow. A surprisingly simple modification of the flow pattern, decreasing the retrograde flow fraction, is sufficient to block valve formation. Building upon this, we show that these same alterations of flow abolish the expression of *klf2a*, a transcription factor that is required for valve formation. Our results present a useful method to control hemodynamic forces during cardiogenesis. This simple approach of monitoring and manipulating heart rate offers a powerful tool for predicting and treating dysgenesis of cardiac valves.

**281. Fast imaging and post-processing techniques to study the beating and developing embryonic zebrafish heart**

*Michael Liebling, Julien Vermot, Scott E. Fraser*

Live imaging has gained a pivotal role in developmental biology since it increasingly allows real-time observation of cell behavior in intact organisms. Microscopes that can capture the dynamics of ever-faster biological events, fluorescent markers optimal for *in vivo* imaging, and, finally, adapted reconstruction and analysis programs to complete data flow all contribute to this success. Focusing on temporal resolution, we reviewed fast imaging techniques that operate with minimal prejudice to spatial resolution, photon count, or to reliably and automatically analyze images [1]. In particular, we showed how integrated approaches to imaging that combine bright fluorescent probes, fast microscopes, and custom post-processing techniques can address the kinetics of biological systems at multiple scales (see Fig. 1).

We developed such a technique for time-lapse imaging of the developing (and beating) heart that allows following its development at any computationally halted heart contraction state [2,3]. The central idea is to image at least one full heartbeat at a fast frame rate, resulting in a two-dimensional plus time data set, and repeat this operation every few minutes over several hours for multiple axial positions.

The acquired data are five dimensional (*X*,*Y*, and *Z* in space, 'fast' and 'slow' dimensions in time). The 2D+T image series is then synchronized to its neighbors in the axial and development time dimensions using a non-rigid registration algorithm (constrained such as to leave all but the fast time dimension unchanged). The algorithm proceeds recursively over the different axial positions and developmental stages. We successfully applied this procedure to image the development of the embryonic zebrafish heart between 32 and 44 hours post fertilization (see Fig. 2).

**Publications**

[1] Vermot, J., Fraser, S.E. and Liebling, M. (2008) Fast fluorescence microscopy for imaging the dynamics of embryonic development. *HFSP J.* **2**:143-155.

[2] Liebling, M., Vermot, J. and Fraser, S.E. Double time-scale image reconstruction of the beating and developing embryonic zebrafish heart, Proceedings of the 5th IEEE International Symposium on Biomedical Imaging: Macro to Nano (ISBI '08), Paris, France, May 14-17, 2008, pp. 855-858.

[3] Liebling, M., Vermot, J. and Fraser, S.E. High-Speed and Long-Term Imaging of Cell Dynamics in the Developing Heart, Experimental Biology, San Diego, CA, April 5-9, 2008. (Conference abstract, invited talk).

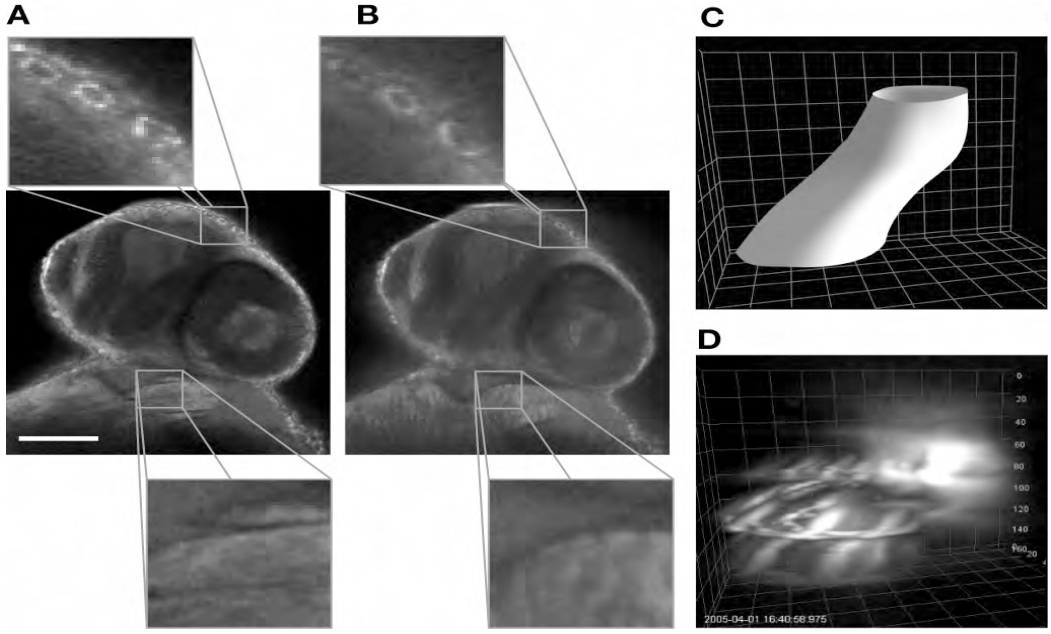



**Figure 1.** Fast Confocal Microscopy and Scanning Artifacts. **A.** While point scanners provide high spatial resolution and axial selectivity (detail, top) fast moving structures such as the heart in this 30 hours post fertilization (hpf) old zebrafish embryo (BODIPY FL fluorescent dye) cannot be captured with sufficient accuracy (detail, bottom). Scale bar is 100 μm. **B.** With frame-rates 10-100 fold more rapid than those of point-scanning microscopes, line-scanning confocal microscopes can capture the actual structure of the beating heart (detail, bottom), while keeping good spatial resolution and optical sectioning ability (detail, top). **C.** Three-dimensional cartoon of the heart-tube and, **D.** as measured by successively imaging planes along the Z direction of the heart in a 30hpf transgenic Tg(*cmlc2*:EGFP) zebrafish. Similarly to Panel A, during the time it takes for scanning along Z (2–3 s), the heart has time to beat 5 times resulting in a corrupted image. More sophisticated techniques are required to capture the actual geometry of the heart tube [2]. Grid spacing is 20 μm.

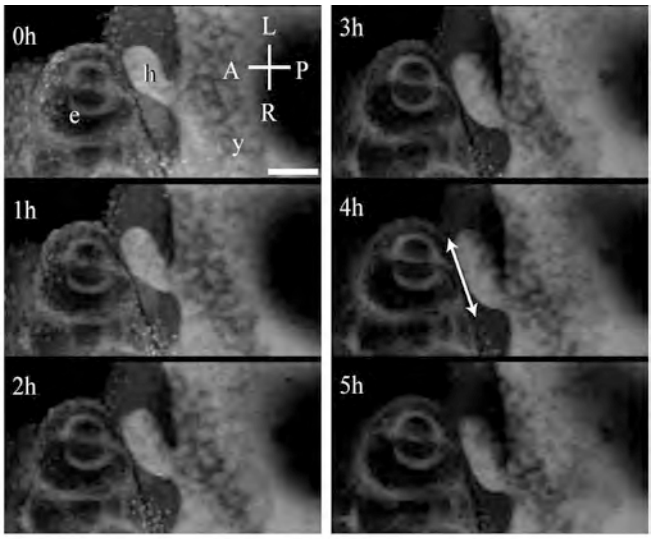



**Figure 2.** 5-hour time-lapse of the developing zebrafish heart (between 28 and 33 hpf), at a fixed point in the heartbeat cycle, after reconstruction with the method described in this paper. The double headed arrows indicates the elongation of the atrium along the left-right axis. Ventral view, maximum intensity projection. e: eye, h: heart, y: yolk sack, L: left, R: right, A: anterior, P: posterior. Scale bar: 100 μm.

**282. Development of two-photon fluorescence imaging technology (Part 1)**

*Thai Truong, Willy Supatto, Amy McMahon, Scott E. Fraser*

(1) Implementation of optical pulse-splitting to reduce photobleaching and phototoxicity for 2PF imaging. We have built an optical setup that passively splits every ultrafast laser pulse into multiple pulses, effectively increasing the repetition rate of the laser. Using the multiple pulses, we have conclusively demonstrated in eGFP-labeled *Drosophila* embryos that photobleaching and phototoxicity were reduced by up to 70-80% at the same fluorescence signal level. Alternatively, the signal could be increased by up to 100% at the same bleaching and toxicity levels. Current efforts are in progress to apply this finding to other biological systems such as zebrafish and mouse embryos.

**283. Development of two-photon fluorescence imaging technology (Part 2)**

*Thai Truong, Scott E. Fraser*

(2) Construction of multi-photon microscope. Given the stringent requirements for Center of Excellence in Genomic Science (CEGS) imaging, and after our characterizations of the various existing microscope platforms, we have concluded that currently no commercially available systems could meet the

requirements for CEGS imaging. We have thus, decided to build our own microscope – this is the best way for optimizing the system specifically, for CEGS' needs, while maintaining the flexibility for future improvements and upgrades as the project evolves. We have identified existing hardware and software platforms, mainly from a robust community of microscopists/neuroscientists, on which we can build our system. We are in the final stages of designing and purchasing parts. Installation of the system should begin in a month, and the system should come on line within a few months.

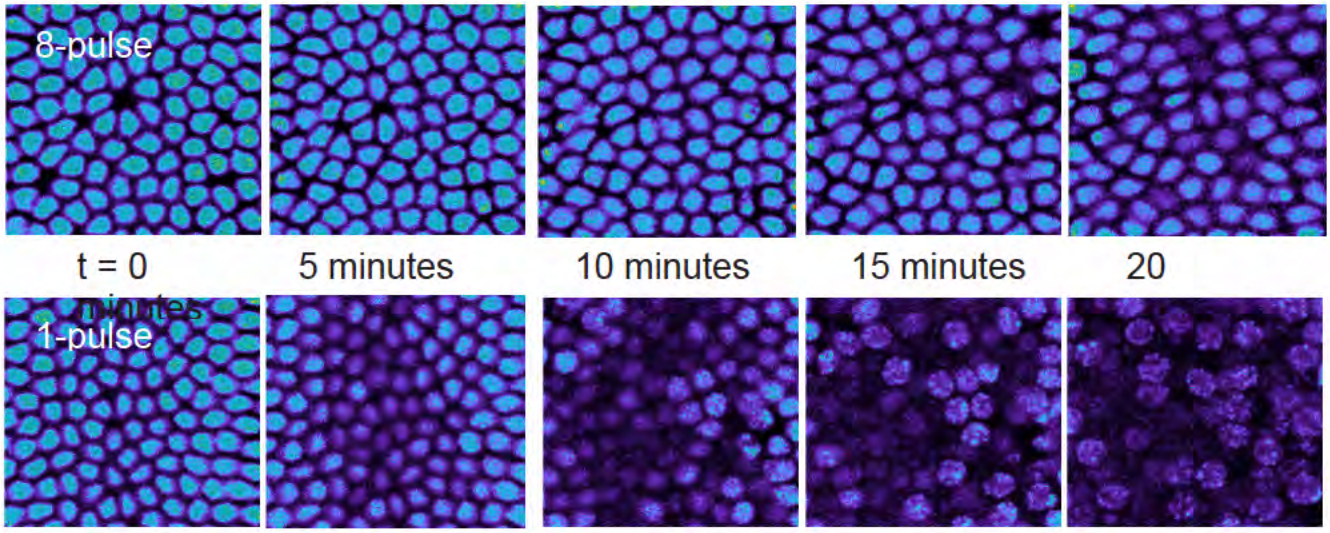



**Figure 1**. Multiple pulses reduce photobleaching and phototoxicity. Two-photon fluorescence images of cell nuclei, collected from live H2B-eGFP *Drosophila* embryos, were taken continuously for time t, using 8 pulses or 1-pulse (which translates to 640 MHz and 80 MHz repetition rate, respectively). Photobleaching is seen as the fading of the signal, and phototoxicity is seen in the disruption of the shape and spatial location of the cell nuclei. The initial signals were approximately the same, but after prolonged illumination, the 8-pulse case yielded much less photobleaching and photoxicity.

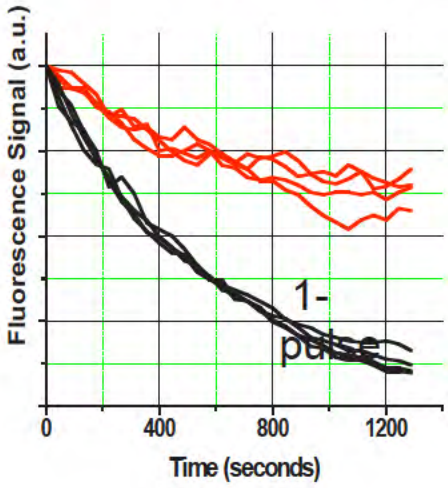



**Figure 2**. Reduced photobleaching by using multiple pulses. Fluorescence curves correspond to signals integrated over different cell nuclei, taken from images similar to those in Fig. 1. Higher photobleaching causes fluorescence to decrease faster as illumination time increases. The signals at time = 0 for different curves were the same within 10%, and had been normalized to one.

**284. Quantitative imaging of the FcRn-dependent transcytotic pathway using high-resolution fluorescence confocal microscopy**

*Galina V. Jerdeva, Devin B. Tesar, Scott E. Fraser, Pamela J. Bjorkman*[*]

Specific delivery of proteins across polarized epithelia is controlled by receptor-mediated transcytosis. The pathways of a model receptor, the polymeric immunoglobulin receptor (pIgR), mediating transport of dimeric and polymeric IgA(dIgA/pIgA) in the basolateral to apical direction are relatively well understood. The neonatal Fc receptor (FcRn) transports maternal immunoglobulin G (IgG) across intestinal or placental epithelial barriers in the apical to basolateral direction to provide immunity to fetal or newborn mammals and serves as a protection receptor for IgG. To investigate FcRn-mediated transport of IgG and to compare it to pIgR-mediated transport of dIgA/pIgA we are using Madin-Darby Canine Kidney (MDCK) cells expressing either one or both receptors FcRn (MDCK-FcRn, MDCK-FcRn-pIgR). The transfected cells, specifically transcytose IgG or Fc and dIgA across polarized cell monolayers when applied apically and basolaterally, respectively. A spinning disk Confocal Imaging System (UltraVIEW ERS) with a sensitive EMCCD Camera allows acquisition of high-resolution 3D imaging volumes of cells after internalization of fluorescently-labeled ligands co-stained with endogenous intracellular markers

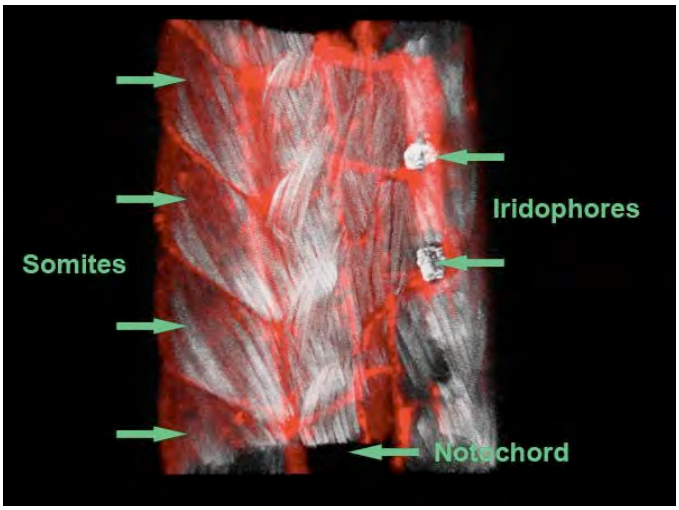
with minimal bleaching of fluorescent probes. We hope to identify at what stages of endocytosis/transcytosis the trafficking routes of Fc and pIgA intermix, and to learn the identities of endosomal compartments involved in receptor trafficking. Quantitative colocalization analysis of 3D volumes by Coloc Module of Imaris software (Bitplane) revealed significant colocalization of apically internalized Fc and basolaterally internalized pIgA after 20 min of chase but not after 5 min as revealed by calculations of the amount of colocalizations between two channels (13.95% + 0.99% at 20 min chase; 1.13% + 0.3% at 5 min chase) and Pearson's correlation coefficients (P) (p=0.38 + 0.01 at 20 min chase; p=0.05 + 0.007 at 5 min chase). A significant subpopulation of ligand-positive endosomes at the 20 min chase were also positive for the early endosomal marker EEA1 (10.9% + 1.30% for Fc; 9.66% + 1.67 for pIgA). P values for Fc /EEA1 and pIgA/EEA1 in regions of interest (ROI) containing endosomes were 0.33 + 0.2 and 0.30 + 0.03 respectively, indicating significant spatial overlap in internalized ligands and the marker. Studies of colocalization with other intracellular markers such as rab proteins will clarify the identity of common and unique endosomal compartments.

[*]*Professor, Division of Biology, Caltech*

**285. Second harmonic generation (SHG) imaging microscopy**

*Periklis Pantazis, William Dempsey, Robert Kaspar, Scott E. Fraser*

Second harmonic generation (SHG) imaging microscopy is an emerging microscopic technique for a wide range of biological and medical imaging. SHG is a second-order nonlinear optical process in which two photons at the frequency w interacting with non-centrosymmetrical media (i.e., material lacking a generalized mirror symmetry) are combined to form a new photon with twice the energy, and therefore twice the frequency (2w) and half the wavelength of the initial photons. In zebrafish, a vertebrate model system, the tissues that produce SHG signal and the conditions required for imaging have not been established. We determined which zebrafish tissues produce SHG, at which developmental stages, and at which wavelengths. The generated optimum wavelength map for various organs at early embryonic stages offers us the possibility to perform long-term *in vivo* imaging without the use of fluorescent markers. This way, mutations affecting developmental processes, as well as function of SHG zebrafish tissue can be analyzed.



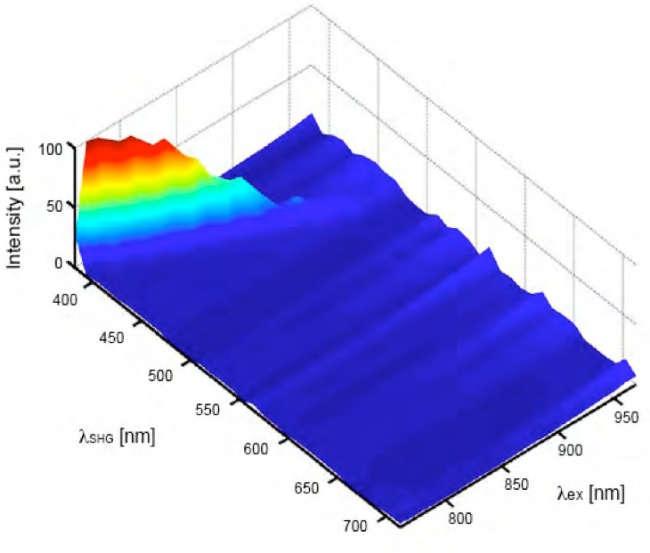

SHG signal of a representative 4dpf zebrafish tail. Signal shown in white is endogenous SHG, while red signal is the BODIPY counterstain. The two circular white clusters indicated on the right are iridophores, and the segments outlined in red along the left side of the tail are distinct somites. Within the somites, repeated white strands form fast and slow muscle patterns.

**286. Second harmonic generation (SHG) nanoprobes for *in vivo* imaging**

*Periklis Pantazis, Ye Pu, James Maloney, Demetri Psaltis*[*]*, Scott E. Fraser*

Fluorescence microscopy has profoundly changed how cell and molecular biology is studied in almost every aspect. However, the ultimate need of characterizing biological targets is largely unmet due to fundamental deficiencies associated with the use of fluorescent agents. Dye bleaching, dye signal saturation and tissue autofluorescence can severely limit the signal-to-noise ratio (SNR). Here, we demonstrate that second harmonic generating (SHG) nanoprobes are suitable for *in vivo* imaging and eliminate most of the inherent drawbacks encountered in classical fluorescence systems. These nonlinear nanocrystals of less than 100 nm are capable of generating second harmonic signals because they do not posses an inversion symmetry and can be detected by conventional two-photon microscopy. Unlike commonly used fluorescent probes, SHG nanoprobes neither bleach nor blink, and the signal does not saturate with increasing illumination intensity. SHG nanoprobes provide a superb SNR in live imaging of zebrafish embryos, and unlike endogenous second harmonic generation their signal can be readily detected both in *trans*- and in epi-directions**.**

[*]*EPFL, Switzerland*




Displayed is the 3D normalized SHG signal data profiles of SHG nanocrystals (signal ranging from 380 to 710 nm) generated by conventional two-photon excitation (excitation ranging from 760 to 970 nm). Note that the SHG signal peaks are always half the wavelength of the incident excitation wavelength. Color code: dark blue corresponds to zero intensity and dark red corresponds to maximal intensity.

**287. Field Resonance Enhanced Second Harmonic (FRESH) signaling biosensors**

*Periklis Pantazis, Nathan Hodas, Ye Pu, Demetri Psaltis[*], Scott E. Fraser*

Signaling regulates embryonic development by providing positional information to cells so that they differentiate properly as they proliferate to build up the final shape. However, defects leading to abnormal activation of signal pathways often underlie most tumorigenic events. In recent years, much has been discovered about the molecular and biochemical characteristics of a variety of signal transduction pathways. In contrast, the cell biology of such signaling events is starting now to be studied. In particular, we are interested in the following questions: 1) When does signaling occur? 2) Where does it take place? and, 3) for how long? We aim to address these questions by developing new biosensors based on field resonance-enhanced second harmonic (FRESH) for following *in vivo* signaling events with large sensitivity increase and high spatial and temporal resolution. We plan to use such biosensors to monitor signal transduction through visualization of conformational changes and protein-protein interaction of components of signaling pathways that will enable us to follow various signaling events in real time. Ultimately, the established biosensors will be used to investigate signaling during zebrafish development and in the manifestation of cancer.

[*]*EPFL, Switzerland*

**288. Examining nano-scale Second Harmonic Generation (SHG)**

*Nathan Hodas, Periklis Pantazis, Rudy Marcus, Scott E. Fraser*

We are opening the door into a new modality of studying molecules. Instead of attaching fluorescent dyes to molecules of interest, we are developing a tag that is observable by shining a laser of one frequency onto it, producing light with a doubled frequency, a process called second harmonic generation (SHG). However, this approach is so new that the fundamental properties of these SHG nano-probes are not well understood. Some of the central questions we have identified so far involve the interactions of the SHG nanoparticles with their environment. We are working with Optiwave Systems, Inc., to develop a 3D FDTD software package capable of simulating SHG in 3D. Using this software, we are creating a sound foundation for understanding some of the macroscopic properties of the nanoparticles. We are designing a microfluidic system for systematically screening the interaction of SHG nanoprobes with FRESH targets. In this way, we can quickly and efficiently establish some of the key properties of FRESH. Although the theory of SHG is well documented, nanometer sized particles have properties that force us to reexamine our assumptions that led to the common derivation. Taking our experimental results as a guide, we are developing a concise model of SHG on the nanometer scale. A clear, simple theory of SHG and FRESH will guide further developments, and we will very likely uncover new and unexpected science. We very much look forward to the scientific program lying ahead.

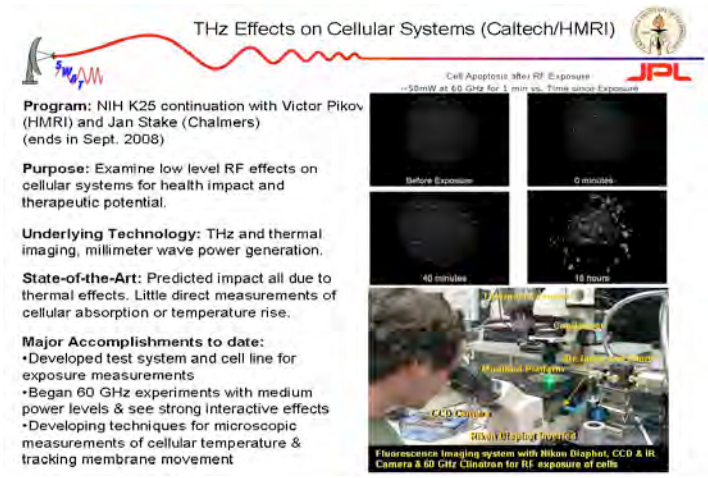
**289. Terahertz effects on cellular systems**

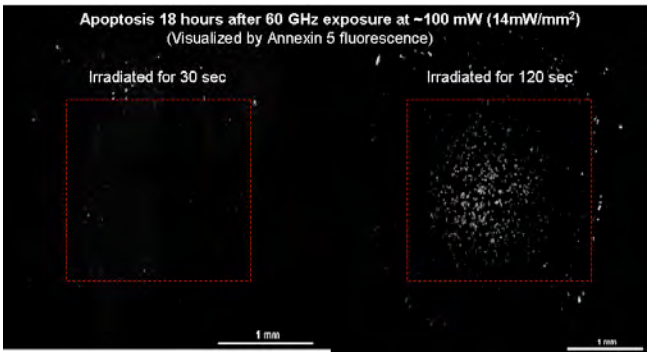
*Peter H. Siegel, Victor Pikov[1], Jan Stake[2]*

This newly started exploratory program attempts to quantitatively examine terahertz (and millimeter wave) radiation impact on cells and cellular processes. It blends biological, optical and RF instrumentation in a novel way to examine RF dosimetry effects while directly monitoring cell lines. It establishes one of the first IR Raman/optical/RF test platforms for microscopic evaluation of thermal and chemical processes at the cellular level. A proposal to continue the work is currently in preparation with collaborator Dr. Victor Pikov, a neurophysiologist at the Huntington Medical Research Institute and electrical engineering Professor Jan Stake, of Chalmers University, Sweden.

[1]*Huntington Medical Research Institute, Pasadena, CA, USA*

[2]*Chalmers University, Göteborg, Sweden*



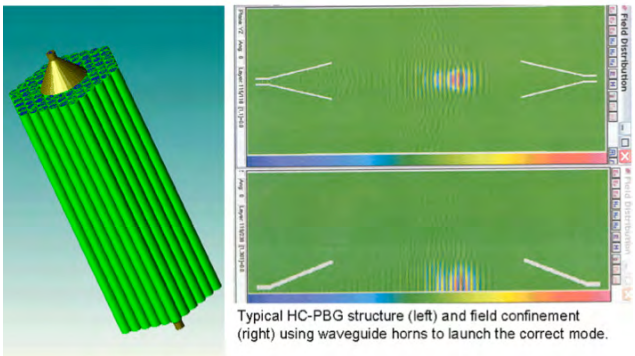




## 290. THz low loss flexible waveguide

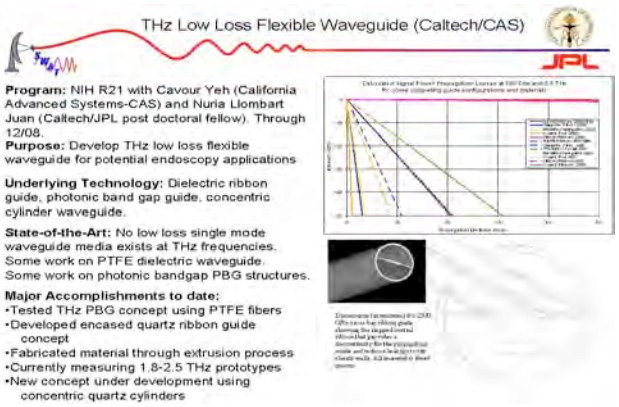
*Peter H. Siegel, Nuria Juan, Cavour Yeh* [*]

This program is a collaboration between Caltech and California Advanced Systems (Dr. Cavour Yeh and Dr. Fred Shimabukura) to develop the first low loss single mode waveguide for THz applications. The work involves analysis, fabrication and testing of several types of new waveguide media specifically tailored for the submillimeter bands: ribbon guide, photonic-band gap waveguide and a new concentric cylinder guide, all based on quartz fiber technology. Applications include simple flexible piping of energy between radio frequency components, inside instruments such as spectrometers and transceivers as well as possible use in endoscopy to perform THz imagery *in vivo*. Dr. Nuria Llombart Juan, a Caltech post-doctoral fellow and a JPL SWAT team member, is collaborating on the task.

[*]*California Advanced Systems, Santa Monica, CA, USA*




Typical HC-PBG structure (left) and field confinement (right) using waveguide horns to launch the correct mode.

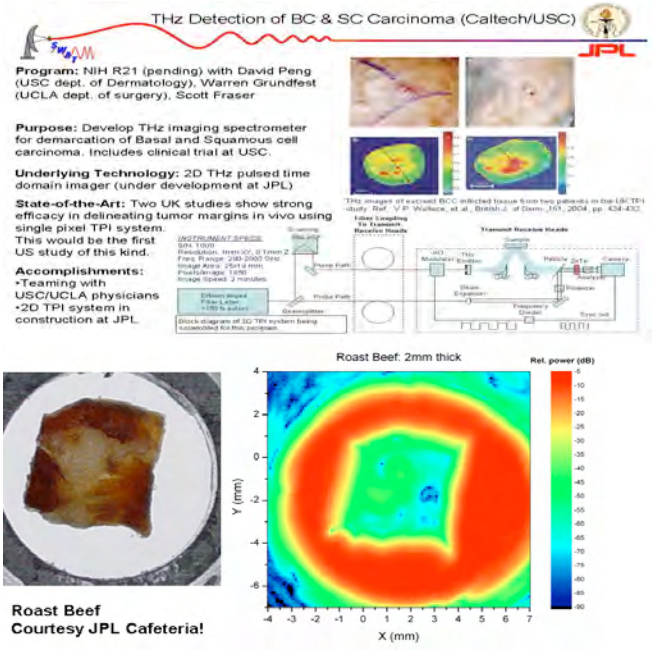



## 291. THz detection of basal and squamous cell carcinoma

*Peter H. Siegel, David Peng*[1]*, Warren Grundfest*[2]

THz imaging has already been proven to be effective in delineating tumor margins on areas of the body that are near the surface, i.e., skin or surgically exposed regions. Our efforts involve establishing real time video imaging instruments in the THz bands (using pulsed time domain techniques) and then applying these in an actual clinical environment – University of Southern California Medical Center – to establish efficacy for diagnosing, and perhaps someday thermally treating, skin lesions, specifically basal and squamous cell carcinoma. This work has been proposed through NIH but has not yet received funding. The work has been ongoing at a low level in the hopes of acquiring sufficient data to bolster future proposal efforts. Collaborators include Professor Warren Grundfest, a surgeon and bioengineer at UCLA, Professor Scott E. Fraser at Caltech and Dr. David Peng, a clinical dermatologist at USC.

[1]*University of Southern California, Viterbi School of Medicine, Los Angeles, CA, USA*
[2]*Department of Surgery, UCLA, Los Angeles, CA, USA*




Roast Beef
Courtesy JPL Cafeteria!

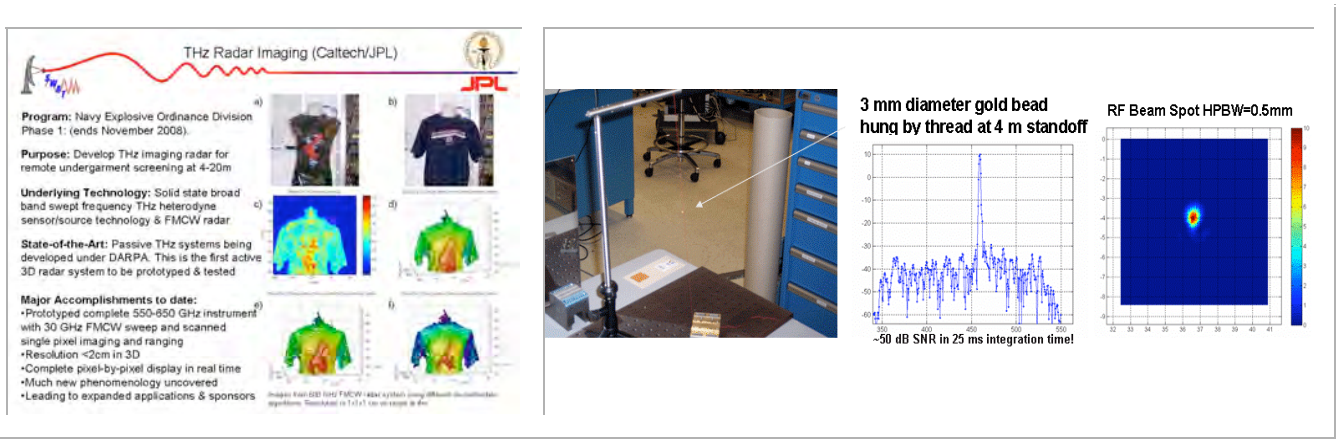
**292. THz radar imaging**

*Peter H. Siegel, Tomas Bryllert, Nuria Juan, Ken B. Cooper*[*]*, Imran Mehdi*[*]*, Goutam Chattopadhyay*[*]*, Erich Schlecht*[*]*, John Gill*[*]*, Choonsup Lee*[*]*, Anders Skalare*[*]*, Robert Dengler*[*]

Using terahertz sensor and source technology we developed at JPL for NASA Earth, planetary and astrophysics applications, we have started the first work on THz frequency modulated continuous wave (FMCW) radar. In this very exciting program we have designed, fabricated and begun testing a 600 GHz FMCW radar imager that can mechanically scan and 3D reconstruct (using radar ranging) objects between 1 and 25 meters distance with cm resolution in all three dimensions. Since THz waves can pass through many dielectrics, the system is being applied to undergarment threat detection.

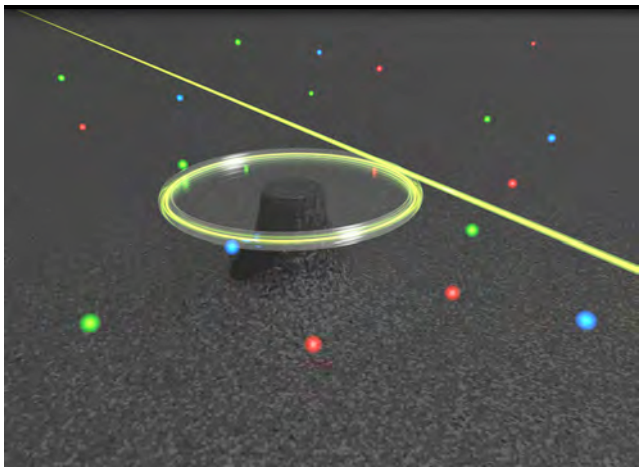
However significant phenomenologic breakthroughs have already been established through the use of this long established technique in this very new frequency range. The ultimate goal of the program is to demonstrate near video rate imaging over a modest angular scene scale. This work is being supported by the DoD, although, applications include medical screening. Most of the instrumentation and staff reside at JPL. Program participants include a number of JPL SWAT team members, particularly Dr. Ken Cooper, Dr. Goutam Chattopadhyay and Mr. Robert Dengler. Other contributors are Caltech postdoctoral fellows Dr. Nuria Llombart Juan and Dr. Tomas Bryllert, as well as JPL's Dr. Imran Mehdi, Dr. Choonsup Lee, Dr. Anders Skalare and Dr. Erich Schlecht.

[*]*Submillimeter Wave Advanced Technology (SWAT) team, JPL, Pasadena, CA, USA*




**293. Label-free detection of cytokines using optical microcavities**

*Andrea M. Armani, Rajan P. Kulkarni, Richard C. Flagan, Scott E. Fraser*



Optical microcavities, whether fabricated from polymeric materials or silica, have applications in quantum optics, sensing, and telecommunications. The benefit of using resonant microcavities revolves around the photon lifetime or photon storage within the cavity, characterized by the quality factor of the cavity.

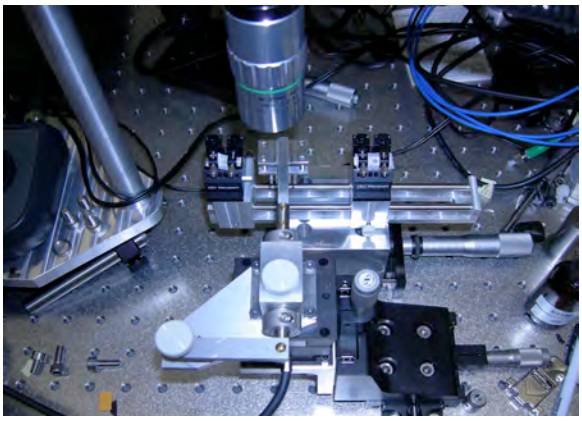
In recent work, ultra-high-Q microresonators (Q>100 million) have demonstrated sensitive and specific chemical and biological detection. The sensitivity is a result of the long photon lifetime inside the cavity, and specificity is achieved through surface functionalization. In an optical cavity with a Q of 100 million, a single photon interacts with a bound molecule over 100,000 times. This increased interaction, when compared to other optical methods such as optical waveguides, results in the cavity's ability to perform label-free, single molecule detection experiments.

Using microtoroid cavities with an antibody surface functionalization, label-free, single molecule detection of Interleukin-2 (IL-2) in fetal bovine serum (FBS) was demonstrated. IL-2 is a cytokine released in response to immune system activation. This detection was the first demonstration of label-free, single molecule detection, and current research is investigating integration of the device with additional components.

**294. Ultra-high-Q microtoroid resonators for single cell proteomics**

*Judith Su, Scott E. Fraser*

A single cell assay is needed to fundamentally understand protein function and expression; however, current detectors cannot sense the small amount of protein present in one cell that is typically on the order of zeptomoles. Recently, microtoroid resonators have been shown capable of achieving label-free single molecule detection. We propose a microfluidic toroid-based assay as a new and simple (fewer processing steps) means to achieve single cell proteomics. We have in the Fraser lab replicated the entire suite of equipments needed for the toroid project (Figure 1). Currently we are working on integrating the toroid into microfluidic channels for high throughput experiments and validating the binding data taken with the toroid by direct comparison with industry standard instrumentation, Biacore Surface Plasmon Resonance (in collaboration with J. Vielmetter, A. West, and P. Bjorkman). In addition, we have developed a new way to sense the binding of molecules to the sensor that is far more robust than has been previously reported (in collaboration with T. Lu and K.J. Vahala).



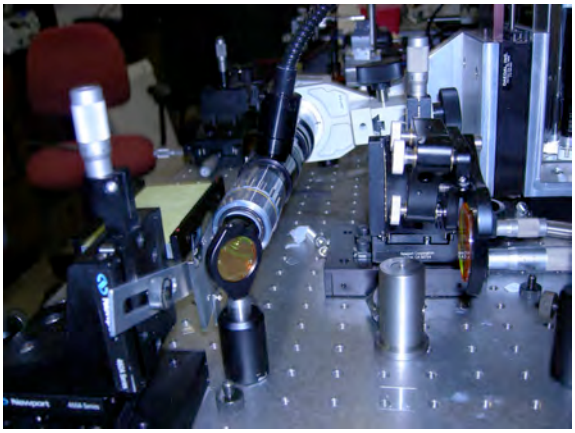


Figure 1: Toroid testing facilities, Fraser Lab (left). Testing set-up (right). Reflow system to create toroids.

**295. Self-assembled monolayers for the study of biological targets**

*Christie A. Canaria, Scott E. Fraser, Rusty Lansford*

Understanding the interactions of biological molecules with solid supports is vital for the development of detection systems and assay platforms. These relationships are frequently quite complex, involving hydrophobic interactions, electrostatic interactions, van der Waals forces, and covalent chemical bonds. We can exploit these interactions in a solid support device by modifying the surface substrate with thin films and monolayers. Self-assembled monolayers (SAMs) are powerful tools for functionalizing and imparting chemical character to surfaces. In my work, alkylthiol reagents are utilized to build SAMs on gold (Au) substrates. My work characterizes and studies monolayer formation, as well as utilizes SAMs to generate surfaces specific for binding proteins, DNA, and cells. The popular biotin-streptavidin motif is used to demonstrate protein binding, as well as characterize monolayer composition as a result of solvent effects. Using biotinylated alkylthiols and triethylene-glycol alkylthiols together on Au generates substrates that bind specific proteins while repelling non-specific ones. We designed and synthesized an additional reagent "DMT-coated controlled porous glass (CPG)" for the generation of custom sequence oligonucleotides. Phosphoramidite syntheses using this modified CPG yield oligos with a 3' alkylthiol modification. SAMs generated with this reagent demonstrate specific-binding of complement strands. Both electrochemical techniques and restriction enzymes (where appropriate) provide methods for releasing monolayer-bound species. Lastly, we employ SAMs to generate substrates amenabl to cell capture and cell adhesion. Binding B- and T-cell lymphocytes is achieved, demonstrating SAM-coated Au as a substrate for cell panning. Chemokine vascular endothelial growth factor (VEGF) is also bound to SAMs, generating surfaces amenable to cell adhesion and motility. Cells plated on higher surface concentrations of VEGF migrate with longer cell displacements, and show the effect is specific to cells with VEGF receptors. Overall, my work explores the formation and utilization of SAMs for capturing and studying biological targets. The findings here may be transferred in the future into bio-sensing devices and arrays.

**296. Dynamic analysis of embryogenesis**

*Rusty Lansford, Scott E. Fraser*

Our research aims to dynamically visualize and characterize embryonic development and stem cell development at sub-cellular resolution. Our primary focus in living animals is determining how the brain and heart forms and develops. We have developed transgenic, fluorescent protein (FP) expressing Coturnix quail as an experimental system. Coturnix quail offer advantages in the small size of its egg, the moderate size of the breeding adults, and its short generation time.

We continue to develop techniques for

performing *in toto* imaging that allow us to systematically image vertebrate embryos throughout their embryonic development at single-cell resolution using *in vivo* time-lapse, multispectral laser microscopy. We are also developing advanced software to recognize and track cells in 4-dimensional, xyzt image sets. Our goal is to image and track all cell movements and divisions during embryogenesis and to digitize this data quantitatively at the level of the cell.

**a. Computational biology of neural cell behavior**

*David Huss, Greg Poynter*

To increase our knowledge of how neural cells and their precursors are patterned in space and time, we are using dynamic computational imaging, *in vivo*, in conjunction with a new array of elegant molecular tools tailored for use in wild-type and transgenic quail embryos. In particular, we are using the synapsin-H2B.GFP and synapsin-GFP transgenic quail to dynamically study forebrain and midbrain formation along with neural crest cell migration and differentiation.

**b. Computational biology of vascular cell behavior**

*Yuki Sato, Jen Yang, David Huss, Greg Poynter*

To increase our understanding of how endothelial cells and their precursors are precisely patterned in space and time, we are using dynamic computational imaging, *in vivo*, in conjunction with a new array of elegant molecular tools tailored for use in wild-type and transgenic quail embryos. In particular, we are using the TIE1-H2B.YFP transgenic quail to study vasculogenesis, angiogenesis, and cardiogenesis and synapsin-H2B.GFP/TIE1-H2B.YFP and synapsin-GFP/TIE1-H2B.YFP double transgenic quail to study neurovascular interactions during development.

**297. 4D imaging of blood vessel formation in transgenic quail embryo**

*Yuki Sato, David Huss, Greg Poynter, Scott E. Fraser, Rusty Lansford*

The circulatory system overcomes the limits of diffusion as a viable system for internal transport. Its size, complexity, and metabolism correlate with those of the animal. The heart is the first functioning organ in the vertebrate embryo and drives convective blood flow for the exchange of gases, nutrients, and wastes. Blood vessels are also thought to play an important role in formation of the nervous system and visceral organs during development. Although a number of molecules required for blood vessel formation have been identified mainly by gene inactivation studies in mice, a detailed understanding of normal blood vessel formation and direct effects of the gene defects have not been well performed from a view of cell behavior, especially in higher vertebrates, due to the difficulty of imaging the living embryos. To take advantage of the easy accessibility to the developing embryos, we generated transgenic quail carrying endothelial cell-specific fluorescent protein gene, Tie1.H2B-EYFP, as a versatile model animal of amniote to study blood vessel formation. It enables us to image the nucleus of every blood vessel endothelial cell in a living embryo by EYFP signals under the fluorescent microscope.

Dorsal aortas, which are primary blood vessels in the trunk, are formed as two separated tubes in lateral areas at an early-stage (Figure 1). They change relative position from lateral to axial, getting close to each other during early development, eventually fusing into a single tube in the ventral midline at later-stages. Because these processes involve various types of dynamic morphogenetic events – cell migration/sprouting, mesenchymal-to epithelial transition, and rearrangement of luminal structure – the dorsal aorta is a good model to study cell behaviors underlying blood vessel formation. We have achieved live imaging of the developing Tie1.H2B-EYFP transgenic embryos by *ex ovo* culture method under the confocal laser microscope. It allows us to track individual endothelial cell behavior and determine gross morphometric changes during the dorsal aorta formation in a spatiotemporally continuous manner. In order to understand molecular mechanisms regulating endothelial cell behaviors, we are trying experimental loss of- and gain-of-function studies of specific genes in the transgenic quail embryo and combining it with DNA electroporation technique.

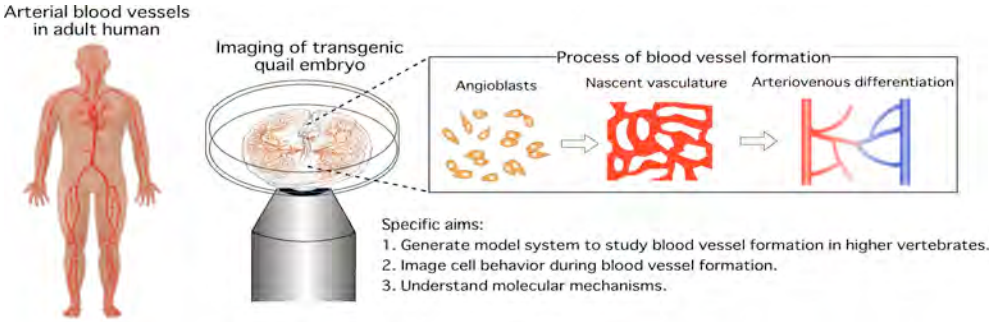



Figure 1. Transgenic quail as a model animal to study blood vessel formation in vivo.

**298. Characterizing cardiogenesis through dynamic fluorescent imaging in the developing quail embryo**

*Jennifer Yang, Scott E. Fraser, Rusty Lansford*

Recent technological advances in dynamic confocal microscopy and four-dimensional reconstruction have shown that the embryonic heart tube in the developing zebrafish functions more as a dynamic suction pump than a peristaltic pump, disputing a theory cardiac physiologists had long held. Along with technological advances, recent research has shown the impact of flow in heart morphogenesis and valve development. As scientific methods and tools improve, previous conclusions of cardiogenesis need to be revised to incorporate new data at the molecular level.

The Japanese quail is an ideal system to study the pump mechanics of the four-chambered heart because it is a warm-blooded vertebrate with easily accessible embryos. All stages of the developing heart during looping and chamber formation are accessible *in ovo* and *ex ovo*, allowing for ease of manipulation of the embryo for dynamic imaging. Knowledge obtained from an avian four-chambered heart can then be applied to the less accessible mammalian and human hearts.

The mechanics of the developing embryonic heart are still poorly understood. Characterization of the interactions of the different layers of the heart will give us information on the contractile wave moving along the heart muscle, the offset of the contractile wave as it transverses the cardiac jelly to appear in the endocardium, and how this affects fluid flow in the heart. In order to characterize the heart, we intend to fluorescently label the cardiomyocytes and endothelial cells lining the heart, and to inject fluorescent beads into the bloodstream to track the flow of blood. By dynamically imaging the endocardium, myocardium, and blood cells, we will be able to study the interplay between hemodynamics and genetics by offering unsurpassed visual access to the morphogenetic events of cardiogenesis and angiogenesis. Future experiments will involve the addition of pharmaceutical drugs to determine their effects on morphology and hemodynamics in cardiogenesis and heart valve development.

**References**

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Hove, J.R., *et al.* (2003) *Nature* **421**(6919):172-177.
Liebling, M., *et al.* (2006) *Dev. Dyn.* **235**(11):2940-2948.

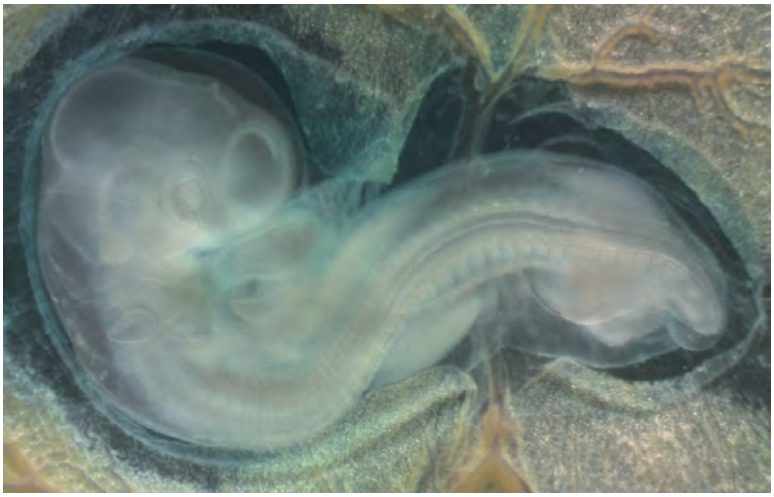


Figure 1: Day 3 quail embryo removed from the egg. The heart is located outside the body just under the developing head.

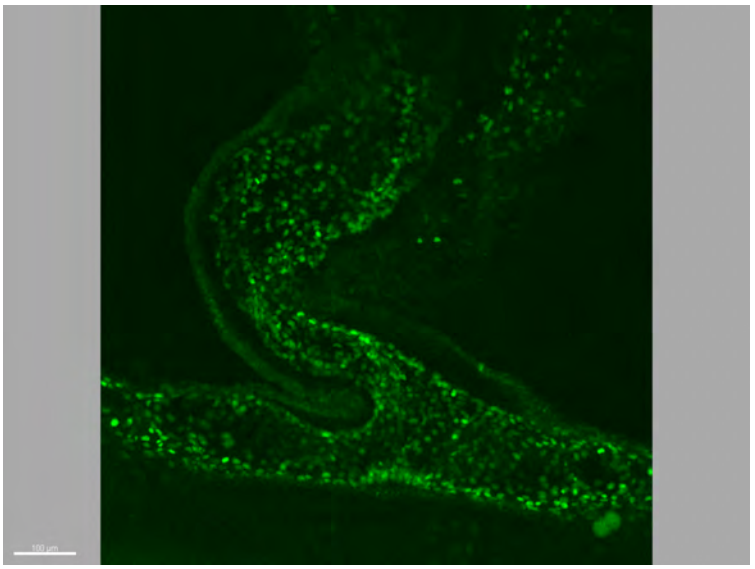
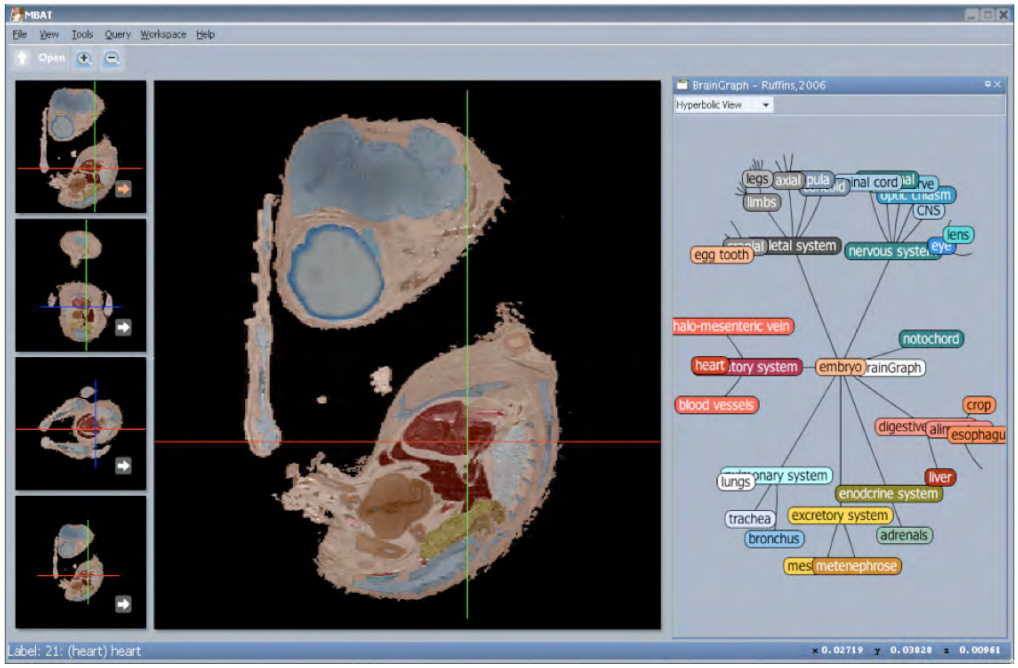


Figure 2: Transgenic quail heart (Tie1-H2B.eYFP) at HH stage 11. The heart is a single tube and has begun to loop. Individual endothelial cells are labeled with a fluorescent protein. The myocardium lining the outside of the heart can be seen by autofluorescence.

**299. Quail developmental atlas**

*Seth W. Ruffins, Melanie Martin, Lindsey Keough, Salina Truong, Scott E. Fraser, Russell E. Jacobs, Rusty Lansford*

Development is a complex process involving a vast number of events occurring over temporal scales from seconds to days, and spatial scales from nanometer to millimeters. The embryonic form provides an intuitive framework for integrating the vast quantities of data generated in the quest to understand development. In the effort to integrate developmental events, we are constructing a series of 3-D embryonic atlases of the Japanese quail (*Coturnix japonica*) based on microscopic Magnetic Resonance Imaging (μMRI). The atlas series contains six *ex ovo* embryos ranging in age from 5 to 10 days of incubation and three *in ovo* embryos at 10, 11 and 13 days of incubation. Currently, the atlases are strictly anatomical but provide a framework for spatially mapping developmental events such as gene expression patterns, the distribution of extra-cellular matrix, fate maps, cell migration patterns, etc. I will discuss the construction of these atlases, how they are being linked to other data sources and how they can be used in education and research.




**300. Spatial regulation of NF-κB signaling in early *Drosophila* embryogenesis**

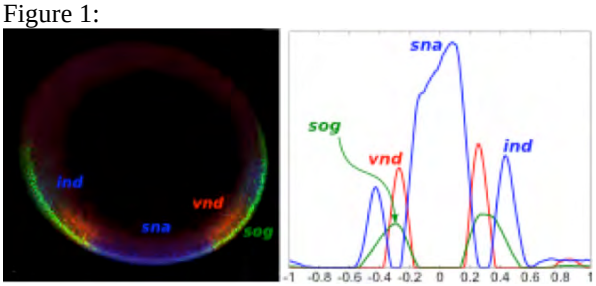
*Marcos Nahmad*[*]*, Greg Reeves*[*]*, Scott E. Fraser, Angela Stathopoulos*

The ability of developing systems to regenerate and preserve proportions with respect to size has fascinated several generations of embryologists and naturalists. Despite the increasing knowledge on morphogenetic pattern formation, the molecular relationships between gradients and size remain elusive. We ask how patterns accommodate natural or artificial variation in the size of a developmental field. We investigate the patterning of the embryonic dorsoventral (DV) axis with respect to feedback mechanisms that ensure its proper scaling despite large variations in the size of the DV domain. The *Drosophila* NF-κB homologue, Dorsal (dl), is the only known maternal source of DV information in the embryo. As such, the ability to generate a well-proportioned insect initially depends on whether or not patterning by dl – and factors downstream of it – scales with respect to the size of the DV axis.

We quantify gene expression data of known dl target genes – such as *sna*, *sog*, *vnd*, and *ind* (see Fig. 1) - in both wild-type and mutant genetic backgrounds. We have presently seen appreciable scaling with respect to natural variations in the size of the embryo for all genes investigated. We also employ a system in which the dl gradient is altered to run along the anteroposterior (AP) axis (Fig. 2). Since the AP axis is 2-fold larger than the DV axis, we use this artificial system to study mechanisms of size-dependent scaling. Remarkably, we see scaling in this artificial system, as well. For example, the width of *vnd* gene expression scales proportionally with the size of the DV axis in both wild-type embryos and in the artificial system (Fig. 3). Previous models have suggested opposing gradients may provide size-dependent positional information. If such an opposing gradient exists in this system, it should depend – at least initially – on dl itself. We are using molecular genetics and mathematical modeling to ask whether it is possible to establish size-dependent positional information in a system with a single independent gradient.

[*]*These authors contributed equally to this work.*

Figure 1:

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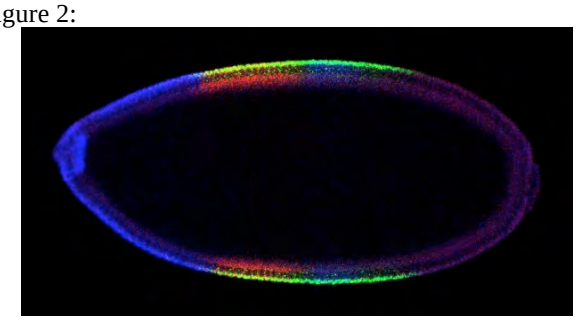
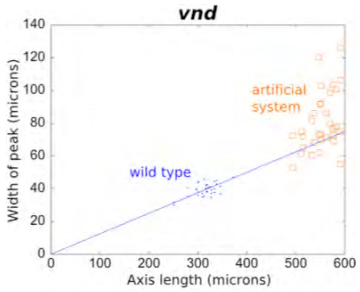


Figure 3:




**301. A dynamic fate map of the neural crest and dorsal neural tube in the chicken gastrula**

*Max Ezin, Scott E. Fraser, Marianne Bronner-Fraser*

In contrast to the classical assumption that neural crest cells in chick are induced as the neural folds elevate, recent data suggest that they are already specified during gastrulation. This prompted us to map the origin of the neural crest in the early avian embryo. Using focal dye injection together with time-lapse imaging, we find that neural crest precursors are present in a broad, crescent shaped arc region of the gastrula, juxtaposed to future neural plate. Surprisingly, static fate maps together with dynamic confocal imaging reveal that the neural plate border is considerably broader and positioned more caudally than expected. Some degree of rostrocaudal patterning, albeit incomplete, is already evident in the gastrula. Time-lapse imaging showed that the neural crest precursors undergo shearing, convergence and extension movements, with a spatiotemporal progression from lateral to medial and from anterior to posterior. It is through those rearrangement and reorganization movements that, gradually, the neural crest precursors become regionally segregated, coming to occupy predictable rostrocaudal positions along the axis. This regionalization occurs progressively and appears to be fixed in the neurula by stage 7 at levels rostral to Hensen's node. Interestingly, we find that the position of the presumptive neural crest broadly correlates with BMP4 and Pax7 expression domains from gastrula to neurula stages.

**302. The regenerative potential and plasticity of the post-otic neural crest in the chicken gastrula**

*Max Ezin, Huaising C. Ko, Scott E. Fraser, Marianne Bronner-Fraser*

Based on our fate map of the neural crest at gastrula stages (see above abstract), we were in a position to determine if the post-otic neural crest has regenerative potential and plasticity in gastrulating chick embryos. In neurulating chick embryos, it is known that post-otic neural crest cannot regenerate after it is ablated. In addition, at those stages, the post-otic neural crest cannot contribute to normal cranial neural crest derivatives after it is transplanted into the head. Our experimental approach to study regeneration at early, gastrula stages consists of removing the post-otic neural crest and dorsal neural tube and re-incubating the embryos until later stages. Preliminary results show that regeneration does occur as indicated by the resurgence of Pax7-positive cells in the dorsal neural tube in the post-otic levels. In order to study plasticity, the cranial neural crest in a chick host is replaced with the presumptive post-otic neural crest from a quail donor. If the post-otic neural crest is plastic at gastrula stages, we expect to see migration and neuronal differentiation of the grafted quail tissue into the cranial region of the chick host. Migration of the cells is monitored by QCPN, an antibody stain marking quail tissue, and HNK1, marking neural crest identity. Neuronal differentiation is indicated by the Tuj1 antibody against neuronal beta-tubulin. Results show that the grafted presumptive post-otic neural crest: 1) takes the fate of neural crest cells, shown by HNK1; 2) migrates and populates the head region, shown by the presence of QCPN in the cranio-facial region; 3) differentiates into neurons, indicated by Tuj1; 4) can form a morphologically normal ciliary ganglion; and 5) cannot contribute to the trigeminal ganglion. In all, during gastrula stages, the post-otic neural crest seems both regenerative and somewhat plastic, and this is in contrast to the state of post-otic neural crest at neurula stages.

**303. Imaging cell behaviors in early mammalian embryos**

*Nicolas Plachta, Shirley Pease*, Juan Silva*, Carol Readhead, Periklis Pantazis, Scott E. Fraser*

Our current knowledge about the early developmental events that pattern the mammalian embryo is based largely on analyses performed on rodent embryos fixed and sectioned at several time points. To gain insights into the dynamic processes underlying early mammalian embryogenesis, which in rodents and human occur mostly inside the uterus, we have recently established techniques for culturing mouse embryos *ex utero*, and for directly observing cell behaviors using different modalities of laser scanning microscopy.

At pre-implantation stages, early blastomeres with equal developmental potentials are sorted into the first differentiated cell lineages of the body: the inner cell mass and the trophectoderm. We are injecting RNAs encoding for fluorescent proteins targeted to specific cellular compartments into single cells of cultured embryos. Embryos are then imaged using confocal microscopy to characterize individual cell behaviors during lineage allocation. We are also testing the effects of mis-expressing transcription factors, previously shown in fixed specimens of mutant animals to be important for proper pre-implantation development, on individual cell behaviors. In addition, by expressing fluorescent proteins targeted to different cell membrane compartments, we are assessing whether there are any links between the allocation of cells to early lineages and the dynamics of distinct membrane domains.

At post-implantation stages, embryonic cells speed up their cell cycle rates and the embryo grows in size dramatically. We have established conditions allowing normal development of post-implantation embryos cultured for >24 hours and imaged using multiphoton microscopy. We are investigating how the orientation of cell divisions, cell migration and cell death, contribute to the shaping of the first primary germ layers during gastrulation stages. Together, these ongoing experiments are expected to reveal dynamic aspects of key processes underlying the patterning of mammalian embryos, and to help linking previous genetic data to defined developmental events.

[*]*Genetically Engineered Mouse Services, California Institute of Technology*

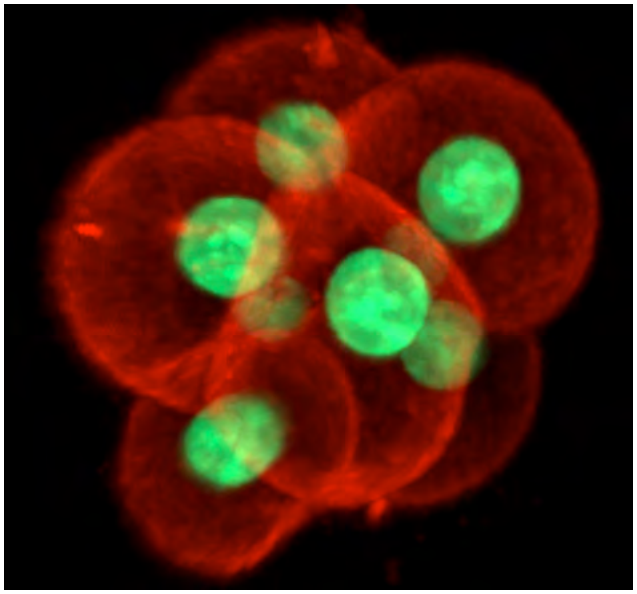


**Figure legend:** Cultured mouse embryo at the 8-cell stage (morula stage) injected with RNAs encoding for histone-tagged green fluorescent protein and for membrane-targeted red fluorescent protein.

**304. Redefining the olfactory necklace in terms of the Grueneberg Ganglion**

*David S. Koos, Scott E. Fraser*

The Grueneberg Ganglion (GG) has recently been recognized as an olfactory organ in most mammals, distinctly located at the tip of the nose where it is ideally situated to be the first olfactory subsystem to interogate inhaled chemicals. In the mouse, the GG projects to the olfactory necklace domain, an unusual sub-region of the olfactory bulb containing interconnected glomeruli that appear like beads on a string encircling the entire caudal olfactory bulb. The olfactory necklace domain also receives afferent input from another olfactory subsystem comprised of GC-D expressing neurons. A major mystery is how these two distinct populations interact and contribute to those unusual necklace glomeruli structures, and whether or not individual necklace glomeruli are innervated by both populations or are exclusively innervated by one or the other. To address this fundamental question, we are microscopically dissecting the olfactory necklace glomeruli. By combining state of the art imaging technologies with genetic labeling, inducible axon tract tracing and whole-mount immunolabeling, we generate large-scale, high-resolution, extended-volume datasets that represent the olfactory necklace axons and their cognate glomeruli. 3D reconstructions of these data allow us to explore the physical relationships between the two different classes of necklace axons, and demonstrate the amount of overlapping glomerular innervation (or lack thereof) among these populations. Our preliminary results indicate that the olfactory necklace domain contains multiple, genetically defined axon necklaces of glomeruli. There appears to be no overlapping glomerular innervation between these populations, even though these necklaces of interconnected glomeruli occupy the same region of the olfactory bulb. This suggests that the GG necklace and the GC-D necklace have their own exclusive glomeruli. This high-resolution connectivity map of the olfactory necklace glomeruli lays the foundation for functional analysis of the olfactory necklace domain's response to odorants and its relation to animal behavior.

**305. Characterizing the receptive structures of the Grueneberg Ganglion**

*David S. Koos, Cambrian Y. Liu, Scott E. Fraser*

The Grueneberg Ganglion (GG) is a mammalian olfactory subsystem located just inside of the nostrils and far forward of the other olfactory subsystems. Morphologically, the GG is unusual because, unlike the sensory neurons of the other olfactory subsystems, GG neurons do not appear to project a dendrite with receptive structures to the luminal surface of the nasal cavity. Furthermore, within the sub-epithelium of the nasal vestibule, the GG neurons are completely ensheathed by glial satellite cells. Given this unusual arrangement, it is a major mystery where the GG's receptive structures are located and the classes of ligands they can access. To address this fundamental question, we are using semi-

serial EM, optical imaging, genetic labeling and immunolabeling to characterize the mouse GG in detail. We find that the neurons of the GG are often decorated with two large distinct bundles of cilia. Each ciliary bundle is composed of several individual aligned cilia. The cilia originate from basal bodies located deep within the GG soma and extend to the cell surface where they follow the surface contour of the soma and appear to remain underneath the ensheathing glia. Although their arrangement is unusual, the ciliary bundles on the GG neurons are suggestive of receptive structures. Providing further support for this proposal, we find that specific particulate guanylyl cyclase receptors are preferentially localized to the ciliary bundles. These findings provide strong support that the ciliary bundles located on the GG soma represent receptive structures. We propose that the unique sub-epithelial location and glial encasement of the GG and its receptive structures may serve as a selective filter that controls the type of chemicals that can access and be detected by this atypical olfactory subsystem.

**306. The Grueneberg Ganglion employs a cGMP-signaling pathway**

*Cambrian Y. Liu, Scott E. Fraser, David S. Koos*

Mammalian olfactory subsystems employ different signal transduction pathways and associated receptor expression repertoires to detect different classes of compounds. We characterized the signaling pathway in the adult Grueneberg Ganglion (GG), a newly-appreciated mammalian olfactory nerve of unknown function located bilaterally at the rostral tip of the nose just inside of the nostrils. The GG expresses the cGMP-stimulated phosphodiesterase 2a and two distinctive particulate guanylyl cyclases, type A (pGC-A) and type G (pGC-G). The GG does not express pGC-D. These results demonstrate that the GG employs a cGMP signal transduction pathway and can be grouped along with the GC-D olfactory subsystem as unusual, cGMP-signaling olfactory subsystems. A differing receptor repertoire suggests that the GG is activated by different ligands than the GC-D subsystem.

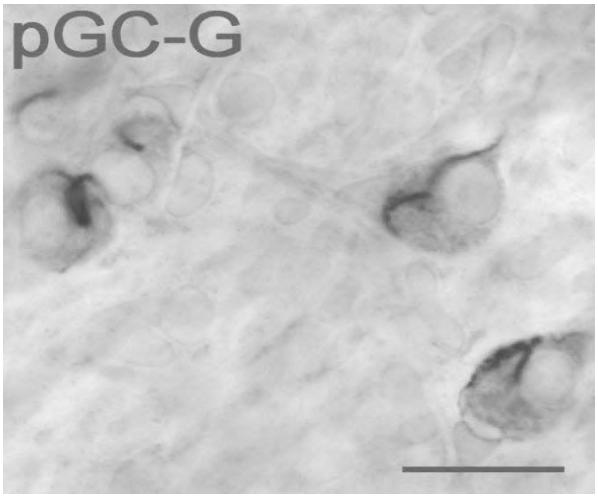



Figure 1: Whole-mount immunostaining with pGC-G antibody shows that GG neurons (light gray cells) express pGC-G (black) on chemoreceptive subcellular domains. Scale bar: 25 μm.

**307. Imaging the organizational structure and composition of neuronal receptors**

*L.A. Wade, D. Lo, H.A. Lester, Scott E. Fraser*

Nicotinic acetylcholine receptors (nAChRs) are found in many central nervous system and nerve-skeletal muscle postsynaptic membranes. The nAChR is a (pseudo)symmetric pentameric structure comprised of homologous subunits. This straightforward structure is complicated by there being five major groups of subunits that can be arranged in a variety of permutations: α, β, γ, δ, and ε. Furthermore the α subunits exist in at least ten different subtypes (α1 through α10) and the β subunits exist in at least four subtypes (β1 through β4). Therefore a remarkable variety of specific assemblages are possible with a corresponding variety of chemical reaction rates.

We've initiated an effort that combines several novel techniques to directly image the microorganization of nAChRs expressed in *Xenopus* oocytes. By expressing different color XFPs in specific AChR α and β subunits, we expect to resolve individual receptors. Furthermore, we hope to discretely identify individual receptors over the field-of-view. This will enable us to simultaneously characterize the large-scale distribution of such receptors, to identify local structures within an imaged membrane surface, and to assay variation of receptor subtypes. The techniques being combined in this experiment include a novel method for repeatedly aligning the atomic force microscope probe and excitation laser with specific substrate locations to within a few 10's of nm, and an ~10 nm resolution, single-molecule sensitive near-field optical microscope. Receptor subunit imaging is enabled by the Lester group's development of specific XFP-labeled AChR subunits.

By developing the ability to directly image each subunit of each AChR expressed in the membrane of *Xenopus* oocytes, we lay the groundwork for future studies of transmembrane proteins, receptors and membrane-embedded proteins in many different types of cells. In particular we will substantially advance our ability to image the microorganization of nicotinic acetylcholine receptors at neuronal synapses.

This work is supervised by Professor Scott E. Fraser in collaboration with Professor Henry Lester.

**Support**: *JPL R&TD and BioNano programs, NASA-PRSGC IDEAS-ER grant, Caltech President's fund, NIH, NSF and Pharmagenomix grants, NS-11756, DA-17279, and Philip Morris External Research Program.*

**308. Fluorescent imaging of prostate cancer during robotic-assisted surgery**

*John M. Choi, David Wert, Andrew Raubitschek, Scott E. Fraser*

We are developing a technique to image the boundary of the prostate during robotic-assisted laparoscopic radical prostatectomy (RALP) surgery. By combining antibodies with fluorescent dyes, only the diseased organ of the prostate can be targeted, allowing the surgeon to see the demarcation between the organ to be removed and the healthy peripheral tissue. Specifically, the anti-PSMA antibody will be coupled to a commercial fluorophore absorbing at the 488 nm laser line. This is particularly important in the case of RALP because the surgeon must rely on a single sensing modality, vision, giving up any haptic awareness. While robotic-assistance has provided advantages by reducing hospital stay and recovery times, this restriction makes it difficult to achieve consistent results across different surgeon experience levels. By presenting a clear, high contrast boundary, it is hoped that the incidence of positive surgical margins will be reduced while still maintaining the quality of life for the patient by preventing lifelong impotence and incontinence. In addition, to the dye-antibody coupling, we will explore other methods to provide image contrast between the prostate, nerves, sphincter muscles, and surrounding tissue that are compatible with the current techniques of robotic-assisted surgery.

**309. Phase sensitive high speed swept source OCT with application in AMD**

*Reza Motaghiannezam, Dan Schwartz, Scott E. Fraser*

A swept source F-OCT system for Age-related Macular Degeneration (AMD), an eye disease that involves a degenerative process of the macula or central retina, has been constructed. We developed and validated a 50 kHz swept source F-OCT with 100% duty cycle at 1040 nm. The filter design of the swept source was constructed using a custom polygon-scanning mirror with 72 facets with individual facet widths of 2.77 mm. We used a relatively high-groove density blazed diffraction grating (830 lines/mm) to decrease the line-width (increase ranging depth) as narrow as possible. The collimator was chosen to produce a 1/e2 beam width of 3 mm to illuminate more lines on the grating. The rotational rate of the polygon could be continuously adjusted up to a maximum speed of 695 revolutions per second, corresponding to a filter repetition rate of 50 kHz. To test the tuning performance of this filter, a unidirectional fiber-optic resonator was constructed using a broad-bandwidth semiconductor optical amplifier (QSA1050) as a gain medium for the laser. Figure 9 shows the swept source laser trace as a function of time. We measured 95 nm tuning range (FWHM~65 nm) corresponding to ~5 μm resolution in tissue. We experimentally characterized the line-width and the system 6 dB ranging depth by measuring the point spread function using a calibrated partial reflector at various depths in the sample arm. Figure 10 shows the measured point spread function over 2.75 mm ranging depth. The 6 dB ranging depth of the system is estimated ~1.75 mm. The design system SNR was measured ~52 dB at ~1 mm and the corrected phase sensitivity was calculated ~ 600 pico-meter (pm). Increased ranging depth enabled us to improve image intensity and phase sensitivity through the sample. Currently, we start working on *in vivo* mouse retina imaging using assembled OCT system.

**310. Consequences of impaired metabolism on cerebral perfusion in glutaric acidemia type I**

*Jelena Lazovic Zinnanti, William Zinnanti, Russell E. Jacobs*

Glutaric Acidemia Type I (GA-1) is an inborn error of lysine, hydroxy lysine and tryptophan catabolism due to a deficiency of glutaryl-CoA dehydrogenase (Gcdh). Affected children may experience an encephalophatic crisis, most frequently between 6 and 18 months of age, leading to an irreversible bilateral striatal necrosis. The lack of diagnostic markers to predict striatal injury in affected GA-1 children has impeded the intervention efforts. The acute encephalophatic crisis in GA-1 children is usually associated with metabolic decompensation (increased catabolism). To mimic metabolic crisis in GA-1 mouse model, we exposed Gcdh-/- mice to increased dietary lysine (3.8% added lysine to a normal diet). Young, 4-week old mice, become encephalophatic within 3-5 days following the onset of high lysine diet, and develop striatal injury (visible on MRI) within 7-10 days (Zinnanti *et al*., 2006). Previously, using this model and combination of MRI and MRS, we were able to measure a decrease in the neurotransmitter levels 48h following increased dietary lysine and 114h before striatal injury became visible on MRI (Zinnanti *et al*., 2007). Now, we hypothesized that the metabolic derangement associated with catabolic crisis, will lead to perturbation of several key metabolites, among them one that impacts cerebral blood flow. Several investigators have proposed cytosolic NADH/NAD+ ratio (equivalent to lactate/pyruvate ratio) to represent the key regulatory mechanism by which metabolic changes in the cellular activity can be translated to changes in the cerebral blood flow (CBF) (Ido *et al*., 2001; Vlassenko *et al*., 2006). We first measured early reduction (within first 36h) in striatal perfusion (~15%), Figure 1. Second, we measured decreased lactate/pyruvate ratio at 48h, Figure 2. In a separate group of Gcdh-/- we were able to correlate early (~36 h) reduction in striatal perfusion with the later development of striatal injury (at 7 days). These findings might be fundamental toward our understanding why GA-1 children experience reduced CBF, and reduced CBF could potentially be used clinically as a marker of impending brain injury.

**Figure 1.**

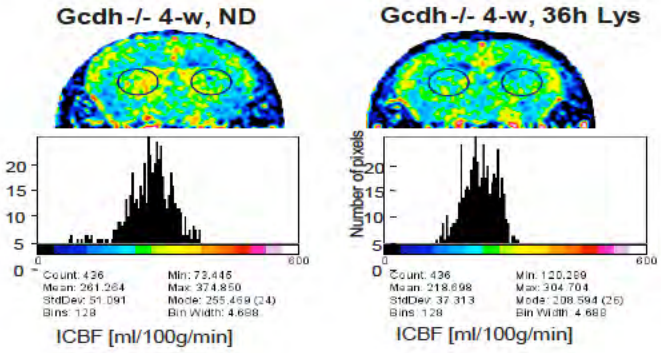



Figure 1. Changes in the index of cerebral blood flow (ICBF) of 4-week old Gcdh-/- mouse after 36h on high lysine diet. A coronal slice at the striatum level is shown. Different values of ICBF are color coded, and corresponding distribution of ICBF within regions of interest are shown as histogram distributions. Black circles outline the region of interest (striatum).

**Figure 2.**

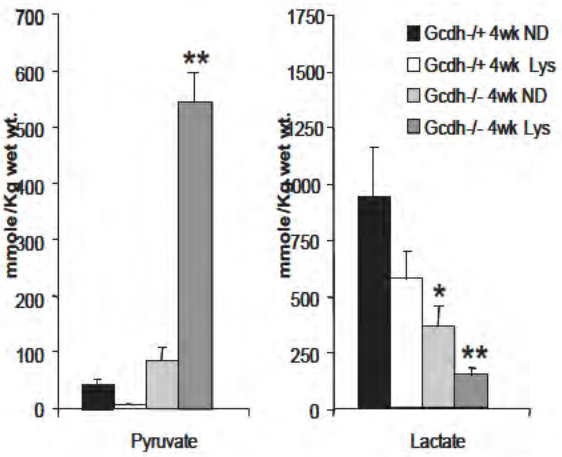



Figure 2. Changes in the brain lactate and pyruvate levels in 4-week old Gcdh-/- and Gcdh-/+ mice on normal diet (ND) and 48h following high lysine diet (4.8% total lysine). (mean ± s.e.m, *p<0.05, **p<0.01, N=3).

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**311. Mapping dopaminergic pathways by pharmacological MRI**

*Adriana T. Perles-Barbacaru, Daniel Procissi, Russell E. Jacobs*

Supervision of animal procedures: Janet F. Baer, Ph.D.

The specific aim of this project is to combine non-invasive high-field functional Magnetic Resonance Imaging (fMRI) with micro Positron Emission Tomography (μPET) of the brain in order to study monoamine neurotransmission abnormalities after cocaine administration in transgenic knockout mouse models.

The animal protocol has been approved by the Office of Laboratory Animal Resources of the California Institute of Technology, and all procedures are carried out under general anesthesia using isoflurane. Respiratory rate and end tidal $CO_2$ are monitored continuously. Radiolabeled glucose will be used to detect the increased energy metabolism of activated brain areas with the MR-compatible μPET scanner operating in a 7T Bruker Biospec/Avance 30 cm horizontal bore magnet in the CalTech Brain Imaging Center. The fMRI acquisition is made sensitive to the cerebral blood volume (CBV) change accompanying neuronal activity, by overpowering the blood oxygenation level-dependent effect (Ogawa *et al.*, 1990; Ogawa *et al.*, 1992) on T2- or T2*-weighted images of the brain with the intravenous injection of a superparamagnetic contrast agent (Berry *et al.*, 1996; Huber and Soliman 1997; Mandeville *et al.*, 1996; Marota *et al.*, 1997). Figure 1 shows a coronal pre-contrast T2* weighted image (a) and a relative CBV map (b) of a healthy mouse brain acquired with a fast gradient echo sequence and a dose of 30 mg/kg MION as contrast agent.

Neuronal activity in specific pathways is elicited by a pharmacological challenge. Figure 2 shows the time-course of the CBV-weighted signal from a subcortical ROI, illustrating a CBV decrease after a 1 mg/kg cocaine dose injected at time frame 130, 50 min after MION injection. Acute intravenous cocaine administration increases the levels of various monoamine neurotransmitters implicated in the mediation of mood, emotions and reward, and responsible for subsequent addictive behavior. The transgenic mice to be studied in this project are the catechol-O-methyltransferase enzyme knockout, and the dopamine, norepinephrine and serotonin transporter knockouts. The neuronal alterations are compared with the corresponding wild-type mouse strain.

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**Figure 1:**

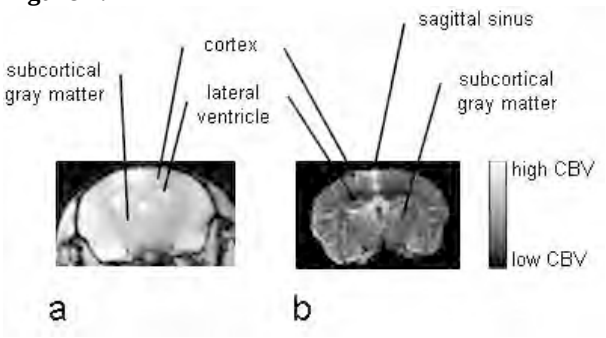
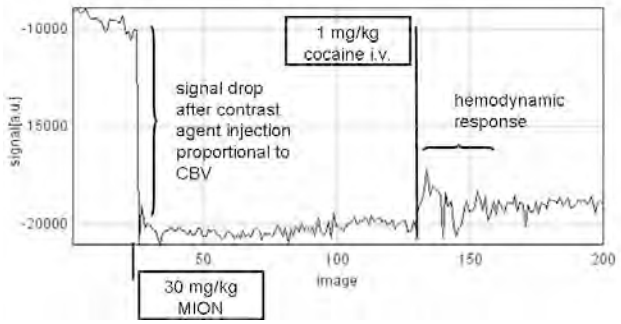


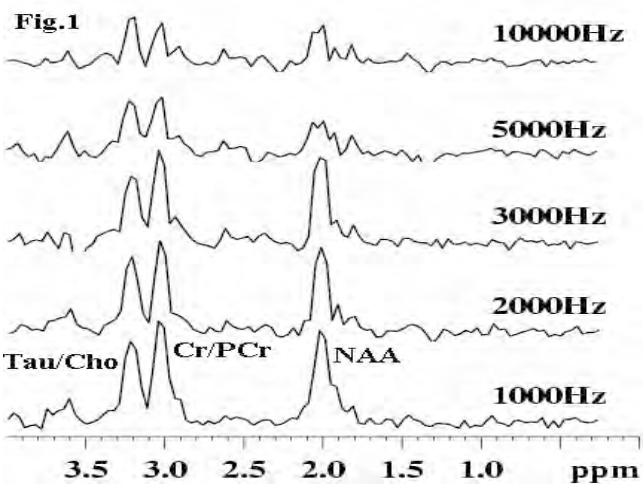

**Figure 2:**

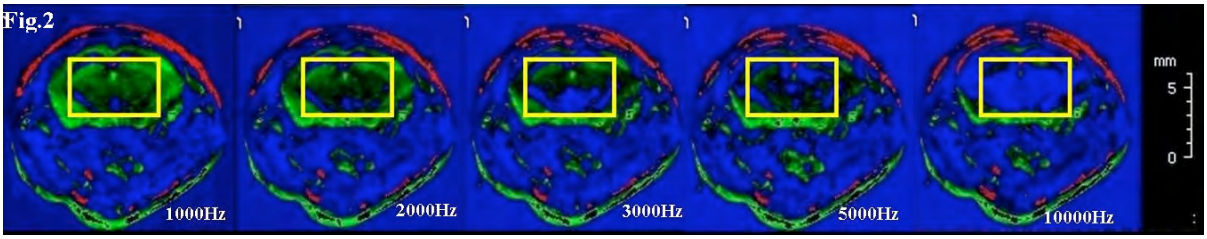



## 312. MT effects in localized IZQ spectroscopy in the live mouse brain

*Benoit Boulat, P.T. Narsimhan, Russell E. Jacobs*

Intermolecular Zero Quantum spectroscopy (IZQ) allows us to record spectra, which exhibit immunity against broadening due to magnetic field inhomogeneities. Spectra of this nature can be recorded due to the mechanisms generated by the distant dipolar field (DDF). In the live mouse brain the DDF arises mainly through the spins of the water molecules. These can be crucially affected by magnetization transfer (MT) from the spins of mostly immobile protons that can be saturated by off-resonance irradiation. We have studied MT effects on IZQ spectra of metabolites in the live mouse brain. The method presented utilizes techniques of two-dimensional NMR spectroscopy and for the DDF part, can be well described within the formalism known as "Intermolecular Zero Quantum (IZQ) coherence" although a more classical description using the Bloch equations including the DDF would provide for the same physical predictions. The $t_1$-modulated signal of the water and of the metabolites in the mouse brain was collected by the RF coil during the time $t_2$. After 2D Fourier transformation of the time domain data, the projection along the $w_1$ dimension provided a high-resolution MT affected IZQ spectrum (MTIZQ) or simple IZQ spectrum. In the setting described above, $w_1$ labels the difference in frequency between the water resonance and the resonances of the metabolites present in the brain of the mouse studied. Fig.1 below shows the 1D projected $w_1$ *in vivo* [IZQ- MTIZQ] difference spectra obtained in a localized 5x5x5mm$^3$ region in the brain of a live C57 wild-type mouse for various offsets with respect to the water line of the radiofrequency inducing MT effects. Well-known metabolites are clearly identified in the difference spectrum; NAA: N-acetylaspartate, Cr: Creatine, PCr: Phosphocreatine, Cho: Choline, Pcho: Phosphocholine. Fig 2. presents for the same offsets, MT affected conventional MR images in a 1 mm slice in the brain of the same live mouse.






**313. Simultaneous PET/MR as an imaging tool: exploring tumor response to therapy**

*Thomas Ng, Daniel Procissi, Yibao Wu[1], Andrey Demyanenko, Andrew A. Raubitschek[2], Simon Cherry[1], Russell E. Jacobs*

The study of biological processes *in vivo* is vital to their proper elucidation. Positron Emission Tomography (PET) and Magnetic Resonance (MR) offer complementary functional and anatomic information [1] that provide unique windows into such processes. Previous studies have shown that PET and MR data combined retrospectively show correlations between information garnered from the individual modalities [2]. Despite the use of careful registration techniques, it can be difficult to analyze multimodal data that are temporally and spatially different. The integrated PET/MR scanning system developed in collaboration with Simon Cherry's lab at U.C. Davis [3-5] overcomes these problems by allowing simultaneous imaging using both modalities. Apart from integrating datasets in space and time, PET/MR offers the potential to perform real time analysis of multi-modal data that can feed back to direct further studies in a single imaging session. The high spatial resolution, capabilities of anatomical MRI, provides the base upon which PET and other MR imaging methods such as MR spectroscopy (MRS) and angiography can be performed. The latter methods can be analyzed immediately to determine the appropriate timing windows and regions of interest (ROI) to pursue.

Ongoing studies in the lab apply this new technology to obtain new insights to clinically relevant questions. We are developing engineering and scientific techniques and protocols to maximize the integrative utility of this multimodal imaging system. This effort ranges from developing software and improving hardware of the system to handle the acquisition and visualization of concurrent data to applying multimodal probes to see multiple functional processes together. In particular, we are currently applying the simultaneous PET/MR instrumentation towards understanding tumor responses to cancer immunotherapy. The non-invasive nature of both imaging modalities is ideal for this, for it allows us to monitor responses in intact animals longitudinally and in areas of anatomy that are not accessible by other means. As proof of concept, we demonstrated the feasibility of using FDG-PET imaging to guide the study of tumor heterogeneity using MRS within a single imaging session (Figure 1). FDG-PET activity was correlated with high choline proliferative activity, with anatomical MRI delineating true tumor volume missed by FDG-PET alone (Figure 2). Techniques and insights derived from the integrated small animal scanner system can be translated efficiently to clinical settings.

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[2]*Dept. of Radioimmunotherapy, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA USA*

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**314. Quantitation and relaxivity of manganese in live brain tissue**

*Jessica Bastiaansen, Russell E. Jacobs*

Manganese enhanced magnetic resonance imaging (MEMRI) uses the magnetic properties of $Mn^{2+}$ to generate contrast. Manganese can enter cells through voltage-gated calcium channels and can be transported along axons. It has been used for visualizing neuroarchitectures not previously detectable by MRI, performing anterograde neuronal tract tracing after and for observing neuronal activation.

Large signal changes are usually measured in MEMRI, but there is still need to improve the detectability of $Mn^{2+}$. One reason for optimization is for lowering the dose since manganese is neurotoxic. The second reason is that axonal transport dilutes its concentration. To improve the detectability, imaging parameters can be optimized according to the expected concentration of the contrast agent and signal to noise ratio SNR.

Manganese lowers the longitudinal relaxation time, $T_1$, which is the decay constant for the recovery of the z-component of the nuclear spin magnetization, $M_z$, towards its thermal equilibrium, $M_0$. Accurate knowledge of relaxation times is necessary for adjustment of MRI parameters to obtain optimal SNR. The relation between

$T_1$ and the contrast agent concentration is, $1/T_{1(CA)} = 1/T_{1,(0)} + R_1 * [Mn]$. The relaxivity, $R$ depends on field strength, temperature, pH and molecular environment. In tissues, it also depends on compartmentalization because of the uneven distribution. When the relaxivity is known, $T_1$ mapping provides the potential to quantify tissue concentration of the agent used. In phantoms, concentrations as little as 2 μM $MnCl_2$ can be detected. However, since $Mn^{2+}$ quantitation has not yet been performed in tissue extracts, the relaxivity for deriving the tissue $Mn^{2+}$ concentration from measured $T_1$ values is not yet known.

This research aims to obtain the relaxivity values for neural tissue, *in vivo* and *in vitro*, and implies the development of a compartmentalization model of manganese in neural tissue. With ICP-MS determined concentrations will be compared with $T_1$ maps and the Mn relaxivity determined as function of the location in the brain.

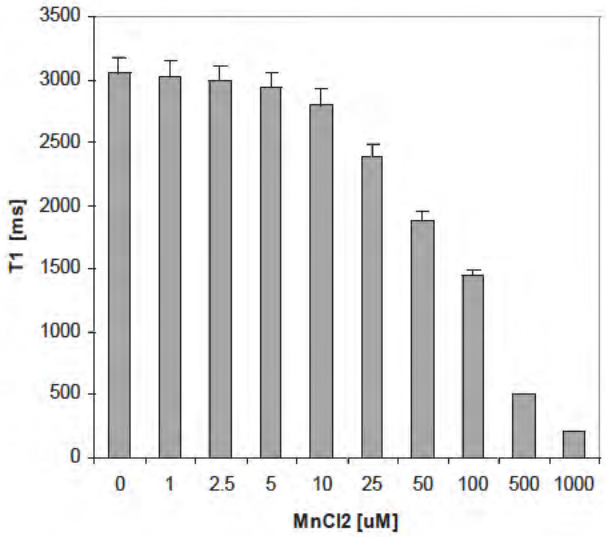


Figure 1. Test $T_1$ measurements on phantoms, NMR tubes with double distilled water doped with $MnCl_2$.

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**315. Mapping carotid arterial strain of atherosclerosis *in vivo* using DENSE MRI**

*Alexander Lin, Han Wen*, Morteza Gharib, Scott E. Fraser*

*In vivo* displacement encoding using stimulated echo magnetic resonance imaging (DENSE-MRI) has been validated in the carotid arteries of healthy volunteers at 1.5T and 3.0T (Figure 1). Atherosclerotic lesions have been shown to localize to regions of excessive stretching of the carotid arterial wall and since these atherosclerotic plaques are the source of emboli and/or thrombi that cause stroke, DENSE-MRI strain measurements may provide early diagnosis for stroke.

The goals of this study are two fold: First, DENSE strain patterns in atherosclerotic plaques will be determined using an *in vitro* model of the carotid arteries. The model will be created using polyvinyl alcohol cryogel that has been shown to mimic the material properties of human arteries. Using a variable blood pump and molds that range in thickness and stenosis, different physiological conditions can be created (Figure 2). These conditions will then be imaged with DENSE MRI to determine their effects on the strain maps.

The second goal will be to expand the study to determine strain patterns in patients with known atherosclerosis and compare these results with age-matched, healthy subjects. Multi-sequence MRI will also be acquired providing structural imaging of plaque such as IMT thickness, plaque load, and degree of stenosis (Figure 3). Patient histories will provide risk factors for stroke (such as age, sex, BMI, BP, HDL count, and clinical history). The hypothesis is that strain maps will be significantly different in patients with plaques and that these strain measurements will significantly correlate with morphological and clinical measures of athero-sclerosis.

The results of the study will define the strain pattern for atherosclerotic plaques in the carotid arteries and provide correlates to risk factors for stroke. These results can be used for patient monitoring to determine therapeutic efficacy and may provide the basis for future studies to establish DENSE-MRI as a diagnostic tool for early diagnosis and answer the critical clinical question of plaque vulnerability.

**National Institutes of Health*

Figure 1

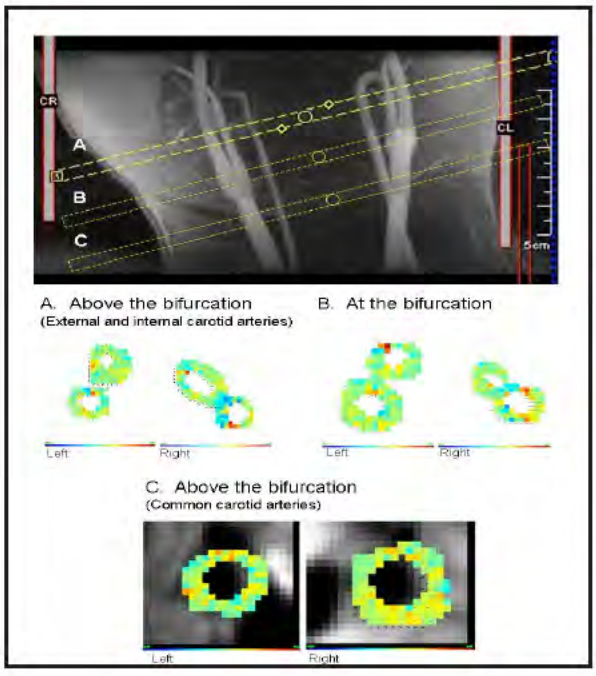


Figure 2

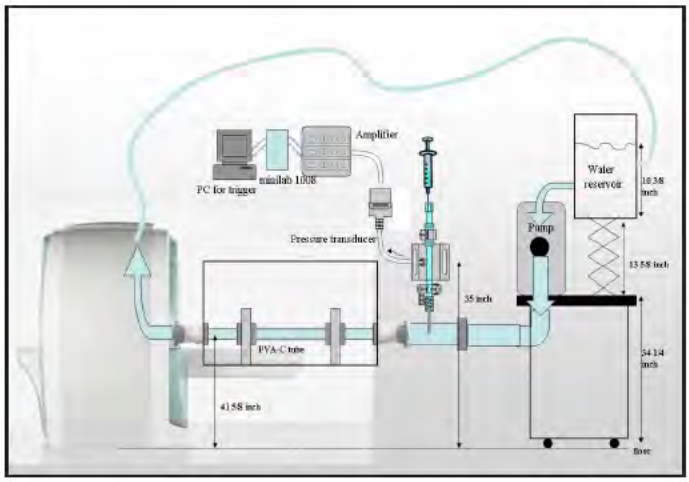
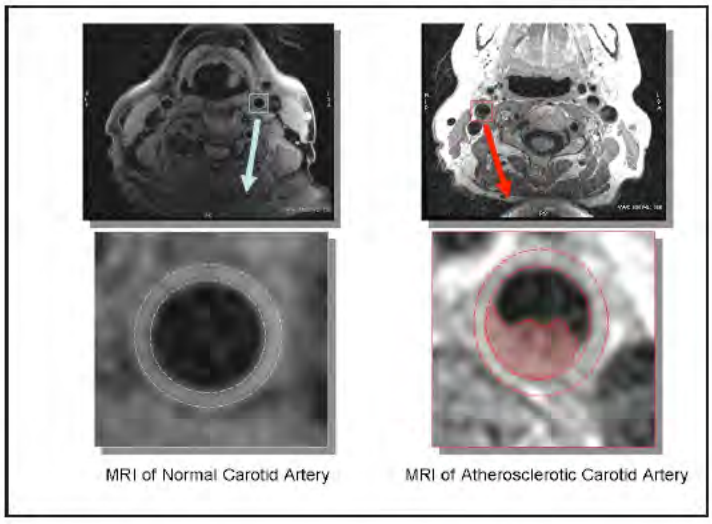



Figure 3




**316. Water permeability of neuronal cell membranes in a frog sciatic nerve model**

*Lin Zhao, Scott E. Fraser, J. Michael Tyszka*

Functional magnetic resonance imaging (fMRI) offers a non-invasive way to study brain activation in response to a task or stimulus. For example, the blood oxygen level-dependent susceptibility (BOLD) contrast mechanism detects the hemodynamic response associated with increased regional neuronal activity. Although it is a powerful tool, this hemodynamic method is an indirect way to detect neuronal cell activities. It monitors the change of the ratio of the deoxyhemogloin to oxyhemoglobin in blood vesicles, not the changes in neuronal cell physiological states. Here, we use an excised nerve tissue -- frog sciatic nerve trunk – as a model to understand the effect of nerve cell excitation on the neuronal cell water MR signal.

In particular, we measure the water permeability changes across cell membranes upon depolarization. In order to be able to study the water exchange process between the intracellular and extracellular compartment, one has to differentiate the intracellular and extracellular water signal. We use a paramagnetic agent (GdDTPA) to shift the extracellular water signal to a different resonance frequency, while intracellular water signal is not directly affected. Fig. 1 shows the separated intracellular and extracellular water $^1$H spectrum. This work lays the foundation for quantitative determination of the water exchange time constant through cell membranes before and after depolarization, with the ultimate goal of non-invasive, direct imaging of neuronal activity.

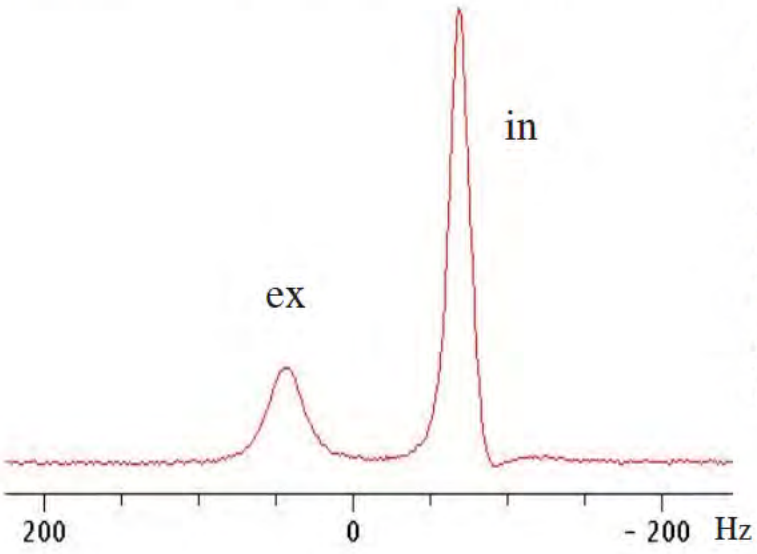



Fig. 1 The separation of the intracellular and extracellular water signal is essential for studying the water exchange process across cell membrane. A water $^1$H spectrum acquired from an excised frog sciatic nerve tissue is shown. The nerve was placed in 5 mM GdDTPA Ringer's solution for 2 hours before MR experiments. The resonance at left is assigned to extracellular water. Depolarization-dependent water transport across the cell membrane can be inferred from changes in the extra- and intracellular resonances.

## 317. Uniplanar MR stage microscopy: Hardware development and proof-of-concept imaging

*Andrey Denyanenko, Lin Zhao, J. Michael Tyszka*

This project develops the idea of a one-sided magnetic resonance microscope analogous to a conventional optical-stage microscope. Uniplanar magnetic field gradient coils have been developed for the X, Y and Z axes and in the current prototype, allow for high amplitude and high slew-rate field gradient generation at significantly higher efficiencies than conventional cylindrical designs. Successful insulation of the sample from heat generated by the gradient module is a prerequisite for MRI of living organisms including developing embryos. During the year since first reporting this design, we have successfully added a new air-cooling system to supplement the existing water cooling system, reducing the temperature rise at the stage surface to less than 0.5°C under typical imaging conditions (Figure 1 Left). The high performance of the uniplanar gradient module opens up several very high speed imaging sequences that have previously remained unused for high resolution MR microscopy. These include echo planar imaging (EPI), capable of acquiring complete 2D images in less than 50 ms. We have acquired proof-of-concept EPI data with an in-plane spatial resolution of 100 μm in 26 ms using the current uniplanar gradient hardware (Figure 1, Right).

The stage geometry not only allows easy access to samples, but also allows much larger medium volumes than would be possible with a conventional cylindrical gradient and radiofrequency coil design. Consequently, imaging of multiple organisms in an environment similar to that typically used for optical microscopy is now possible with the MR-stage microscope. To support this goal, we have acquired the first proof-of-concept images of live, developing African clawed frog (*Xenopus laevis*) embryos using the stage. Animal-cell signal was enhanced using injection of a commercial MR contrast agent. A group of seven embryos was imaged using a conventional 3D gradient echo imaging sequence over the course of three days from approximately developmental stages 11 through 26 (Figure 2).

*This work is funded in part by a grant from the National Science Foundation (DBI 0552396).*

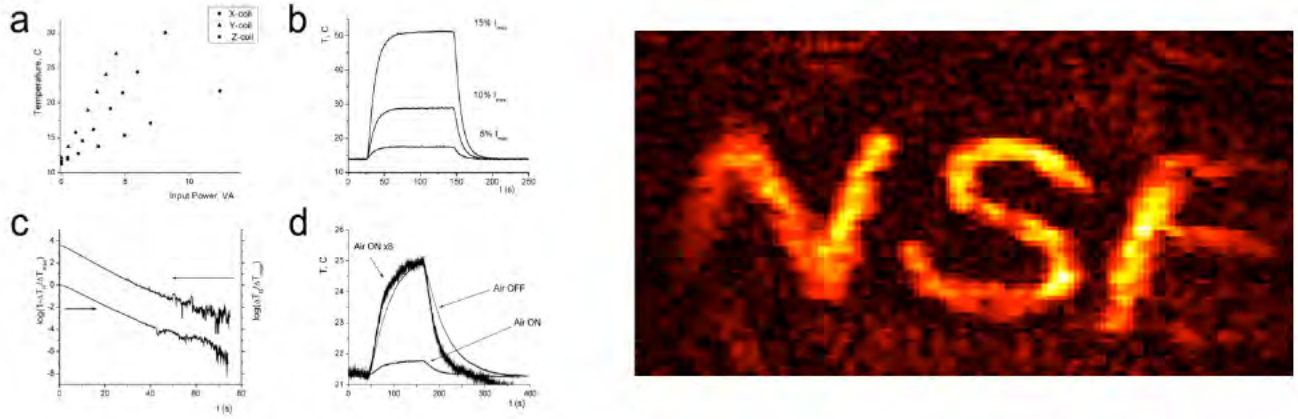



*Figure 1. (Left) Resistive heating effects for the prototype MR-stage microscope gradient set. Airflow insulating/cooling effect. Adding airflow reduces the temperature rise on the surface of RF coil to less than 1.0 C. (Right) High speed MR microscopy. Gradient echo EPI acquired using the prototype MR-stage microscope. The image represents a 500 μm slice through a plastic phantom with hollow character spaces filled with 1mM Prohance solution. Nominal in-plane resolution is 100 μm x 100 μm with a total imaging time of 26 ms.*

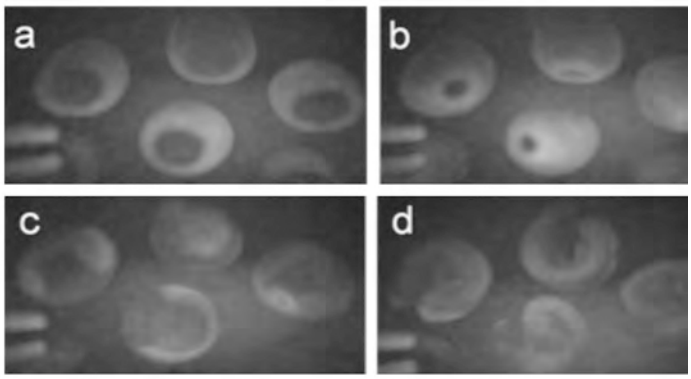



*Figure 2. Volume renderings of selected time-points in a serial study of developing frog embryos enhanced with 1mM Prohance at embryo-stage 11 (a), 12.5 (b), 22 (c) and 26 (d). Each image was acquired in one hour with four signal averages. The nominal isotropic spatial resolution is 50 μm. Bright signal arises from the animal cells in these T1-weighted gradient echo images. Embryos are approximately 1mm in diameter.*

**318. Hydrodynamic synchronization among eukaryotic 9+2 and pseudo 9+0 cilia**

*David Wu, Scott E. Fraser*

Eukaryotic cilia (or flagella) are bundles of microtubules (usually in an arrangement of nine circumferential outer doublets together with or without an inner core of two doublets) that are antennae-like appendages that cells use to either sense the surrounding chemical/mechanical environment or as a means of propulsion in an inherently low Reynolds number environment. Synchronization of neighboring cilia is a known phenomenon that is useful in the bulk transport of fluid; this occurs in diverse organisms such as the single-celled *Opalina*, in which coordinated cilia motion helps movement, the feeding organ of *Stentor*, for the transport of metabolites, to the muco-ciliary escalator of the lungs of air-breathing vertebrates, for the removal of particulates. In short, without synchronization, fluid transport would be slower, since every ciliary beat includes a backwards force. However, the experimental parameters that underlie synchronization have yet to be fully explored since cilia preparations are confounded by the organisms themselves. We, thus propose to study synchronization in an *in vitro* setting by observing the purified cilia of a set of wild-type (9+2) and *pf* (pseudo 9+0) mutants of *Chlamydomonas reinhardtii* placed in a regular, variably-spaced array. By using this novel *in vitro* technique, we hope to uncover the relevant parameters behind the synchronization of cilia independent of their natural spacing and the underlying cellular metabolism.

**319. A trajectory approach to two-state kinetics of single particles on sculpted energy landscapes**

*David Wu, Kingshuk Ghosh, Mandar Inamdar, Heun-jin Lee, Scott E. Fraser, Ken Dill, Rob Phillips*

We study the trajectories of a single colloidal particle as it hops between two energy wells *A* and *B*, which are sculpted using optical traps. Whereas the dynamical behaviors of such systems are often treated by master-equation methods that focus on *particles* as actors, we analyze them here instead using a *trajectory*-based variational method called Maximum Caliber. We show that the MaxCal strategy accurately predicts the full dynamics that we observe in the experiments: from the observed averages, it predicts second and third moments and covariances, with no free parameters. The covariances are the dynamical equivalents of Maxwell-like equilibrium reciprocal relations and Onsager-like dynamical relations. In short, this work describes an experimental model system for exploring full trajectory distributions in one-particle two-state systems, and it validates the MaxCal approach as a useful way to understand trajectory-based dynamical distribution functions in this system.

**320. Real-time visualization of λ-phage DNA ejection into *E. coli***

*David Wu, Hernan Garcia, Arbel Tadmor, Rob Phillips*

The study of infection of bacteria by bacteriophage led to some of the earliest biophysics experiments, as well as one of the first examples of interest in stochastics and noise at the single cell level of decision-making. From a 1:1 ratio of phage to bacteria concentration, time to lysis is anywhere from 30 to 90 minutes. This time includes binding of phage to bacterium, injection of DNA into the host, production and assembly of phage precursors, and finally bursting from the cell. We focus here on the initial events in the infection process during which the viral genome is translocated into the host bacterium. While DNA packaging of lambda phage and phi29 and the rate of DNA ejection have been investigated *in vitro*, these processes (in addition to lysogeny, lysis) have yet to be studied at the single molecule level *in vivo*. We report here on progress towards visualizing the ejection of phage DNA at the single molecule level into its host *in vivo*. Recent advances in visualization of protein-binding onto DNA, via a gated-imaging technique, allow us to see a single molecule of DNA as it translocates into a cell. Additionally, we also demonstrate a method to control the initial time of infection to measure the waiting time between phage-binding and DNA ejection. One of the aims of this work is to test simple theoretical models on the rate of DNA ejection and to examine questions such as: how does genome-length effect ejection? What are the binding dynamics of a bacteriophage to its receptor?

**321. Synthetic *cis* regulatory modules**

*Roee Amit, Frances Arnold, Scott E. Fraser*

We would like to introduce a new paradigm for synthetic biology – Synthetic *Cis* Regulatory Modules (SCRM). Namely, rather than design circuits – design and implement non-protein coding regions of DNA that regulate gene-expression in a precise 5-D pattern (time, space, and intensity). In nature, CRMs are the large non-coding genomic regions that occupy broad segments of the genome (sometimes tens of kb), and are implicated in the regulation of the precise 5-D expression of many genes. The precise regulation that is associated with these regions is due to a handful of transcription factors (typically 5-10), DNA-binding proteins, and chromatin remodeling complexes that bind at several (typically clustered) binding sites each. It is the purpose of this project to construct synthetic regulatory regions in an attempt to mimic some of the logic that is presumed to exist.

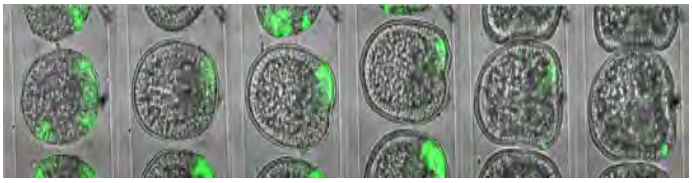
In attempting to construct SCRMs, one must first assume some general mechanistic design principles that can – in theory – generate similar regulatory phenotypes to what is observed in natural systems. To that end, we will argue that one needs a) a precision or noise-minimizing transcription apparatus and b) a set of biophysical/biochemical design principles that will allow us to hard-wire the complex logic operations carried out in CRMs in a predictable fashion. In this case, both requirements are met by utilizing a specialized transcription apparatus that relies on the biophysical property of DNA that allows it to form loops (and thus, bring distal segments of DNA together), and the ability of DNA-binding proteins to change that bending rigidity of the DNA – thus, having the capability to precisely affect the looping process itself.

This model will be tested initially on a bacterial platform with the σ54 transcriptional apparatus, which satisfies the above model and is considered in the literature to be "eukaryotic-like." The goal will be generate expression-level patterns reminiscent of CRM controlled genes that can be predicted through the above looping/bending rigidity model. This will be done by designing *ab-initio* spacer regions containing binding sites for DNA-binding proteins in some distribution, whose level in the cell can be controlled by external means (i.e., TetR, TraR). The distribution and number of binding sites will be altered using a directed-evolution type of approach, until a desired feature will be obtained (in this case, well-spaced discrete expression states).

**322. Quantitative *in vivo* imaging of the dynamics of gene regulatory networks**

*Mat E. Barnet, Eric H. Davidson, Scott E. Fraser*

The fundamental question of developmental biology is how a single fertilized egg cell gives rise to a complex organism containing thousands, millions, or billions of highly integrated cells. As large-scale genome projects continue to reveal the striking genetic similarities among different species, it is becoming more and more clear that interspecies variation results not from major differences in the sets of genes different organisms possess, but from differences in the regulation and expression of those genes. To better understand how gene regulatory networks govern embryonic development, we are collaborating with Eric Davidson's lab, using sea urchins as a model system. The size, shape, and optical transparency of sea urchin embryos makes them ideal for study by light microscopy. We are developing fluorescence imaging tools and techniques to enable *in vivo* quantification of the dynamics of the gene regulatory network responsible for specification of the sea urchin endoderm and mesoderm. One technique involves quantitatively imaging expression of a reporter gene (e.g., green fluorescent protein (GFP)) in the three-dimensional embryo using confocal laser scanning microscopy (CLSM) or two-photon laser scanning microscopy (TPLSM). By quantifying gene expression patterns in three dimensions over time, we aim to better understand how the dynamics of gene regulatory networks govern developmental processes. Another technique involves fusion proteins consisting of a photoactivatable or photoconvertible protein fused to a protein whose dynamics are of interest, such as a transcription factor. Such fusions allow for *in vivo* pulse-chase type experiments, in which we aim to quantify protein production and turnover dynamics. By our quantitative approaches, we hope to generate kinetic data that can be applied to the current, largely qualitative, gene network models, to enable the models to make quantitative predictions of regulatory dynamics, thereby helping to further our understanding of how complex organisms develop from single cells.



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**Graduate Students:** Kelly J. Dusinberre, Catherine M. Ward
**Undergraduate Students:** Jessica Su, Annie Hong, Margaret Chiu
**Collaborators:** H.-A.J. Müller[1], M. Guo[2], Rollie Clem[3], Yigong Shi[4], S.J. Yoo[5]
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**Support:** The work described in the following research report has been supported by:
National Institutes of Health
Moore Center for Biological Circuit Design
Foundation for NIH Research/Bill and Melinda Gates Foundation

**Summary:** We are interested in multiple questions in basic and applied biology. For further information on Hay lab research consult our web page (http://www.its.caltech.edu/~haylab/). One goal of our work is directed towards understanding the genetic and molecular mechanisms that regulate cell death, proliferation, innate immunity and spermatogenesis. We use *Drosophila melanogaster* as a model system to identify genes that function to regulate these processes. Important cellular regulatory pathways are evolutionarily conserved; thus, molecules identified as regulators of these processes in *Drosophila* are likely to have homologs in vertebrates and the pathways that link these molecules are likely to be regulated similarly. A second goal of our work addresses three questions in population biology. 1) Can we bring about reproductive isolation (speciation) between populations of plants or animals that otherwise freely interbreed? Answers to this question have application to the growing number of situations in which plants and animals are engineered to show specific traits. In brief, we would like to be able to limit gene flow between engineered organisms and their wild counterparts. 2) Can we engineer the genetics of populations so that they drive themselves to local extinction? For example, invasive non-native plants and animals cause substantial economic losses. A number also cause substantial environmental damage, leading in many cases to extensive range reduction and/or extinction of unique, endemic species. Our goal is to develop genetic tricks that drive local extinction of invasive species. 3) Can we drive genes into wild populations so that all individuals express a trait of interest. With regard to this last aim, we are particularly interested in developing transgenic insects that will prevent transmission of mosquito-borne diseases such as malaria and dengue fever. More than 500 million people are infected with the malaria parasite each year, resulting in 1-3 million deaths. Dengue, a mosquito-borne virus infects more than 100 million people each year, resulting in more than 25,000 deaths. Effective vaccines for these diseases do not exist, and in the case of malaria, the causative agent, the parasite *Plasmodium falciparum* has acquired resistance to many drugs. Vector suppression through the release of sterile males, the use of insecticides, or modification of the environment provides an important tool for limiting mosquito-borne disease. However, each approach has limitations. Release of sterile males provides only transient population suppression, insecticides affect many non-target species and mosquitoes often evolve resistance to these compounds, and wholesale modification of the environment may not be feasible, or desirable in many situations based on ecological concerns. Our goals are two-fold: to develop transgenic insects that lack the ability to transmit these pathogens; and to develop genetic tools for driving these genes into wild populations of insects, thereby blocking disease transmission.

### 323. *Drosophila* models of human neuro-degenerative diseases

*Ming Guo (and the Guo lab), Haixia Huang, Bruce A. Hay*

In collaboration with the Guo lab at UCLA we are studying *Drosophila* models of the two most common neurodegenerative diseases, Alzheimer's disease and Parkinson's disease.

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### 324. Gene activation screens for cell death regulators: MicroRNAs, small non-coding RNAs, define a new family of cell death regulator

*Chun-Hong Chen, Haixia Huang*

We have carried out several screens for cell death regulators in the fly and have identified a number of new molecules. Among these are multiple microRNAs, small noncoding RNAs that function by inhibiting translation of target transcripts. We are interested in determining when and where these molecules regulate death, as well as the nature of their targets. We are also designing microRNAs that target known cell death regulators as a way of probing the function of these proteins in specific contexts.

### 325. Cell death, caspases and IAPs

*H. Arno J. Müller, Bruce A. Hay, Chun-Hong Chen*

In flies and vertebrates most, if not all, cells can undergo apoptosis in the absence of new gene expression, indicating that the components required to carry out apoptosis are present and ready for activation. The core of the cell death machine consists of members of a family of proteases known as caspases, which become activated in response to many different death signals. Active caspases then cleave a number of different cellular substrates that

ultimately lead to cell death and corpse phagocytosis. Most if not all cells constitutively express caspase zymogens (inactive precursors) sufficient to bring about apoptosis. Thus, the key to cell death and survival signaling revolves around controlling the levels of active caspases in the cell. Several basic strategies are used to regulate caspase activity, and the core proteins that drive caspase-dependent death are evolutionarily conserved. In *Drosophila* many cells experience chronic activation of the apical cell death caspase Dronc. If unrestrained, active Dronc cleaves and activates downstream effector caspases that bring about cell death. Cells survive because they express the IAP DIAP1, which suppresses Dronc activity, as well as that of caspases activated by Dronc. One major pathway through which caspase-dependent cell death in flies is induced is through the regulated expression of pro-apoptotic proteins that disrupt DIAP1-caspase interactions through several different mechanisms, each of which has the effect of unleashing a cascade of apoptosis-inducing caspase activity. We are interested in several questions. 1) What are the signals that lead to caspase activation in cells that would normally live? 2) How do IAPs regulate caspase activity and when and where does this regulation define points of control? 3) How is IAP activity regulated? 4) And finally, as discussed further below, how do caspases, IAPs and their regulators work to regulate non-apoptotic processes? We are using both genetic screens and biochemical approaches to identify the critical molecules.

**326. Caspases and their regulators in a non-apoptotic process, spermatid differentiation**

*Haixia Huang, Jun R. Huh, Jean Edens*

We have found that multiple caspases, acting through distinct pathways, acting at distinct points in time and space, are required for spermatid individualization, a process in which spermatids (which develop in a common cytoplasm) become enclosed in individual plasma membranes and shed most of their cytoplasm*. Spermatid individualization is an evolutionarily conserved process, but little is known about how it is brought about. Several questions are of interest to us: 1) What are the upstream signals that drive caspase activation? 2) What are the nonapoptotic targets that facilitate differentiation? 3) How is cell death prevented in the face of high levels of caspase activity that would normally be associated with cell death? 4) Do caspases play similar roles in promoting spermatid differentiation in mammals?

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**327. Cell death and the innate immune system**

*Chun-Hong Chen, Ming Guo, Bruce A. Hay*

As discussed above, many IAP family proteins inhibit apoptosis. IAPs contain N-terminal BIR domains and a C-terminal RING ubiquitin ligase domain. *Drosophila* DIAP1 protects cells from apoptosis by inhibiting caspases. Apoptosis initiates when proteins such as Reaper and Hid bind a surface groove in DIAP1 BIR domains via an N-terminal IAP-binding motif (IBM). This evolutionarily conserved interaction disrupts IAP-caspase interactions, unleashing apoptosis-inducing caspase activity. DIAP2 overexpression also inhibits Rpr- and Hid-dependent apoptosis, but little is known about DIAP2's normal functions. We generated *diap2* null mutants, which are viable and show no defects in developmental or stress-induced apoptosis. Instead, DIAP2 is required for the innate immune response to Gram-negative bacterial infection. DIAP2 promotes cytoplasmic cleavage and nuclear translocation of the NF-κB homolog Relish, and this requires the DIAP2 RING domain. Increasing the genetic dose of *diap2* results in an increased immune response, while expression of Rpr or Hid results in down-regulation of DIAP2 protein levels. Together these observations suggest that DIAP2 can regulate immune signaling in a dose-dependent manner, and that DIAP2 is regulated by IBM-containing proteins. Therefore, *diap2* may identify a point of convergence between apoptosis and immune signaling pathways.

**Reference**

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**328. Driving genes for disease refractoriness into wild pest insect populations**

*Chun-Hong Chen, Haixia Huang, Catherine Ward, Jessica Su, Nikolai Kandul, Geoff Pittman, Bruce A. Hay*

An attractive approach to suppressing mosquito-borne diseases involves replacing the wild-insect population with modified counterparts unable to transmit disease. Mosquitoes with a diminished capacity to transmit *Plasmodium* have been identified in the wild and created in the laboratory, demonstrating that endogenous or engineered mosquito immunity can be harnessed to attack *Plasmodium*. However, a critical unanswered question is how to spread these effector genes throughout the areas inhabited by disease-transmitting insects. Epidemiological and modeling studies suggest that it will be necessary to rapidly replace a large percentage of the wild mosquito population with refractory insects in order to achieve significant levels of disease control. Because insect disease vectors are spread over wide areas and can migrate significant distances, mass release of refractory insects associated with simple Mendelian transmission of effector-bearing chromosomes is unlikely to result in a high enough frequency of transgene-bearing individuals. Compounding this problem, enhancement of immune function in insects is often costly, requiring tradeoffs with other life history traits such as longevity and fecundity that decrease fitness. Therefore, it is likely that insects carrying effector transgenes will be less fit than their wild counterparts, resulting in a decrease in the fraction of individuals carrying genes for refractoriness over time. These observations argue that population replacement will require coupling of genes conferring disease refractoriness with a

genetic mechanism for driving these genes through the wild population at greater than Mendelian frequencies.

Maternal-effect lethal selfish genetic elements have been described as genetic entities in the flour beetle *Tribolium casteneum*. The molecular nature of these elements (known as *Medea* elements) is unknown, but their genetic behavior makes them attractive candidates to mediate drive. This is because when present in a female, they must be inherited in the next generation in order for the offspring to survive.

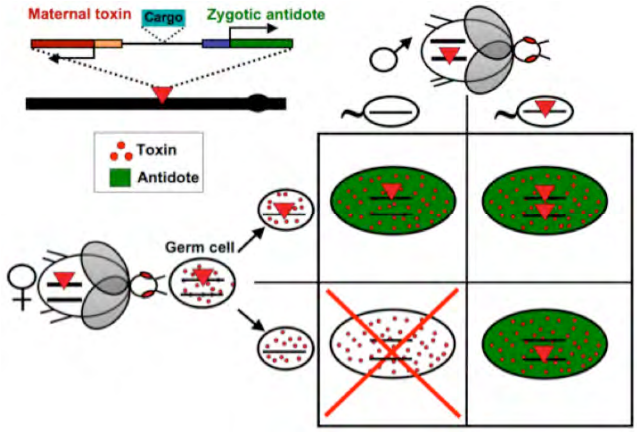


**Figure 1.** *Medea* is a "spiteful" selfish genetic element that enhances its transmission from generation to generation by causing the death of offspring that fail to inherit it. Mothers that carry a *Medea* element express a toxin (red dots) that is inherited by all oocytes (small ovals). Embryos (large ovals) that do not inherit *Medea* die because toxin activity (red background) is unimpeded (lower left square). Embryos that inherit *Medea* from the mother (upper left square), the father (lower right square) or both (upper right square), survive because expression of an antidote early during embryogenesis (green background) neutralizes toxin activity. *Medea* is comprised of two closely linked genes (upper left). One consists of a maternal germline-specific promoter that drives the expression of an RNA or protein that is toxic to the embryo. The second locus consists of a zygotic (early embryo) promoter that drives expression of an antidote.

This behavior is predicted to lead to rapid spread of the element within the population even if it carries an associated fitness cost because the chromosome that carries it gains a transmission advantage relative to counterparts that do not. Since the molecular biology of endogenous *Medea* elements is unknown, we created synthetic elements in *Drosophila* that can drive population replacement and that are resistant to recombination-mediated dissociation of drive and effector functions. The genetic and cell-biological principles utilized, which utilize microRNA-mediated silencing of a maternally-expressed gene essential for embryogenesis, coupled with early zygotic expression of a rescuing transgene, should be generally applicable to a number of other animal and plant species and have the potential to allow for iterative cycles of population replacement. We are now expanding this work into the mosquito system.

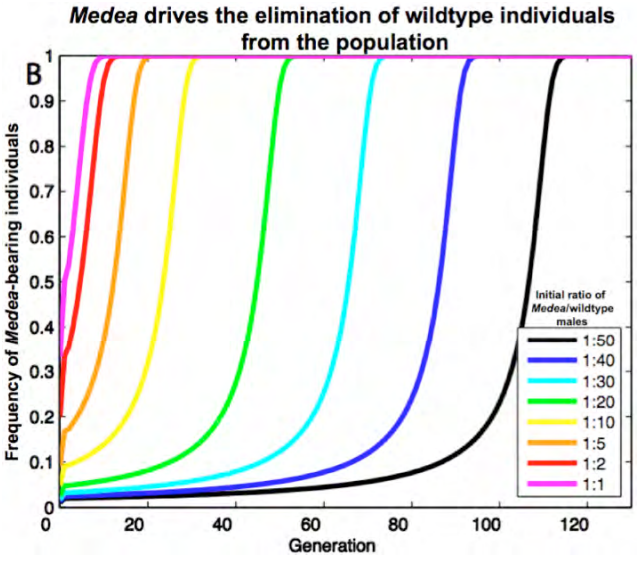


**Figure 2.** When *Medea*-bearing males are introduced into a population consisting of wildtype males and females, wildtype individuals are eliminated from the population. The greater the initial ratio of *Medea* to wildtype males, the more rapidly this elimination occurs.

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**329. Sensing and killing dengue and yellow fever virus-infected cells in their insect host**

*Kelly J. Dusinberre*

Dengue and yellow Fever virus infect mosquitoes during a blood meal. The virus must enter and replicate inside mosquito midgut cells, disseminate throughout the body and ultimately infect the salivary gland (7-14 days later), in order to be transmitted to a new individual during a subsequent blood meal. Our goal is to develop transgenes that are phenotypically neutral when expressed in uninfected individuals, but that kill virus-infected cells and/or the mosquitoes themselves. The virus encodes several activities that are not present in uninfected host cells. These include a viral polyprotein protease, and RNA-dependent RNA polymerase. We are developing molecules that sense these activities and cause the death of cells and insects in which they occur, thereby preventing disease transmission to humans.

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**Research and Laboratory Staff**: Sara McBride

**Support**: The work described in the following research reports has been supported by:
Crohn's and Colitis Foundation of America
Damon Runyon Cancer Research Foundation
Searle Scholars Program
Weston Havens Foundation

**Summary:** The Western world is experiencing a growing medical crisis. Epidemiologic and clinical reports reveal a dramatic increase in immune disorders: inflammatory bowel disease, asthma, type 1 diabetes, and multiple sclerosis. Emboldened by the 'hygiene hypothesis' proposed two decades ago, scientists have speculated that lifestyle changes (vaccination, sanitation, antibiotics) have predisposed developed societies to these disorders by reducing bacterial infections. However, the hypothesis remains without explanation as our exposure to most bacteria does not result in disease. Mammals are colonized for life with 100 trillion indigenous bacteria, creating a diverse ecosystem whose contributions to human health remain poorly understood. In recent years, there has been a revolution in biology toward understanding how (and more importantly, why) mammals harbor multitudes of symbiotic bacteria. We have recently demonstrated for the first time that intestinal bacteria direct universal development of the immune system; thus fundamental aspects of mammalian health are inextricably dependent on microbial symbiosis. Furthermore, it is now clear that all of the diseases in question astonishingly involve a common immunologic defect found in the absence of symbiotic bacteria. As we have co-evolved with our microbial partners for eons, have strategies used against infectious agents reduced our exposure to health-promoting bacteria, ultimately leading to increased disease? We propose that the human genome does not encode all functions required for health, and we depend on crucial interactions with products of our microbiome (collective genomes of our gut bacterial species). Through genomics, microbiology, immunology and animal models, we wish to define the molecular processes employed by symbiotic bacteria that mediate protection from disease. Advances in the past year have now made it possible to mine this untapped reservoir for beneficial microbial molecules. Ultimately, understanding the immune mechanisms of these *symbiosis factors* may lead to natural therapeutics for human diseases based on entirely novel biological principles.

**330. Dynamic surface variation by symbiotic bacteria is required for host colonization**

*Sung-Eun Lee, Janet Chow, Rue Shen, Julie Huang*

Bacterial surfaces represent functional organelles decorated with molecules that mediate critical interactions between the microbes and their milieu. These environments may be on or within another organism; not uncommonly, that organism is a mammal. Capsular polysaccharides are abundant external structures of bacteria, and the capsules of many prokaryotic pathogens have been found to be important virulence factors during mammalian infection. Unlike pathogens, commensal bacteria establish a life-long co-habitation with their mammalian hosts. However, the molecular mechanisms employed to establish this beneficial relationship remain almost entirely undescribed. The unique identification of multiple surface polysaccharides in the important human symbiont *Bacteroides fragilis* raised the critical question of how these molecules contribute to commensalism. Herein, we report that mutation of the master regulator of *B. fragilis* polysaccharide expression results in a global reduction of capsule. Surprisingly, attempts to completely eliminate expression of capsule are not tolerated and result in abrogation of bacterial growth. Subsequently, the organism acquires a spontaneous mutation that restores growth and production of at least one capsular polysaccharide. We identify an alternative pathway by which *B. fragilis* is capable of re-establishing capsule production. Most importantly, mutants expressing single, defined surface polysaccharides remain defective for intestinal colonization of animals compared to bacteria that express a complete polysaccharide repertoire, a process mediated by specific interactions between bacteria and intestinal mucus. The extensive surface diversity and multiple layers of regulation suggest a profound evolutionary requirement for capsular polysaccharide during host-bacterial symbiosis.

**331. Host-bacterial mutualism by a microbial symbiosis factor prevents inflammatory disease**

*June L. Round, Juscilene Menezes, Sara McBride*

Colonization of humans with multitudes of commensal species creates an ecosystem harboring members of five of the six kingdoms of life. Bacteria in particular dominate this ecologic niche; the gastrointestinal tract is resident to an astounding $>10^{14}$ microorganisms with a diversity of approximately 2,000 species. This consortium of gut bacteria represents an integral factor in mammalian biology. Germ-free animals, born and raised under sterile conditions, exhibit profound defects in the development of intestinal tissues. Many reports have shown that both gastrointestinal and systemic immune responses are deficient in the absence of commensal microoroganisms. Surprisingly however, the gut is stably colonized by both beneficial and potentially pathogenic microorganisms; the reasons for this phenomenon remain unclear. Moreover, imbalances in the composition of the

bacterial microflora, known as dysbiosis, are thought to be a major factor in human disorders such as inflammatory bowel disease (IBD). We report herein that the ubiquitous human symbiont, *Bacteroides fragilis*, protects animals from experimental colitis induced by the pathogenic commensal, *Helicobacter hepaticus*. Most importantly, this beneficial activity requires a single bacterial molecule (Polysaccharide A or PSA). Animals harboring *B. fragilis* not expressing PSA develop disease and produce pro-inflammatory cytokines in colonic tissues similar to *H. hepaticus* colonization alone. Purified PSA administered to animals protects from experimental colitis and wasting disease by inducing anti-inflammatory responses, both *in vivo* and *in vitro*, and activation of interleukin 10-producing $CD4^+$ T cells. These results reveal the first molecule of intestinal commensal bacteria that mediates the critical balance between health and disease. As incidence of IBD have dramatically increased over the last several decades, harnessing the immunomodulatory capacity of *symbiosis factors* such as PSA may ultimately provide novel therapeutics for human inflammatory disorders.

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**Research and Laboratory Staff:** Arnavaz Garda, Carolyn Ohno, Daphne Shimoda

**Support:** The work described in the following research report has been supported by:
Balzan Foundation
DOE
Gosney Postdoctoral Fellowship
Helen Hay Whitney Memorial Fund
Human Frontier Science Program
Lund University
NIH
NSF

**Summary**: The work of our laboratory is directed to the goal of understanding how plants grow. While this is an intellectual problem of great depth, it is also important for practical reasons, as our food, fiber, and even the oxygen we breathe comes from plants. We are concentrating on understanding the causal relations between gene activity, protein location, and cell division in the growing tip of the flowering shoots of the laboratory plant *Arabidopsis thaliana*. At the shoot tip is a collection of stem cells called the shoot apical meristem. This meristem is the source of all of the cells that make the above ground part of the plant; only the hypocotyl and root system are formed from cells that do not originate in the shoot apical meristem.

The shoot meristem is also the source of a number of mysteries that derive from its structure and activities. The meristem is composed of different regions, defined by unique and persistent patterns of gene activity. The dividing cells of the meristem move through these regions, starting in the Central Zone at the tip of the meristem, which are the pluripotent stem cells that give rise to stem, leaves and flowers. Cells depart from the Central Zone to either the Peripheral Zone, which is the region where leaves and flowers form, or to the Rib Meristem, where the cells of the stem originate. One question is how the spatial location of the meristematic zones remains constant (for centuries in some plants) while the cells that comprise them are ever-changing. Another is how the cells in the Peripheral Zone and Rib Meristem form ordered tissues, with many cell types in proper relation to each other. Yet another is how the leaves and flowers are formed in the proper pattern around the meristem, the phyllotactic pattern, such as the spiral pattern characteristic of *Arabidopsis* leaves and flowers, and of pine cones and pineapples. One final mystery that we are working to solve is the developmental origin of shoot apical meristems – they self-organize spontaneously in masses of cultured plant cells called callus, by processes that are only now beginning to be observed.

We have made progress with answering all of these questions, as detailed in our published papers, and in the abstracts that follow.

**332. Investigation into the role of mechanical signals in regulating microtubule orientations**

*Marcus G. Heisler, Olivier Hamant[1], Pawel Krupinski[2], Henrik Jonsson[2], Jan Traas[1]*

During the development of multicellular organisms, the regulators of growth and patterning must interact with the mechanical properties of individual cells to generate specific tissue morphologies. Using a combination of physical, mathematical and biological approaches, we have investigated the influence of mechanical signals on microtubule orientation in meristem epidermal cells. We find that microtubule orientations correlate with stress patterns, as predicted by the Finite Element Method (FEM) both during normal development as well as in response to cell ablation. Direct mechanical perturbation also results in a reorientation of microtubules such that they align perpendicular to an applied compression and parallel to observed extension. These results are consistent with a proposal that cell anisotropy resists and feeds back on local stress patterns.

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**333. Investigation into co-alignment of PIN1 polarity and microtubule orientation**

*Marcus G. Heisler, Olivier Hamant[*], Carolyn Ohno, Jan Traas[*]*

Through simultaneous imaging of multiply colored GFP markers, we have found that the polarity of the auxin efflux carrier PIN1 correlates with interphase microtubule orientations in the epidermis of the *Arabidopsis* meristem. This correlation also holds in terms of response to the ablation of nearby cells. We are investing the causal basis of this correlation and whether PIN1, like microtubules can be influenced by mechanical perturbation. So far we have determined that the protein kinase PINOID alters PIN1 such that the PIN1 response to cell ablation is significantly reduced; whereas, we find that microtubules respond normally in a *pid* background. If PIN1 and the microtubules are responding to a single upstream factor to affect their correlation, our data indicates that PID functions downstream of this signal in a

PIN1-specific manner and that PID does not act as a simple binary polarity switch, as previously proposed.

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**334. Establishment of adaxial/abaxial patterning of lateral organs in *Arabidopsis***

*Marcus G. Heisler, Carolyn Ohno*

A fundamental patterning process in plant and animal development is the positioning of lateral organs and their partitioning into distinct cell types. In plants the dorsoventral boundary of organs such as leaves defines an axis of symmetry through the centre of the organ separating the upper (dorsal or adaxial) and lower (ventral or abaxial) organ tissues. We have found that auxin acts to promote adaxial cell fate by turning on REVOLUTA (REV) expression. However, this can only occur in cells not already expressing miRNA 165 or 166, both of which are antagonistic to REV and other HD-ZIP genes. By expressing REV throughout the meristem region, we have determined that these miRNAs are expressed in a concentric domain surrounding the meristem and that when auxin accumulation occurs to specify new primordia, this occurs on the edge of this domain. This means those cells accumulating auxin on the central (adaxial) side of the miRNA domain end up expressing adaxial genes whereas, those located more peripherally which are already expressing the miRNAs, are fated to be abaxial. Lastly, we have observed ectopic *REV* expression in *kanadi* (*kan*) triple mutants and that KAN1 is expressed in a similar domain to the miRNAs. Together these data suggest the KAN genes somehow coordinate miRNA activity in developing primordia and at present we are investigating this possibility in more detail. Overall our data demonstrate that plant organ cell type patterning along the adaxial/abaxial axis is directly inherited from a meristem pre-pattern and that organ position is somehow coordinated with the radial patterning of meristem cell types.

**35. Function of a lipid/sterol-binding domain in HD-Zip transcription factor GLABRA2**

*Kathrin Schrick, B.P. Venkata[1], Sara A. Klemin[1], B.P. Srinivas[2], Martin Hülskamp[2]*

In plants, putative lipid/sterol binding domains include members of the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) family. START domains are integral components of various signaling proteins in multi-cellular eukaryotes. Mammalian START proteins bind sterols, phospholipids or sphingolipids, and exhibit diverse functions and sub-cellular distribution, including nuclear localization. Despite this, their function in transcription is not clear. In plants, START domains are found in homeodomain leucine zipper (HD-Zip) transcription factors, consistent with the hypothesis that ligand binding to the START domain may regulate transcription factor activity of these proteins. In *Arabidopsis*, GLABRA2 (GL2) is a representative family member that acts in a complex regulatory circuit controlling patterning and cell-type differentiation of the epidermis. We are using site-directed mutagenesis and deletion of the START domain of GL2 to address its presumed function as a regulatory module within the transcription factor. Our results show that although deletion of the START domain does not affect GL2 mRNA expression, protein levels are greatly reduced. Thus, the START domain appears to be required for stable post-translational expression of the GL2 transcription factor, perhaps via ligand binding. The $gl2^{\Delta START}$ mutant protein, although weakly expressed, is found in the nucleus during embryogenesis, indicating that the START domain is not essential for nuclear localization. In addition, several missense mutants that alter key residues within the predicted ligand-binding pocket result in reduced GL2 function. An example is the $gl2^{R384L}$ mutant, which corresponds to $StAR^{R182L}$, a mutation affecting a conserved arginine residue in the human *StAR* gene from lipoid congenital adrenal hyperplasia (CAH) patients. Plants expressing the $gl2^{R384L}$ missense mutation exhibit reduced levels of protein expression concomitant with defects in epidermal cell differentiation. Although the putative ligand for the GL2 START domain is not yet known, our experiments test a model in which the transcriptional activity of the HD-Zip protein is regulated by a positive feedback loop via binding to its START domain.

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**336. Genome-wide expression profiling of *Arabidopsis* sterol biosynthesis mutants**

*Kathrin Schrick, Marta Bjornson[1], Vijaya Kumar[2], José Luis Riechmann[2]*

Several sterol biosynthesis mutants of *Arabidopsis* exhibit striking defects in embryonic patterning, but direct evidence for the roles of steroid hormones other than brassinosteroids in plant signaling is lacking to date. The aim of this project is to identify components of sterol signaling pathways that are differentially expressed under conditions of aberrant sterol composition. In our genome-wide microarray experiment, *fackel (fk)*, *hydra1 (hyd1)* and *cephalopod (cph)* sterol biosynthesis mutants deficient in sterol C-14 reductase, C-8,7 isomerase, and C-24 methyltransferase, respectively, were examined in conjunction with *gurke (gk)* sphingolipid synthesis mutants that are deficient in acetyl CoA carboxylase. All four mutants display a similar dwarf seedling phenotype, while only the three sterol biosynthesis mutants exhibit a primary defect in sterol production. The mutant gene expression profiles were compared to a wild-type control. We identified a set of 218 genes that were differentially expressed in the sterol biosynthesis mutants but not in *gk* ($\geq$2-fold change, $P<0.05$). Among the up-regulated sterol-biosynthesis specific mRNA set, there is an overrepresentation of

membrane proteins and transcription factors, suggesting a critical role for transcription in sterol response. Included are two members of the homeodomain leucine zipper (HD-Zip) StAR-related lipid transfer (START) transcription factor family, as well as a gene encoding a transcription factor that interacts with members of this family. The presence of START domains in the class III and IV HD-Zip transcription factors has implicated their involvement in regulation by lipid/sterol ligands, and our data are consistent with this hypothesis. In addition, the genome-wide expression results reveal an extensive overlap between mRNAs differentially expressed in sterol and sphingolipid synthesis mutants. We identified shared up-regulation of two genes that are implicated in sterol or lipid binding: *SCP2*, which encodes sterol carrier protein-2, and *GLABRA2* (*GL2*), which codes for a class IV HD-ZIP transcription factor. These data indicate that common mRNA expression pathways are influenced coordinately by sterol and sphingolipid composition.

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**337. Investigation of the mechanism of *MODIFIER OF B FUNCTION***

*Carolyn Ohno, Cory Tobin*

The *MODIFIER OF B FUNCTION (MOB)* gene is required for proper specification of floral organ identity during flower development. MOB encodes a broadly expressed, nuclear-localized protein with a candidate DNA binding domain. MOB is essential for repressing the B function petal and stamen organ identity gene *PISTILLATA (PI)* even prior to the onset of reproductive development, since plants lacking *mob* function express abnormal random patches of *PI* transcripts in vegetative tissues such as cotyledons, leaves and stems, as well as in reproductive organs that do not normally express *PI*. To assess if repression of *PI* transcription is directly mediated by MOB, we are employing recent advances in chromatin immunoprecipitation (ChIP) technology to assay for *in vivo* interaction of the MOB protein with regulatory regions of the *PI* promoter. A functional MOB-GFP fusion protein has been immunoprecipitated with anti-GFP antibody from both floral and vegetative tissues, in case tissue-specific cofactors are required for a stable MOB protein-DNA interaction. At the same time we are surveying possible histone modifications at the *PI* locus that might be enriched or depleted in plants deficient for *MOB*. In parallel studies we have generated transgenic plants such that we can monitor the expression of a *PIp::dsRED-N7* B function reporter gene in response to inducible expression of *MOB* in order to determine the relative timing and spatial specificity of MOB's ability to repress *PI*. These ongoing studies are to investigate the mechanism of MOB's role in the timely maintenance of whorl-specific expression of petal and stamen organ identity genes.

**338. *Arabidopsis* flower transcriptome analysis at single-cell-type resolution**

*Yuling Jiao*

We are developing and will apply methods for RNA extraction and chromatin immunoprecipitation from specific cell types in developing *Arabidopsis thaliana* flowers. The principal deficit in current microarray and chromatin immunoprecipitation experiments on *Arabidopsis* shoots, including our own, is that the RNA and DNA used comes from a mixture of different cell types. As cell-type-specific data are necessary to infer gene regulatory networks, current types of data are inadequate to understand flower development. We are adapting existing methods for expressing epitope-tagged ribosomal proteins, and new constructs and methods to drive cell-type-specific expression, and will apply methods we have developed for obtaining large amounts of floral tissue at defined developmental stages, followed by immunoprecipitation of polysomes, to perform cell- and stage-specific transcriptome analysis of 12 different floral cell types at multiple critical developmental stages.

**339. Transcriptome-wide analysis of decapped mRNAs in *Arabidopsis* reveals regulation through decapping**

*Yuling Jiao, José Luis Riechmann*

The composition of the transcriptome in eukaryotic organisms is regulated by both mRNA transcription and degradation. One route for mRNA decay is through 5' decapping, which can be initiated by decapping enzymes and small RNAs. Although decapped RNAs are an important intermediate for mRNA decay, their identity and abundance have never been studied at a large scale. We have developed a novel experimental method for transcriptome-wide profiling of decapped mRNAs. We applied the method to study the prevalence of decapped transcripts during the early stages of *Arabidopsis thaliana* flower development. Decapped transcripts were identified for the majority of expressed genes, although at different levels. By comparing decapped RNA levels with steady-state overall transcript levels, our study provided evidence for widespread decapping-mediated mRNA degradation control in numerous biological processes and for genes of varied molecular functions, implying that mRNA decapping is a dynamically regulated process. Sequence analyses identified structural features of transcripts and *cis*-elements that were associated with levels of decapping.

**340. Cytokinin perception in the shoot apical meristem**

*Sean Gordon, Vijay Chickarmane, Elliot M. Meyerowitz*

The hormone cytokinin is known to be an activator of biological processes such as cell proliferation and cell senescence. Experiments over the last several years have shown that the biochemical network for cytokinin perception is essentially composed of a two-

component signaling network involving histidine kinases, and the type A & B *Arabidopsis* response regulators. The network responds to cytokinin by using signaling and gene regulatory interactions that have the ultimate effect of inducing negative feedback of cytokinin activity. We seek to understand the functionality of this network. Towards this end, we have developed a computational model of the signaling-genetic circuit that suggests that the network functions as a switch, such that beyond a certain threshold level of the input concentration of cytokinin, the output is strongly induced in a switch-like manner. One of the central questions which we address here is how the regulatory circuit for cytokinin signaling leads to a specific patterning of cells, which ultimately maintain stem cells in the shoot apical meristem. We are using confocal microscopy and gene expression profiling to test specific predictions made by the model.

**341. How does the shoot apical meristem achieve homeostasis of stem cell numbers?**

*Vijay Chickarmane, Sean Gordon, Paul Tarr, Henrik Johnsson, Eric Mjolsness, Elliot M. Meyerowitz*

The shoot apical meristem, which is located at the tip of the shoot, houses stem cells. It is a veritable fountain of youth, since it provides differentiated cells to form lateral organs, as and when they are required by the plant. It is a fascinating question, as to how the number of stem cells is maintained, by signaling and regulatory interactions between these cells and other more differentiated cell populations. Genetic studies in our laboratory have revealed one piece of this puzzle, which sheds light on a negative feedback mechanism, whereby cells located below the stem cells (organizing center--OC), maintain stem cells (forward loop), and where the stem cells down-regulate the activity of the OC cells (backward loop). Specifically, cells re-specify from neighboring cell populations back into stem cells, when the backward loop of the feedback mechanism is interrupted.

To uncover other possibilities of regulation and general principles of homeostasis, we are employing a computational approach. We have developed population based models that keep track of the number of cells of each population, where the rate of transitions from one population to the other are defined by the nature of the regulation. Through these population models we seek to identify which types of regulatory interactions can give rise to homeostasis. This provides a hypothesis, which we will use to test against the results of genetic experiments. The population models are amenable to a stochastic treatment, which would answer the question, as to the mean exit time for extinction of the meristem, i.e., when the cell numbers go to zero. The next goal of this project is to scale this to a full spatial model, based upon a lattice, where each cell can be tracked independently. Such a combined computational/experimental approach. we believe, will significantly improve our understanding of the general paradigm of stem cells and their interactions with the niche.

**342. Identification of interactors of the GATA-like transcription factor HANABA TARANU during *Arabidopsis* development by mutagenesis**

*Xiang Qu, Elliot M. Meyerowitz*

*HAN* (*HANABA TARANU*) encodes a GATA-like transcription factor that is essential for floral development in *Arabidopsis*. All four identified *han* mutants (*han-1*, *han-2*, *han-3*, and *han-4*) display dramatic floral phenotypes, with fused sepals and reduced organ number throughout the four floral whorls. The expression pattern of *HAN* in the shoot apical meristem (SAM) and floral meristem is distinctive, with strong expression at the boundaries between the meristem and its newly initiated organ primordia, and at the boundaries between different floral whorls. Although *han* mutations have minor effects upon vegetative SAMs, *han;clv* double mutants display highly fasciated SAMs. Along with the observation that the WUS expression pattern is altered in *han* mutants, our data suggest that HAN is also involved in SAM development. To understand the molecular mechanism by which HAN regulates flower and SAM development, in collaboration with Dr. Wolfgang Lukowitz at Cold Spring Harbor Labs, we have conducted a second-site screen for suppressors from the EMS-mutagenized *han-2* plants. About 20,000 mutagenized plants from 40 pools were screened, among which, 64 repressors and 25 enhancers were identified. One identified enhancer line contains a point mutation within the coding region of *AP2*. The *ap2; han-2* double mutant displays severe floral phenotypes, lacking the outer three whorls including sepal, petal and stamen. Further analysis and mapping other mutants is currently ongoing.

**343. Functional analysis of CLV2 in SAM maintenance**

*Xiang Qu, Elliot M. Meyerowitz*

Located at the growing tip of stems, shoot apical meristems (SAMs) are actively dividing, embryonic tissues responsible for all the aerial organs in plants. A balance between meristematic cell division and differentiation is required to maintain a functional SAM. In *Arabidopsis*, the *CLAVATA* (*CLV*) genes encode important elements in SAM maintenance. Single loss-of-function mutations of the *CLV* genes (*clv*) result in a progressive enlargement of shoot and floral meristems. Among these genes, the *CLV2* gene contains a single exon that encodes a 720-amino acid LRR-RLP (Leucine-rich repeat receptor-like protein). It was hypothesized that CLV2 forms a heterodimer with CLV1 so as to stabilize the CLV1 signaling complex at the plasma membrane. However, recent finding that *clv1-11 clv2-1* closely resembles *clv3-2* (with a much stronger meristem enlargement phenotype than *clv1-11*, a loss-of-function allele, alone) indicates that *CLV2* has functions other than as a *CLV1* partner. We have now been using a

combination of methodologies, including biochemical, genetic, and cell biological approaches, to gain information in molecular detail on how the receptor-like protein CLV2 functions as a key element to maintain a functional SAM. We have generated constructs that allow us to visualize the CLV2 protein directly under a confocal microscope and/or detect it indirectly by chemi-luminescence. We are also testing now functions of various domains of CLV2 during SAM maintenance using both a tobacco transient expression system and stable *Arabidopsis* transgenic lines.

**344. Identification of additional signaling components within the CLV pathway**

*Xiang Qu, Elliot M. Meyerowitz*

Signal transduction in cells typically requires protein-protein interaction. However, little is known the composition of the CLV signaling complex, other than the direct interaction between the peptide ligand CLV3 and the receptor kinase CLV1. CLV1 has been previously reported to form two distinct complexes with respective molecular masses of 185 kD and 450 kD. The presence of CLV3 is required for CLV1 to form the 450-kD complex. In addition, CLV1 monomer elutes upon treatment with reducing agent, suggesting that intermolecular disulfide linkages might be important for formation of the CLV1 signaling complexes. However, these published data contradict our recent findings on the stability of CLV1, which show that CLV1 is unstable and undetectable in the absence of CLV3. In addition, CLV1 and CLV2 act independently to regulate the SAM based on the recent study of CRN and our *clv1;clv2* double mutant analysis. Thus, we are reanalyzing the CLV signaling complex by use of both genetic and biochemical approaches. We are conducting a sensitized genetic screen for CLV1-interacting genes. We are also conducting a series of experiments to examine the CLV1/CLV2 protein complexes in the SAM and further to identify novel components that directly interact with CLV1/CLV2 by *in vivo* protein complex purification and mass spectrometry.

**345. Boundary specification and maintenance between the shoot apical meristem and organ primordia**

*Xiaolan Zhang, Elliot M. Meyerowitz*

Boundaries serve the purpose of delimiting regions of gene activity and of separating distinct organs as they develop. M-O boundaries (meristem-organ) are formed to separate plant organ primordia from the shoot apical meristem (SAM), whereas O-O boundaries (organ-organ) develop between individual floral organs to create space between them. Loss of boundaries can result in abnormal organ fusion, failure of SAM initiation and defects in SAM maintenance. However, little is known of when and how the boundaries are specified and maintained during plant development. There are three hypotheses for M-O boundary formation: (1) cells that downregulate the meristematic marker *SHOOT MERISTEMLESS (STM)* immediately acquire boundary identity, which acts as a barrier allowing their progeny to gain organ primordial identity; (2) juxtaposition of *STM* and primordial marker *ASYMMETRIC LEAVES1 (AS1)*-expressing cells is required to form a boundary; and (3) *STM* and *AS1* expression transiently overlap, and the overlapping cells become the boundary. To test which hypothesis is true, I will use fluorescent tags to label STM (*pSTM::STM-VENUS*), AS1 (*pAS1::AS1-CFP*) and boundary gene CUP-SHAPED COTYLEDONS2 (*pCUC2::CUC2-GFP*) simultaneously, then track their expression dynamics during new primordial initiation using live imaging. To explore how boundaries are maintained, dexamethasone-induced transient overexpression constructs of *STM, AS1* and CUC2 have been made, and changes in boundary formation will be tracked at different times after ectopic induction. Similar experiments will be performed using inducible inactivation lines *(p35S::-STM ds RNAi-GR, p35S::AS1 ds RNAi-GR* and *p35::-CUC2 ds RNAi-GR)* to validate results from the above experiments. Furthermore, three additional boundary genes (*ASYMMETRIC LEAVES 2-LIKE 9, LATERAL ORGAN BOUNDARY* and *JAGGED LATERAL ORGANS*) can be used to identify boundary cells when *CUC2* is being manipulated.

**346. Characterization the causal relations between HANABA TARANU, WUSCHEL and CLAVATA3 by live imaging**

*Xiaolan Zhang, Elliot M. Meyerowitz*

*HAN* (*HANABA TARANU*) encodes a GATA transcription factor that is expressed at both the M-O and O-O boundaries. *HAN* knockouts display small and flat SAMs, fused sepals and reduced numbers of floral organs. Mutation of *HAN* also leads to an abnormally diffuse domain of WUSCHEL (*WUS)* expression (normally a marker of the center of the SAM) and enhancement of the expanded meristem phenotype of *clv* mutants, suggesting that *HAN* regulates SAM expansion through the *WUS-CLV* pathway. However, it is completely unknown how boundary-expressing *HAN* affects floral development and SAM organization, and in particular, how *HAN* interacts with *WUS* and *CLV*. In order to understand the causal relations between HAN, WUS and CLV activity, following four experiments will be performed with the use of live imaging: (1) to test whether the *WUS* expression pattern undergoes dynamic change or remains relatively stable in the misshapen *han* mutant meristem, *pWUS::GFP-ER* will be observed in *han* homozygous mutant plants; (2) to elucidate how HAN regulates meristem organization, a *pCLV3::GFP-ER; pWUS::dsRED-N7* line will be crossed to inducible *p35S::HAN-GR* plants, and changes of *CLV3* and *WUS* expression will be monitored upon ectopic *HAN* activation. Moreover, to test whether *HAN* affects meristem formation through cell division, meristem reorganization or both, co-detection of *pCLV3::mGFP-ER* and *p35S::YFP29-1* (a general nuclear marker that allows all cell divisions to be seen) upon *HAN* induction will be performed as well; (3) to monitor the action of WUS and CLV on *HAN*, *pHAN::GFP-ER* will be tracked upon

transient induction of *WUS* and *CLV3*; and (4) despite the expression domains of both *WUS* and *HAN* being expanded in the *clv* mutant SAM, the temporal order of expansion is unknown. Live imaging of *HAN* and *WUS* upon removal of CLV function will answer this question. The appropriate strain will be made by crossing a *pWUS::dsRED-N7*; *pHAN::GFP-ER* line to a *CLV3* inactivation line (*p35S::GR-LhG4; p6XOP::CLV3 ds RNAi*).

**347. Identification of protein interactors and direct targets of boundary genes CUP SHAPED COTYLEDONS2 and HANABA TARANU**

*Xiaolan Zhang, Elliot M. Meyerowitz*

Boundary genes play essential roles in boundary formation, meristem regulation, and floral organ development. A long list of boundary regulators has been identified in *Arabidopsis thaliana*, including *CUP-SHAPED COTYLEDONS 2* (*CUC2*) and *HANABA TARANU* (*HAN*). *CUC2* and *HAN* encode transcription factors that may regulate downstream genes whose nature will lead to hypotheses for boundary gene function. The distinct morphology of M-O boundary cells may: 1) increase their surface area to facilitate signal transduction; 2) better serve as a physical barrier to protect the stem cell niche from developing leaf and flower primordia; or 3) divide in their unusual pattern to shape the meristem and primordia. To better understand the mechanism of how boundary genes lead to boundary cell morphology and division pattern, we plan to identify direct targets of, and interactors with, boundary genes at a genome-wide scale. *CUC2* is expressed extensively in cultured callus, which enables the use of callus as starting material to identify *CUC2* target genes by chromatin immunoprecipitation with precipitated DNA identified using microarrays (ChIP-on-chip). Antibodies against *CUC2* will be generated and used for ChIP using induced callus from a *pCUC2::CUC2-GFP* line. Alternatively, the precipitated DNA will be made into a library and sequenced using an Illumina DNA sequencer. Further molecular and reverse genetic analysis will be performed to validate the expression domains of identified candidates in growing plants. Similar ChIP-on-chip experiments will be performed for *HAN* if its expression is throughout callus. Otherwise, whole genome oligonucleotide microarrays will be used to identify *HAN* target genes using a *p35S::HAN-GR* line. Floral buds upon 0, 12 hours and 3 days after DEX treatment will be collected for microarray hybridization. Expression profiling will also be performed comparing floral buds of *han2* mutants and those of wild type. Common candidate targets of *HAN* identified by the above microarray analyses will be further verified by ChIP-PCR and reverse genetic assays. To identify protein interactors of CUC2 and HAN, I will use yeast two hybrid assays to screen for candidates, followed by GST pull down and co-IP to verify the interactions *in vitro* and *in vivo*, respectively.

**348. Characterization of callus formation from the *Arabidopsis* root**

*Kaoru Sugimoto, Sean Gordon*

Plant cells have been recognized as totipotent because they are competent to regenerate the full array of plant tissues, unlike most animal cells. In the commonly used *in vitro* plant regeneration system, first, a mass of growing cells (callus) is formed from a small piece of tissue (explant) on callus-inducing medium. Subsequent culture of the callus on shoot- or root-inducing medium causes the cells to be specified and differentiate into shoot or root tissues, respectively. Because cells are thought to acquire competency and proliferate as they form callus, callus formation might be a key event in plant regeneration. However, little is known about what kind of cells comprise callus and what differentiation status they go through when they divide to form the callus. We have therefore started experiments to determine the nature of the cells that form callus. As the first step, we characterized callus formation from root explants, which are widely used as a tissue source for the *Arabidopsis* regeneration assay, by observing fluorescent root tissue markers. We find that callus is not a random cell mass but a somewhat organized tissue similar to a lateral root primordium, which suggests callus is formed neither by the simple proliferation nor de-differentiation of some specific type of cells, but rather by the somewhat ordered differentiation and proliferation of internal cells that resembles lateral root primordium formation.

**349. A unified description of the nature of callus tissue in *Arabidopsis***

*Kaoru Sugimoto*

A wide variety of plant tissues have been shown to form callus (a mass of growing cells) and regenerate whole plants under proper culture conditions. However, it remains unknown whether calli derived from different tissues are equivalent to each other in their differentiation status, and what elements provide callus with its regeneration ability. In addition to roots (mentioned in the above corner), we induced callus from cotyledons and petals and observed florescent reporters at a few time points. Although roots, cotyledons, and petals are distinct in their position, origin, and differentiation in plant development, to our surprise we find a quite similar reporter gene expression pattern in the callus derived from each of these tissues. When callus is formed, the cells differentiate to express root tissue markers regardless of the tissue of origin, even if these root markers were not expressed in the original organs. The calli from all three tissues form a somewhat organized tissue like a lateral root primordium. We verified this up-regulation of root genes in callus derived from the three organs using whole-genome microarrays. Microarray data also gives us the information at a genome-wide scale about whether callus is similar to any tissues other than lateral root primordia, such as shoot meristematic, embryonic, or vasculature tissues, which give rise to new organs in normal plant

development. The results suggest, so far, that callus resembles to none of these other tissues and only the root primoridium. Now we are repeating the microarray experiments and more detailed analysis will be done on it. These findings raise the next questions whether callus forms under the same genetic controls as lateral root formation and whether callus growth and its regeneration ability depends on the function of root meristem genes in just the same way as a developing root. To answer these questions, we will analyze mutant lines, which are defective in lateral root formation or root meristem formation.

**350. Dissection of the flower initiation pathway using genetic and genomic approaches**

*Wuxing Li*

Understanding of the regulation of fate determination and patterning in organisms including plants and animals requires insight into their genetic regulatory networks. The shoot apical meristem of *Arabidopsis* provides an excellent system to study processes in cell division, cell differentiation, and cell fate determination. Knowledge of the regulation of the flowering process is of significant agricultural importance and also answers fundamental questions in biology. I propose to systematically characterize the process of the floral transition utilizing both genetic and genomic approaches. The transcription network will be examined during the floral transition using expression profiling and dynamic changes patterns of global transcription will be analyzed. Genes that either promote or repress the floral transition will be incorporated to build models mathematically to explain the floral transition process. Chromatin status during this process will also be examined and the involvement of genes, either the regulators of chromatin status, or genes that whose expression is regulated at the chromatin level will be determined.

**351. Genetic studies of the expression pattern of LEAFY**

*Wuxing Li*

Recent studies have identified the *Arabidopsis* gene *LEAFY* as a master regulator in specifying floral meristem identity. I propose an in detail characterization of this gene: (1) factors that control temporal and spatial pattern of *LEAFY* expression; (2) upstream components of *LEAFY* function; and (3) genes that interact genetically or biochemically with *LEAFY*. The expression pattern of *LEAFY* has been observed in different mutant background and an auxin function-related gene was found to potentially regulate *LEAFY* spatial expression. Through a mutagenesis study in the background of a weak *lfy-5* allele, I have obtained several putative novel genetic modifiers, and further characterization of these modifiers is expected provide new insight into the floral initiation pathway. The above mentioned auxin function-related gene was also found in the mutagenesis study and further analysis is being carried out to dissect its interaction with *LEAFY*. Several lines of fusion protein transformants are available and will be applied to screen for *LEAFY*-interacting partners. It is also expected that yeast two-hybrid experiments will elucidate new components in the pathway of *LEAFY* function. This research will advance our understanding in fundamental questions such as cell fate determination.

**352. The timing of endoreduplication controls relative cell size**

*Adrienne H.K. Roeder, Vijay Chickarmane*

About 100 years ago, Boveri and colleagues found that generally the size of a cell correlates with the DNA content or ploidy of its nucleus. The DNA content of a cell increases through endoreduplication, a specialized cell cycle in which the cell grows and replicates its DNA but does not divide, and as a consequence becomes enlarged. Endoreduplication is common in plants and insects, but is also found in specialized mammalian cell types such as megakaryocytes that generate platelets for the blood. In *Arabidopsis*, the epidermis contains cells in a wide range of sizes and corresponding ploidies. We have developed the epidermis of the sepal, the outermost green leaf-like floral organ, as a model system for studying cell size and cell fate. The sepal epidermis contains giant cells stretching as far as a third the length of the sepal and small cells, which stretch about $1/30^{th}$ the length of the sepal. To determine how this pattern of cells with diverse sizes forms, we imaged individual living sepals every 6 hours during their development. We found that giant cells terminate division and start endoreduplicating extremely early in sepal primordia. Simultaneously, the smaller cells continue to divide as many as four times. In addition, the area of one giant cell is approximately the same as the area of all the progeny of a neighboring cell suggesting that the cells are growing at the same rate. Therefore, we propose that there is a trade off between mitotic cycles and endocycles and that each progenitor cell has a limited number of cycles, which can be apportioned between them. Once a cell endoreduplicates, it can no longer divide, so the time at which a cell starts to endoreduplicates controls the number of endocycles it completes. The more endocycles a cell completes the larger it becomes. We have created a computational model for the development of the sepal epidermis in which cells decide to divide or endoreduplicate in each cell cycle which a given probability. In this model, cells enter endoreduplication at different times, and recreate the pattern of diverse cell sizes we observe in the sepal.

**353. Cell cycle inhibitors control giant cell formation**

*Adrienne H.K. Roeder, Vijay Chickarmane, Aida Sun, Alexandre Cunha*

We have tested our computational model of the sepal epidermis by changing the probability with which cells endoreduplicate to determine if the simulated sepals match the sepals of plants in which endoreduplication is

promoted or inhibited. First, if we increase the probability of endoreduplication, the simulated sepals are largely covered by giant cells similar to the sepals of *ATML1::KRP1* transgenic plants (kindly provided by Dr. Keiko Torii) in which the cell cycle inhibitor *KRP1* is expressed throughout the epidermis. Conversely, if we decrease the probability of entering endoreduplication early in sepal development, our computational model produces sepals without giant cells. In a screen for *Arabidopsis* mutants that fail to produce giant cells in the sepal epidermis, we isolated the *loss of giant cells from organs* (*lgo*) (think small blocks) mutant. The sepals of *lgo* mutants are completely covered by small cells, however, the overall size of the sepals is slightly larger than wild type, showing that the growth of the sepals is not affected. Endoreduplication is not generally affected in the *lgo* mutant, since the highly endoreduplicated hair cells, or trichomes, form normally. Upon positional cloning, we found that *LGO* encodes a small putative cell cycle inhibitor in the plant-specific *SIAMESE* family. We conclude that cell cycle inhibitors are likely to regulate the timing of endoreduplication and consequently the cell size pattern.

**354. The interplay between cell cycle control and cell fate in the sepal epidermis**

*Adrienne H.K. Roeder, Carolyn Ohno*

To form an organ, the processes of cell division and developmental specification of each constituent cell must be tightly coordinated. Recently, a cell cycle inhibitor has been shown to promote cell type specification in the *Xenopus* retina suggesting that cell cycle regulators can also influence cell fate (Ohnuma, Philpott *et al*., 1999). The epidermis of the *Arabidopsis* sepal contains cells in a range of sizes and a corresponding range of ploidies generated through regulation of the cell cycle. Do cells of different sizes also have distinct identities or are they all have the same fate and merely differ in size. To address this question, we examined the expression pattern of enhancer trap lines in the developing sepal. One enhancer trap line is expressed specifically in small cells, suggesting that these cells have a unique molecular identity. Conversely, another enhancer trap line, YJ158, is expressed in the giant cells of sepals and leaves as well as the leaf margins. (Eshed, Izhaki *et al*., 2004). YJ158 is not expressed in trichomes indicating that it is not a marker for highly endoreduplicated cells. A 1 kb enhancer element upstream of the YJ158 insertion site is sufficient to produce a giant cell-specific expression pattern in the sepals. Surprisingly, the ectopic giant cells formed in *ATML1::KRP1* sepals also express the giant cell marker suggesting that early entry into endoreduplication is sufficient to trigger giant cell specification. On the other hand, the giant cell enhancer is expressed in many of the small cells in the *loss of giant cells from organs* (*lgo*) mutant sepals suggesting that extensive endoreduplication is not necessary for giant cell identity. One possibility is that the *lgo* cells expressing the giant cell marker are the first to endoreduplicate in these sepals, even though they endoreduplicate later than normal giant cells. We will further investigate the specification of giant cells to determine whether the relative timing of endoreduplication controls specification.

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**355. Cell biological, genetic and chemical approaches to studying the Clavata1 receptor kinase**

*Zachary L. Nimchuk, Xiang Qu, Elliot M. Meyerowitz*

Proliferation of cells in plants is restricted to specific regions called meristems. These regions give rise to all the above ground organs of plants (shoot and floral meristems) and below ground organs (root meristems). Studies have shown that shoot meristems are organized into functionally distinct cell populations: the central zone (CZ), peripheral zone (PZ), and the rib zone (RZ). The CZ is composed of stem cells that divide slowly relative to cells in the flanking PZ. The RZ subtends the CZ and gives rise to the ground tissues of the stem. In addition, the shoot and floral meristems also give rise to flanking primordia that develop into various aerial organs. This process continues throughout the life of the plant and contributes to overall growth and body plan development. Meristems must balance the number of generated and departed cells in order to maintain themselves. A component of this process includes the regulation of proper spatial initiation of primordial outgrowth on meristem flanks. This process of balanced proliferation and patterning is strictly controlled and is defined genetically by *Arabidopsis* mutants affecting meristem regulation and formation. Mutations in *CLV1* lead to a hyper-accumulation of stem cells in both shoot and floral meristems resulting in stem fasciation, club-shaped siliques and extra floral organs. *CLV1* encodes a receptor-like kinase (RLK) with extracellular leucine-rich repeats and a cytoplasmic serine threonine (ser-thr) signaling domain that may interact with the dodecamer peptide ligand CLV3. We have been able to complement *clv1-11* mutations with a new series of double GFP tagged CLV1 constructs driven by the native *CLV1* promoter. Combined with an analysis of different mutant CLV1-2XGFP constructs we have been able to define a role for different CLV1 domains in CLV1 function at the cellular and genetic level. In addition we are exploring the role of other components of the putative CLV1 signaling machinery on the function of various CLV1-2XGFP constructs. Our analysis indicates a key role for CLV1 downregulation in the proper maintenance of the *Arabidopsis* stem cell population.

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**University of Cambridge, UK (SURF)*

**Support:** The work described in the following research reports has been supported by:
Arrowhead Grubstake Fund
DNA Sequencer Patent Royalty Funds
Edelman Discovery Fund
Mrs. Laura Frlan
Louis A. Garfinkle Memorial Lab Fund
Juvenile Diabetes Research Foundation
National Institutes of Health
Vanguard Charitable Endowment

**Summary:** The Rothenberg group studies the molecular mechanisms that induce and guide the choice of blood stem cells to develop into T lymphocytes. We focus on identifying the transcription factors and signaling events that induce T-lineage gene expression in an uncommitted precursor and determining how they work to force the cell to relinquish other developmental options. This process is an illuminating example of a stem-cell-based developmental strategy, which provides unique insights due to the excellent distinctions that can be made between developmental intermediates, the ease with which cell fate can be manipulated, and the great number of molecular markers that are available to define every step toward full T-cell identity. The approaches used in the lab are a combination of *in vitro* developmental biology, high-resolution characterization of individual cell developmental states, and molecular genetics of gene regulation. Kinetic dissection of this complex process using *in vitro* differentiation systems and retroviral perturbation make it possible to solve the roles of individual regulatory molecules in successive, highly defined developmental contexts. In addition, the group is investigating the subtle variations in this pathway that may predispose to autoimmunity in genetically diabetes-prone mice. Emerging results emphasize the exceptionally delicate control of cell fate by quantitative shifts in signaling during the very first T-cell receptor-dependent developmental checkpoint.

Over the past few years we have concentrated on dissecting a complex interplay between the key regulatory factors PU.1, GATA-3, and Notch, which counterbalance each other in a precisely titrated way during the early-stages of T-cell lineage choice. The quantitative effects of these factors, now in hand, can serve as a framework for understanding the overall dynamics of the early T-lineage developmental progression. We have now begun to formalize these relationships in explicit gene regulatory network form. This network model is a substantial extension of our first draft network organization published several years ago and provides a map of known inputs into many early T-cell genes that may now be tested for explanatory power against the observed developmental expression patterns. Regulatory gene effects on targets can be either direct or indirect, and the actual connections in the network may be much simpler when tested by direct molecular biological methods. To help locate the key *cis/trans* interactions, in collaboration with the Wold group we have begun a genome-wide mapping study to identify all sites of active and repressive chromatin marks both in cells just beginning the T-cell development process (DN1 cells) and in cells culminating the T-lineage commitment process (DN3 cells). This approach is being combined with bioinformatic site predictions to help design the verifications of network linkages. Recent work also sheds light on two other parts of the network that are worth much closer investigation. One is the remarkably T-lineage specific transcription factor Bcl11b, which we are now trying to situate both relative to its own target genes in early T-cell development and relative to the inputs that activate its expression. The other is the relationship between Runx1 and an emerging group of collaborating factors as stage-specific regulators of PU.1 itself.

Finally, as a close relationship between developmental programs of T cell and innate-immune system cells has emerged, we are also seeking clues to the evolutionary origins of both the T and B lymphocyte developmental programs by analysis of immune gene regulation in basal vertebrates. Recent results delineate key regulatory elements that may be recognized both in lymphocytes of jawed and jawless vertebrates. This opens a way to a direct regulatory comparison that may resolve the homology relationships among these vital cell types.

### 356. Origins of the lymphocytes

*Jonathan Moore*

Until recently, the origin of the vertebrate adaptive immune system could be seen as an extraordinary evolutionary explosion. Immunoglobulins, TCRs, MHCs, and the RAG genes are not found outside of the jawed vertebrates, and the branches between the jawed vertebrates and their close relatives are quite short, suggesting rapid evolutionary invention or cooption. Four years ago, a plausible intermediate step in the evolution of the adaptive immune system became evident; a novel, somatically recombining locus encoding an immune receptor molecule, the VLR, was discovered in the jawless vertebrates, the sister taxon to the jawed vertebrates who also possess an adaptive immune system. This begged the question as to whether these systems evolved

independently or whether they share a single common origin.

In many diverse jawed vertebrate clades, the transcription factors involved in discrete aspects of immune function are conserved. We are seeking to discover which factors regulate the VLR, since if these are shared in common with jawed-vertebrate immune genes, this would be evidence of a common origin to both adaptive immune systems. To this end, we have used a variety of approaches, including bioinformatics, heterologous biochemical and functional assays, and homologous biochemical assays.

This past year has been quite productive, due largely to the adoption of two catfish cell lines for heterologous assays. In these lines, transcription reporter constructs using putative regulatory sequences from the VLR produce differential results, and the regions implicated in these assays coincide with those suggested by the bioinformatic calculations. One of these regions has only begun to be investigated, but further investigation by EMSA of one 150-bp region close to the promoter has revealed 70 bp that are bound extensively by catfish transcription factors. Within this region, the catfish complexes with predicted Ets and octamer-binding sites are competed away by their respective mammalian control probes. Another complex is competed away by a C/EBP-control probe and another by E4F and CREB control probes; the specificities of the binding proteins in other complexes are currently a mystery. Transcriptional reporter assays with mutations at some of these sites have highly specific effects on expression, with some mutations giving no statistical change in expression while others completely eliminate the up-regulation due to the presence of the 150-bp region. The most dramatic effects are caused by mutations in the region of the putative E4F/ CREB site.

EMSAs using VLR-expressing and VLR non-expressing lamprey lymphocyte-like cells agree with some of the results from catfish. The putative catfish Octamer-binding regions were bound by extracts from VLR- cells but not from VLR+ cells, suggesting lack of function or a negative regulatory function. In contrast, both putative Ets sites are bound by VLR+ cell extracts but not VLR- cell extracts, and a neighboring complex about whose nature we are unsure is similarly bound by VLR+ and not VLR-. Regarding the competition with control probes and mutant lamprey sequence probes, the parallels between the catfish lymphocyte and VLR+ lamprey lymphocyte protein-DNA binding at these sites are truly striking.

In addition, we are just beginning to collaborate with Le Trinh and Scott Fraser to test VLR regulatory sequence function in a wider range of jawed vertebrate cell types, by injecting VLR-mCherry reporter constructs into zebrafish embryos. It may also be possible to collaborate with the Eric Davidson group to do similar experiments in sea urchin embryos, to examine the recognition of VLR elements in a non-vertebrate deuterostome. This will allow us to confirm or refute the results from catfish cells, observe our results in an untransformed *in vivo* system, and learn how specifically the VLR regulatory sequences are interpreted in the context of a whole embryo instead of the very limited survey which we can do in cell culture.

**357. Loss of alternative fate potentials during T lineage specification and commitment**

*Mary Yui, Ni Feng*

Small populations of multipotent stem cells migrate from bone marrow to the thymus in post-natal mice and begin a protracted period of proliferation, specification and commitment to T cell lineages. These cells pass through a series of CD4 and CD8 double-negative (DN) stages defined by cell surface markers, passing from early thymic precursors (ETP or DN1), through the DN2 stage, to the DN3a stage, when the cells become dependent upon T cell receptor (TCR) signaling for survival and further differentiation. DN2 is a key transitional stage in terms of rapid proliferation, as well as T lineage specification and commitment, encompassing major changes in gene expression and loss of alternative lineage potentials. Although DN2 cells are most rigorously defined as $CD44^{hi}CD25^{hi}cKit^{hi}$, many published studies and all older reports have used a less stringent definition of DN2 populations ($CD44^{+}CD25^{+}$, sometimes $cKit^{+}$) leading to variable gene expression and *in vitro* developmental results between studies. To more precisely determine the regulatory and developmental changes that occur from the DN2 to DN3 stages, the traditionally defined DN2 stage was divided into two distinct sub-populations based upon the levels of surface cKit (stem cell factor receptor): DN2a cells with very high surface cKit and DN2b cells expressing intermediate amounts of cKit. In addition to the previously reported distinct gene expression profiles between DN2a and DN2b, we found that, in the presence of Notch ligand, the DN2a cells undergo rapid proliferation, generate more cells overall, and a yield a higher percentage of γδT cells, while the DN2b cells proliferate less and differentiate more rapidly to DN4 and DP cells, consistent with characteristics of a more differentiated population. When alternative lineage potentials were assayed in the absence of Notch signals on OP9-control cells, DN2a cells generated ~10 times fewer dendritic cells (DC) than ETP/DN1 progenitor cells, but similar numbers of natural killer (NK) cells. DN2b cells generate almost no DC and NK cells. These results demonstrate that DC potential may be attenuated before NK potential and that both are extinguished after the earliest down-regulation of surface cKit in the DN2 stage.

**358. Mapping proliferation and transcriptional programming in early T-cell precursors derived from *in vitro* CP9-DL1 stromal cultures**

*Deirdre Scripture-Adams*

The advent of the OP9-DL1 stromal culture system has tremendously improved our ability to replicate T-cell development in easily manipulable *in vitro* cultures. While giving unprecedented access and numerical advantages, little data is available to confirm that T-cell precursors derived through culture in this system truly mirror real thymocytes in their developmental capacity and mechanisms of progression. Our lab is particularly interested in transcription factor control of T-cell development, and in order to make proper use of this highly desirable culture system, it is necessary to determine whether expression of the transcription factors controlling T-cell development are altered in OP9-DL1 culture derived pro-T cells as compared to real, thymically-derived pro-T cells. To begin to address this broad question we have first examined the pattern of developmentally regulated gene transcripts expressed across the early developmental stages of DN1, DN2, DN3 and DN4 using real time quantitative RT-PCR. An initial assessment strongly suggested an overall pattern of similarity between FLDN expression patterns and "real" freshly isolated fetal thymocytes, with an appropriate set of indicator transcripts increasing gradually as development proceeds to DN3, then declining. Additionally, genes such as PU.1 with sharply contrasting patterns (declining dramatically over the course of development) also seem to mirror normal thymocyte patterns of expression. However, the degree to which the *in vitro* system matches the *in vivo* program is time-dependent, and some cells appear to undertake aberrant programs, superficially resembling retrograde development, as the incubation is extended. One possible mechanism for the generation of these aberrant cells is that the OP9-DL1 coculture system appears to sustain a lower level of Notch target gene expression in the early T cells than development in the fetal thymus *in vivo*.

At timepoints that match physiological development, this culture system enables us to investigate the role of proliferation as it relates to developmental progression and fate choice; for instance whether a developing precursor must divide a particular number of times prior to achieving the next developmental stage, and whether certain numbers of divisions are tied in any way to availability of access to non-T fates. Some of these questions can be addressed with the use of single cell sort purified colony cultures derived from cells previously labeled with CFSE to determine initial division status. As described in last year's Annual Report, we are therefore using fetal liver precursors to begin to address these questions by sorting individual precursors directly on to OP9-DL1 stromal layers and culturing them while monitoring precursor frequencies, expansion, divergence, and progression along the T pathway.

**359. *In vitro* systems for the proliferation of early-stage T lymphocytes**

*Amy A. Ross, Rochelle A. Diamond, Ellen V. Rothenberg*

A key element in the study of gene expression in developing T lymphocytes is the sustainability of targeted cell populations *in vitro*. Our laboratory is analyzing gene expression in early-stage T lymphocyte development from T specification through β-selection. To aid in these analyses, we are exploring a variety of *in vitro* systems that will allow for sustained cell proliferation in early-stage T lymphocytes (so-called double-negative DN1, DN2, DN3 cells) from normal C57BL/6 and pre-weaning C57BL/6-Rag-2 knock-out mice. The goal is to achieve adequate *in vitro*-derived cell populations in stages DN1-DN3 to allow for gene transfection assays using the Amaxa protocol. The lab has had extensive success using fetal-liver derived hematopoietic precursors as a source of input cells, but this imposes inflexible logistical constraints on experimental design. Also, innate immune system-linked features of multilineage hematopoietic precursors may interfere with the ability to use siRNA in these cells. These problems could be overcome if adult early-stage thymocytes could be expanded sufficiently instead.

Our *in vitro* system utilizes the mouse bone marrow feeder cell line OP9-DL1 that induces Notch signaling in cocultivated hematopoietic precursors. This signaling pathway is crucial in T-cell development. We have compared the *in vitro* growth of thymocytes derived from pre-weaned and adolescent C57BL/6-Rag-$2^{-/-}$ mice with that of cell-sorted thymocyte sub-populations from normal adolescent C57BL/6 mice. Our preliminary data indicate that in the presence of cytokines Flt-3 (5 ng/ml) and IL-7 (5 ng/ml) on the OP9-DL1 stroma, it is possible to sustain the proliferation of early-stage thymocytes (DN2-DN3) from both strains of mice up to 14 days *in vitro*. Further, it appears that our culture conditions favor the *in vitro* growth of FACS-sorted early-stage thymocytes (DN2-DN3) from C57BL/6 mice in good condition for subsequent gene transfection. Sorted DN1 thymocytes yield significant populations of differentiated DN2 and DN3 cells, while DN3 and DN4-sorted cells progress to CD8-positive cell populations. Preliminary data indicate that transfection by Amaxa Nucleofection® is optimal in DN1-derived DN2 and DN3 cells. On-going studies will optimize our *in vitro* system for the proliferation of these early-stage cell populations for si-RNA transfection with genes of interest in T lymphocyte development.

**360. Principal regulatory components and a gene regulatory network for T-cell specification**

*Constantin Georgescu, William J.R. Longabaugh*[*]*, Ellen V. Rothenberg, Hamid Bolouri*

Choice of a T-lymphoid fate by hematopoietic progenitor cells depends on sustained Notch-Delta signaling combined with tightly-regulated activities of multiple transcription factors. To dissect the regulatory network connections that mediate this process, we have used high-resolution analysis of regulatory gene expression trajectories from the beginning to the end of specification; tests of the short-term Notch-dependence of these gene expression changes; and perturbation analyses of the effects of overexpression of two essential transcription factors, namely PU.1 and GATA-3. The data inputs for this project have been drawn primarily from studies in the Rothenberg laboratory, including some measurements by Elizabeth-Sharon David-Fung, Mary Yui, Deirdre Scripture-Adams, and Mark Zarnegar that are described elsewhere. Quantitative expression measurements of >100 transcription factor and marker genes have been used to calculate the principal components of regulatory change through which T-cell precursors progress from primitive multipotency to T-lineage commitment. Distinct parts of the path reveal separate contributions of Notch signaling, GATA-3 activity, and downregulation of PU.1. This analysis highlights the opposing net influences of PU.1 and Notch/Delta signals in determining the "position" of the cells along the trajectory from ETP/DN1 to DN3 stage. Using BioTapestry software (http://www.biotapestry.org), these results and results from the literature have been assembled into a draft gene regulatory network for the specification of T-cell precursors and for the choice of T as opposed to myeloid/dendritic or mast-cell fates. The current version of the network is presented in regularly updated form, together with the source data for each link, at our web site http://www.its.caltech.edu/~tcellgrn/. This network accommodates effects of basic helix-loop-helix E proteins and mutual repression circuits of Gfi1 against Egr-2 and of TCF-1 against PU.1 as proposed elsewhere. However, to account for the observed pattern of gene expression change it also requires three additional functions that remain unidentified. Distinctive features of this network structure include the intense dose-dependence of GATA-3 effects; the gene-specific modulation of PU.1 activity based on Notch activity; the lack of direct opposition between PU.1 and GATA-3; and the need for a distinct, late-acting repressive function or functions to extinguish stem and progenitor-derived regulatory gene expression.

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**361. Global mapping of chromatin modifications during early T-cell development**

*Jingli Zhang*

Early T cell development includes two different components, specification and commitment. Progressing from the early T-cell progenitor (ETP, also named DN1) stage to double-negative 3 (DN3) stage, cells sequentially acquire T-specific characteristics, while gradually losing non-T cell developmental potentials including myeloid, mast cell, DC and NK cell alternative fates. As they reach the DN3 stage, cells have lost all alternative lineage options, and become committed T-lineage cells.

The step-wise specification and commitment are mediated by a distinct combination of functions of multiple T-specific genes and non-T-specific genes at individual stages. Gene expression studies have shown changes in transcription factor expression profile during this developmental process. While a few T-lineage transcription factors, such as GATA3 and MYB, are expressed throughout the ETP to DN3 stage, many transcription factors are stage-specific. For example, PU.1, an ETS family factor, is required for ETPs, but must be downregulated at DN2 stage. On the other hand, Bcl11b, a T-lineage specific zinc finger transcription factor, is dramatically turned on at DN2 stage and kept on afterwards. Study of how these genes are lineage and/or stage-specifically regulated will help us to better understand how this complicate network functions during early T cell development.

Chromatin modifications often provide information for gene expression status and/or to identify and characterize imprinting *cis*-regulation elements of corresponding genes. For instance, acetylated H3 and trimethylated H3K27 can discriminate genes that are actively expressed or repressed, respectively, while dimethylated H3K4 suggests genes that are poised for expression or regions that are potential *cis*-regulatory elements. Chromatin immunoprecipitation (ChIP) followed with QPCR to assay DNA enrichment has been used and continues to be used to study regulation of several essential T-developmental genes, including IL2, GATA3 and PU.1, in our lab. The major limitations of this method are: 1) it requires a large amount of DNA and creates a large workload if a big genomic region or multiple genes are studied; 2) normally, it does not target genes of possible interest that are previously unknown to be involved in T-cell development. One method efficiently to map chromatin modifications genome-wide is ChIP-Seq, or to sequence all the ChIP-selected DNA and identify regions that are over-represented relative to a control. This has become accessible to us through a new collaboration with the Wold laboratory.

ETPs and DN3s have been differentiated *in vitro* and purified from fetal liver hematopoietic stem cells in the OP9-DL1 co-culture system. ChIP chromatin samples enriched for acetylated H3, dimethylated H3K4 and trimethylated H3K27 have been collected from ETPs and DN3s for amplification and analysis on the Illumina/Solexa Genome Analyzer. The first results show

highly specific, localized chromatin marks at known and novel regions of non-coding DNA around important genes for T-cell lineage choice and development. By comparing the epigenetic modification patterns across the genome in ETPs and DN3s, we will be able to globally decipher T lineage-specific and stage-specific gene regulation, identify potential *cis*-regulatory elements, and may even discover novel genes that play important role in regulating early T-cell development.

**362. Identifying the role of Bcl11b in T cell development**

*Long Li, Mark Leid*[*]

Hematopoietic stem cells (HSC) commit to the T-lineage by gradually losing developmental plasticity and gaining T cell identity. A recently discovered transcription factor, Bcl11b, is expected to be a key regulator in this process. In hematopoietic tissue, the expression of Bcl11b is restricted to the T-lineage. Its mRNA level increases dramatically once HSCs commit their fate to T-lineage. Loss-of-function studies revealed that Bcl11b was essential for T cell development beyond the DN3 stage. We have found its expression pattern is opposite to that of PU.1 (*Sfpi1*), which appears to maintain cellular potential to differentiate into myeloid lineages as long as it is expressed, and opposite to other stem cell genes such as *SCL*, *Id2* and *Gata2*. However, Bcl11b is apparently a repressor in most cases, not an activator, because it interacts directly with histone deacetylases. Based on this evidence, it is rational to speculate that Bcl11b could be one of the genes for which we have been looking that stimulates T cell development by inhibiting developmental plasticity of T cell precursors. The objective of this project is to address the role of Bcl11b and the mechanism that turns it on in T cell development.

Our data so far has shown that one transcript of Bcl11b, exons 1-3-4 (Bcl11b 1-3-4), may stimulate T cell development *in vitro*. Fetal liver HSCs infected with retroviral Bcl11b (MigR1-Bcl11b 1-3-4) were co-cultured with OP9-Delta1 (OP9-DL1) bone marrow stromal cells or OP9 control cells. OP9 control cells have been shown not to support the development of HSC to T-lineage, because the cells do not produce an essential T cell development signal, the Notch ligand Delta1. Our data showed that after 4 days of co-culturing HSCs with OP9 control cells, empty vector-infected HSCs showed no developmental progression: almost all cells were c-Kit$^{+}$ CD25$^{-}$. However, MigR1-Bcl11b 1-3-4 transfected cells reached the c-Kit$^{+}$, Thy1$^{+}$ and CD25$^{+}$ DN2 stage on OP9 control cells which otherwise occurs only on OP9-DL1 cells. Our data thus revealed that Bcl11b may stimulate T cell development and may even have a novel relationship to Notch. We are going to study the functional role of all four transcripts of Bcl11b (Bcl11b 1-2-3-4,1-2-4, 1-3-4 and 1-4) in early T cell development by using retroviral gene delivery and a Bcl2 transgenic mouse model, which helps keep cells healthy through periods of developmental reprogramming.

The other way to study the functional role of Bcl11b in T cell development is through a genetic loss-of-function approach. To do this, we have introduced human nerve growth factor receptor (NGFR)-Cre, delivered with a retroviral vector, into cells of a conditional Bcl11b knockout mouse model developed by our collaborator, Dr. Mark Leid. The data show that Bcl11b$^{-/-}$ HSCs may develop to DN3 pre-T cells *in vitro* as they do *in vivo*, but they reach this stage more slowly than control cells. To determine whether Bcl11b-deleted DN3 cells are irreversibly committed to the T-cell lineage as are control DN3 cells, we sorted these cells from wildtype and Bcl11b-floxed cells expressing Cre and found that after 7 days of co-culture with OP9-DL1, the Bcl11b-deleted cells generated a CD44$^{+}$ c-kit$^{Hi}$ Thy1$^{Hi}$ CD27$^{+}$ CD25$^{-}$ population of cells quite different from any derivatives of the control cells. This population could represent an abnormal retrograde differentiation pathway or a loss of normal limitations on growth in a DN1-DN2-like stage. Another striking finding was that after 9 days of co-culture with OP9-control, they gave rise to a population of Thy1$^{Hi}$ B220$^{+}$ NK1.1$^{+}$ Mac1$^{-}$ population of cells, which probably is an NK cell progenitor. Currently, we are determining the identity of these two populations.

[*]*Oregon State University*

**363. Determining the transcriptional regulation of Bcl11b in T-lineage development**

*Long Li*

The expression pattern of Bcl11b is unique: Bcl11b is silent in HSCs, immature B cells and myeloid cells; it is first activated in DN2 pro-T cells and up-regulated mostly during the transition from DN1 to DN2. Bcl11b is the only gene we have found so far that showed such developmental stage, as well as T-lineage-specific expression pattern. Understanding of how Bcl11b is regulated will dramatically increase our knowledge on T-lineage commitment. We propose that *cis*-regulatory elements and epigenetic mechanisms are involved in the regulation of Bcl11b. The silent state of Bcl11b in stem cells, B and erythromyeloid lineages may be maintained by repressors, DNA hypermethylation and condensed chromatin structure. During T-lineage commitment, the combination of DNA demethylation and histone hyperacetylation may be triggers to open chromatin around the gene, allowing additional transcription factors to up-regulate Bcl11b expression in a stage-specific manner. We have so far mapped DNA methylation of promoter region in Bcl11b by using bisulfite DNA sequencing. Apparently, there is an unmethylated window from -900 to -300 bp in Bcl11b expressing P2C2 cells. We have made several promoter-luciferase reporter-3'UTR constructs, and stable transfections of P2C2 cells (DN3 like pre-T cells) and Raw264 cells (pre-myeloid cells) are currently ongoing to map chromatin markings, conserved enhancers/repressors, 3'-UTR microRNA-binding sites, and transcription factors that regulate the expression of Bcl11b in T-lineage. Our preliminary data has shown that an enhancer element at -8.1 kb to -7.5 kb of Bcl11b may

be involved in its up-regulation at the DN1 to DN2 transition. This enhancer contains two-binding sites for GATA3, a critical transcription factor for T cell development, so we are currently testing the role of GATA3 in the regulation of Bcl11b.

**364. *cis*-Regulatory analysis of the hematopoietic transcription factor PU.1**

*Mark Zarnegar*

Development of committed T cells requires precise regulation of transcription factors to drive forward the T-cell fate while preventing alternative fate choices. Some factors must be turned on, while others must be silenced. One transcription factor that plays dual roles, contributing to T-cell development, yet also hindering commitment, is PU.1. This Ets family transcription factor is needed at the earliest stages of T-cell development (DN1 and DN2), but must be turned off or else the developing pro-T-cells become arrested. Too much PU.1 expression can drive the formation of a myeloid phenotype, whereas too little can block T-cell development from occurring. By studying the molecular mechanism through which PU.1 is silenced just as T cells become lineage committed (DN3), we hope to understand more fully the entire network of factors driving formation of T cells.

As described in previous years' Annual Reports, multiple genome alignments allowed us to locate several conserved non-coding regions upstream of the transcription start sight of PU.1 which could potentially contain *cis*-regulatory function, termed conserved elements (CE)1-9. Using cell type-specific mapping information from chromatin immune precipitation (ChIP) and DNase hypersensitivity data, we postulated that region CE8-9 may contain a non-specific regulatory element, while CE3-7 may contain a myeloid specific enhancer element, and/or a T cell-specific negative regulatory region. These predictions were upheld by experiments with various luciferase reporter constructs driven by different combinations of CEs. When transiently transfected into PU.1 expressing myeloid cells and PU.1 non expressing pro-T cells, CE5 functions as an enhancer only in myeloid cells while CE4 contains a T lineage-specific silencer region. In contrast to the cell type specific effects seen with CE5 and CE4, CE9-8 behaves as a general enhancer. The CE4 region was determined to contain two partially redundant silencing elements (CE4A and CE4B), both contributing to repression. Stable transfections have confirmed the contribution of the CE4A and CE4B regions to the cell type-specific silencing of a reporter. Mice harboring combinations of the CEs have been produced, and are being analyzed to establish their role in an *in vivo* developmental context.

Much of our recent work has sought to identify the transcription factors mediating the repressive function of these redundant elements. Fine-scale scanning mutagenesis has located two distinct 6-bp regions within CE4A that are crucial for its repressive activity. Gel shift analysis has revealed important contributions from multiple families of transcription factors. Of particular interest, we have found two context-dependent transcription factor families can bind to both CE4 regions *in vitro*. These transcription factor families, Runx and Ikaros, are known critical regulators of T cells, and have been shown to negatively regulate other target genes in developing T cells. We have evidence that they may be acting in either a combinatorial or cooperative fashion, and through non-consensus binding sites. Perturbations of these factors can impair the function of CE4A and CE4B, in our transient transfection system. We are continuing to examine CE4A and CE4B for other transcription factor family contributions to their function. Scanning mutagenesis of the CE4A region has also revealed a potential non-Runx, non-Ikaros binding region that may provide important contextual information directing those factors to play a repressive role. Further analysis is underway.

**365. Modification of PU.1 activity by Notch signaling in early T cells**

*Marissa Morales Del Real*

T-cell development is dependent on the combinatorial expression of transcription factors and environmental signals. Early pro-T cells retain lineage plasticity and can be diverted to myeloid/dendritic and NK lineages by the enforced expression of certain transcription factors. PU.1 is a transcription factor expressed in several cell lineages including cells of the myeloid lineage like macrophages. PU.1 is also necessary for early T-development, but antagonistic to the development of T cells if its expression extends beyond the DN2 stage as described in the previous abstract. Enforced expression of PU.1 in early pro-T cells causes a block before the β-selection checkpoint and can also drive the cells from the T-lineage to the myeloid lineage. However, the ability of misexpressed PU.1 to cause a block in T development and diversion to the myeloid fate is attenuated by Notch signaling.

It seems that Notch signaling is modifying the activity of PU.1 so that T-lineage fidelity can be conserved. Notch signaling does not directly repress PU.1 expression at the RNA level but interferes with its effectiveness. The mechanism by which Notch can mediate this modulation can identify key players in the early decisions of pro-T cells to avoid diversion even in the stages when PU.1 expression is high. Possible modifications of PU.1 activity in response to Notch signaling can occur at the protein level or at the level of a required positive or negative regulator of PU.1 activity. Preliminary studies addressing differential degradation or modifications of the PU.1 protein in the presence or absence of Notch signaling have been conducted. These studies suggest that degradation does not play a major role in the modification of PU.1 activity by Notch signaling. Currently, studies are underway to knock down Notch target genes or other candidate genes that may enhance or antagonize PU.1 effects, to dissect how responses to PU.1 overexpression or Notch signaling may be altered.

**366. Transcriptional regulation of GATA-3**

*Jingli Zhang*

GATA-3 is a T-lineage-specific transcription factor. It is required for T cell development from the earliest stages. It also plays an "instructive" role for mature peripheral Th2 cell development. However, while over-expression of GATA-3 in naïve CD4 T cells favors Th2 differentiation and blocks Th1 differentiation, abundant GATA-3 blocks the initiation of the T-cell developmental program. Under certain circumstances it even drives T-cell precursors to a non-T lineage fate. Thus, in order for early T-cell precursors to undergo normal and successful T-lineage development, GATA-3 transcription must be tightly regulated.

Like other members of the GATA family, both mouse and human GATA-3 genes contain two distinct promoters and alternative first exons, a biological mechanism to help gene expression to be flexibly regulated in a tissue or lineage-specific manner. The distal exon1 (exon1a), which is highly conserved between mouse and human, is ~9.5kb 5' to the proximal exon1 (exon1b). In stimulated Th2 cells, Notch1 has been shown to regulate the induction of GATA-3 exon1a directly, and this promoter appears to include sites that could mediate GATA-3 positive autoregulation, as well. This kind of positive feedback could be detrimental to early T-cell development as it could tend to raise GATA-3 expression beyond a T-lineage-promoting level. This raises the question of how GATA-3 expression is turned on and controlled during the Notch-dependent initiation of T-cell development.

Using the OP9-DL1 *in vitro* T-cell development system, we have grown double-negative (DN1-4) cells from fetal liver hematopoietic stem cell precursors for RNA and chromatin structure characterization. mRNA analysis indicated that only exon1b is expressed in DN1-3 stages; only after β-selection, DN4 cells start to up-regulate exon1a transcription in addition to exon1b transcription. To understand how GATA-3 transcription is programmed to be so stage specific, chromatin immunoprecipitation (ChIP) assays were used to study histone modification patterns of GATA3 gene locus during early T-cell development. Our data show that, in agreement with the expression profile, in both fetal liver precursor derived DN1 and DN3 cells the promoter of exon1b, intron1 and intron3 have increased acetylated H3. In contrast, the promoter of exon1a has increased trimethylated H3K27 and little histone acetylation, suggesting that this region is under active repression. These patterns are specific to the natural early T cells, since a transformed pro-T cell line that expresses high levels of GATA-3 from both promoters shows a much less polarized modification pattern.

If Notch and its associated transcription factor cannot gain access to the promoter of exon 1a, how may GATA-3 be turned on? Based on the ChIP result, several regions, including promoter1a-exon1a, promoter1b-exon1b-intron1b, intron3 and promoter1b-exon1b-intron1b-intron3, have been selected as potential *cis*-regulatory elements, and have been constructed into luciferase reporter plasmids. They will be transfected into fetal liver-derived DN3 cells to examine their regulatory potential. Since Notch1 directly regulates exon1a expression in stimulated Th2 cells but has not been thought to work on exon 1b, it is essential to investigate what role the Notch/Delta pathway may play in regulating GATA-3 expression during early T-cell development. We are seeking to use anti-Notch1-mediated ChIP to detect *in vivo* Notch-binding site(s) on the GATA-3 locus in DN3 cells.

**367. GATA-3 is required for the DN2 to DN3 transition**

*Deirdre D. Scripture-Adams*

GATA-3, expressed in all thymocyte stages, is also essential for embryonic development, and thus, its role in T-cell development cannot be studied fully using knockout mice. Although *Gata3*$^{-/-}$ cells cannot contribute to the T lineage in complementation experiments in chimeric animals, the nature of the requirement for GATA-3 in the early DN stages has not been proven, nor has any fine delineation been proposed for its role(s) at any stage before T lineage commitment.

Using retrovirally expressed shRNA to target GATA-3 in fetal liver precursors, we have identified a developmental delay from DN1 to DN2, and a developmental block from DN2 to DN3 in cells with experimentally downregulated GATA-3. The DN2 to DN3 block has been confirmed with FL precursors cultured as individual clones. These developmental progression defects are accompanied by loss of viability occurring in DN2 and DN3 stage cells which is not overcome by providing transgenic Bcl-2. Using *Gata3* conditional knock out mice, we confirmed the DN2/DN3 block in FL precursors, and found that c-Kit$^{++}$ DN1 precursors which had excised *Gata3* before initiating T-cell development never progress beyond DN1. New single cell experiments done by seeding individual thymocytes at the DN1, DN2 or DN3 stage of development into individual wells have now confirmed that loss of *Gata3* reduces seeding frequency and expansion when these clonal thymocytes are cultured under T cell conditions. This shows a specific T-lineage requirement because, when fetal thymic DN1 cells are cultured without Notch signaling to promote non-T lineage development, seeding frequency and expansion differences between Gata-3 knockdown and control cells are eliminated.

To explore the mechanism(s) of the developmental arrest and reduced viability seen in the absence of GATA-3, we have sorted pure populations of DN1 and DN2 stage thymocytes in which a GATA-3 specific shRNA has been expressed, and which we know to be blocked in development. Most reproducible of the effects is an upregulation of PU.1, with subsequent upregulation of additional PU.1 target genes coding for Mac-1 and M-CSF-R. If GATA-3 is normally responsible for reining in PU.1 expression, the "unleashing" of PU.1 may play a substantial role in derailing T cell development

at the DN2 stage simply by pushing the DN2 thymocyte toward a more myeloid transcriptional profile.

In an alternative strategy for studying the effects of loss of GATA-3 function, we have constructed a dominant-negative repressor fusion of GATA-3, using the *Drosophila* engrailed repression domain attached to the GATA-3 DNA-binding domain. In fetal liver precursors transduced with this obligate repressor form of GATA-3, we observed a block between DN2 and DN3, similar to that seen with GATA-3 knockdown achieved by expression of a GATA-3 specific shRNA, and to experiments done with conditional knockout animals. Cells expressing the dominant-negative GATA-3 were inhibited in growth, but survivors showed a strong shift toward upregulation of Mac-1, a PU.1 target consistent with the PU.1 upregulation observed in knockdown experiments. Our analysis of Gata-3-engrailed overexpression in a DN3 like cell line has identified multiple candidates for indirect repression including *Hes1*, *Heb-alt* and *Ptcra*, as well as some candidates for indirect activation (*Gfi1b*, *Aiolos*). We are using the dominant-negative Gata-3, and simultaneously within the same populations, using the Gata-3 shRNA retroviral constructs to map out the activation and repression of Gata-3 target genes within the broader gene framework controlling T cell development, and to begin to define the molecular mechanism of the development arrest caused by loss of Gata-3.

**368. TCRαβ and TCRγδ T lineage choice in non-obese diabetic (NOD) mice**

*Mary Yui, Ni Feng*

The development of autoimmune Type 1 diabetes in humans and NOD mice is dependent upon a balance between pathogenic and protective T cell responses to self-antigens. This balance is determined in part by T lineage choices and selection events during early thymic T development and in peripheral tissues. All T cell lineages, including subsets of TCRαβ and TCRγδ cells, originate from the same population of stem cell precursors that migrate from the bone marrow to the thymus, where they are induced to undergo T cell developmental programming. We have found *in vivo* and *in vitro* abnormalities in NOD mice, in the selection or survival of αβ T cells at the earliest T cell receptor (TCR) checkpoint controlling differentiation from $CD4^-CD8^-$ double-negative (DN) to the $CD4^+CD8^+$ double-positive (DP) cell stages. Using the OP9-DL coculture system, which normally allows differentiation to the DP cell stage, NOD early T cell precursors in long-term culture generate or accumulate DP cells very poorly, while γδ T cell differentiation appears to be enhanced. In addition, we have found that several costimulatory molecules, that are normally upregulated at both β- and γδ-selection and are likely involved in normal proliferation and survival, are poorly upregulated in NOD T cells undergoing β-selection, while γδ-selected cells are relatively normal. Because γδ-T cell development is favored by strong TCR signals and αβ-T cell development favored by weaker TCR signals, these results suggest a defect in TCR signaling, a possibility supported by our previous finding of a spontaneous β-selection checkpoint defect in Rag-deficient NOD DN cells, even in the absence of a TCR signal.

**369. Analysis of TCR signal pathway sensitivity in early T cells from NOD mice**

*Mary Yui, Chen Yee Liaw, Ni Feng*

Immature T cells are dependent upon signaling at multiple stages through their rearranged T cell receptors (TCR) for survival and further differentiation. Our lab has several lines of evidence suggesting that NOD T cells have altered sensitivity to T cell receptor (TCR) signals at their earliest stages, which may have implications for tolerance induction and autoimmunity. We have previously found that: (1) NOD.*Rag*$^{-/-}$ early T cells spontaneously break through their first TCR-dependent checkpoint in the absence of the normal trigger, successful TCR rearrangement; (2) NOD.*Rag*$^{-/-}$ early T cells appear to be more sensitive to anti-TCR (CD3) and phorbol myristate acetate (PMA) stimulation *in vivo* and *in vitro*, giving more vigorous survival and differentiation responses than control mice; and (3) *in vitro* differentiation of early T cells from wild-type NOD mice, using Notch-ligand expressing OP9 stromal cells, favors the development of $\gamma\delta TCR^+$ cells, which require strong TCR signals, over $\alpha\beta\text{-}TCR^+CD4^+CD8^+$ DP cells, which require weaker TCR signals, when compared with T cell differentiation from control mouse cells. Our results with NOD cells suggest that the threshold of TCR activation may be lower in NOD mouse early T cells as compared with those from control mice, and that only a subset of the entire TCR signaling cascade may be involved. For this reason we are seeking to determine which of the TCR signaling pathways differ between NOD and control mouse T cells with and without *Rag*-deficiency. We are using antibodies specific for active phosphorylated forms of molecules in several signaling pathways downstream of TCR signaling (e.g., p38, pErk1/2, Akt, etc.) to determine the dose responses of activation, initially comparing NOD.*Rag*$^{-/-}$ and B6.*Rag*$^{-/-}$ early T cells in response to varying levels of anti-CD3 antibody crosslinking or chemical stimulation (PMA/calcium ionophore). Because of the low numbers of available early T cells, these assays are being developed for multi-parameter flow cytometry, using intracellular staining of the signaling molecules and cell surface phenotyping.

**370. Genetic mapping of the early T cell checkpoint breakthrough in NOD mice**

*Mary Yui, Ni Feng, Justine Chia*

More than 20 Type 1 diabetes susceptibility loci have been mapped through various genetic crosses, but few of the genes involved in disease susceptibility have been identified due to their complex interactions. Based upon our previous research showing that immunodeficient NOD-*scid* and –*Rag*$^{-/-}$ mice, which cannot rearrange T cell receptors, spontaneously break through the first T cell developmental checkpoint, β-selection, we sought to determine if this defect in NOD T cells maps to one or more known diabetes susceptibility genetic regions. This checkpoint violation appears to be a relatively simple and fully penetrant T cell phenotype, which should be amenable to genetic analysis. As previously reported, a limited linkage analysis was conducted using PCR-based microsatellite repeat polymorphisms to map the gene(s) responsible for the trait in a small (NOD X B6) XNOD-*scid/scid* backcross. Preliminary results suggested that the trait maps to genetic intervals on chromosomes (chr) 4 and 2, each region containing multiple major diabetes susceptibility loci. The chr4 genetic region was of particular interest as the 2-3 diabetogenic genes within it are thought to specifically affect T cell-functions. However, after creating a *Rag*-deficient NOD congenic strain with diabetes-resistant alleles in the chr4 region, we found that these mice were not resistant to the β-selection checkpoint violation, demonstrating that the checkpoint violation in NOD.*Rag*$^{-/-}$ mice does not require chr4 diabetes-susceptibility loci. We are currently carrying out a more extensive F2 intercross between NOD.*Rag*$^{-/-}$ and B6.*Rag*$^{-/-}$ mice. We are phenotyping these mice for the breakthrough trait and genotyping using PCR-based microsatellite polymorphisms linked to known diabetes-susceptibility loci.

**Publications**

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**Research Staff:** Tuomas Brock, Anil Ozdemir

**Support**: The work described on the following reports has been supported by:
Beckman Institute
Edelman Foundation
Gordon and Betty Moore Foundation
Jane Coffin Childs Memorial Fund
Keck Foundation
March of Dimes
National Institute of General Medical Sciences
National Institutes of Health
Searle Scholar Funds

The unifying goal of the studies being conducted in my laboratory is to understand *how* genes are orchestrated to control development. To date, the basis for our studies relates to a gene regulatory network (GRN), which describes what is known of dorsal-ventral patterning in the early *Drosophila* embryo. Within this GRN, information regarding genetic interactions and *cis*-regulatory control of ~60 genes is detailed. These genes interact to specify patterning along the *Drosophila* dorsal-ventral axis, to control cell movements that drive gastrulation, as well as to influence the subsequent differentiation of cells into different tissue types. We have used this extensive knowledge base to provide mechanistic insights into the development of embryos: (1) to understand how genes are expressed with proper spatial precision to pattern the embryo; and (2) to define the functions of these differentially expressed genes in controlling morphogenesis and differentiation.

***cis*-Regulatory design: dynamic interpretation of transcription factor levels**

We have contributed to a gene regulatory network (GRN) that describes how ~60 genes interact during gastrulation to specify dorsal-ventral patterning and subsequently to control differentiation of cells in *Drosophila*. However, even after such an extensive analysis, it remained unclear how the transcription factor Dorsal can regulate the expression of genes in a broad lateral domain, where the levels of this factor decrease dramatically. We have conducted an analysis of the *cis*-regulatory sequences supporting expression in the broad lateral domain of embryos, and found evidence that cooperation between Dorsal and a ubiquitous activator is required for this threshold response, sensitive to the lowest levels of the Dorsal transcriptional activator (Liberman and Stathopoulos, in revision). Our approach combined evolutionary analysis, site-directed mutagenesis, and synthetic construct design to support this model. Furthermore, we provide evidence for flexibility in the composition and organization of sites required to support expression within this domain.

ChIP-chip and ChIP-seq analyses will be analyzed to identify *cis*-regulatory sequences, not yet identified, for genes that are differentially expressed along the dorsal-ventral axis. Our prediction would be that different examples of *cis*-regulatory design will be identified, which would explain why we had not been able to identify these regions in the past using standard bioinformatic methods and our prior knowledge (Ozdemir *et al*., in preparation).

Furthermore, one of the most striking properties of some developing systems is the ability to re-organize their developmental program and apparently give rise to normal adults when the size or shape of the embryo is altered. We are currently using genetic and computational approaches to understand how patterning is controlled by morphogen gradients (Nahmad *et al*., submitted).

***Cell movement coordination during migration of cells: high-level spatial organization***

The function of many genes differentially expressed along the dorsal-ventral axis of *Drosophila* embryos is to coordinate the cell movements that are driving gastrulation. We are analyzing the mechanism by which mesoderm spreading is accomplished. With technical advances in imaging and novel quantitative analyses, we have shown that mesoderm migration is a directed process; that cells move from ventral-most to dorsal-most regions of the ectoderm in a coordinate fashion (McMahon *et al*., in revision). High-level spatial organization within the moving population of cells was visualized. Cells at the leading edge originate from a particular position within the invaginated mesoderm; cell divisions are regulated temporally and spatially; and intercalation events contribute to monolayer formation of the migrating collective. We aim to determine whether such spatial organization is required for collective cell migration in general, a process that makes essential contributions to embryonic development.

We also investigate FGF signaling mechanisms using *Drosophila* as a model system. We demonstrate that the FGF-8 homologous proteins, Pyramus and Thisbe, are not redundantly functioning ligands but instead these genes have distinct functions, due in part to differential range of action and in part to differential expression (Kadam *et al*., in revision). In addition, our work has suggested that FGF signaling is important for collective cell mesoderm migration during gastrulation, but that it is not absolutely required. In the absence of FGF signaling, those cells in contact with the ectoderm *are* competent to migrate in a directional manner; those that cannot contact the ectoderm exhibit random movements, and lose the ability to move directionally. In the future, we will determine if these

ligands differ in range of action (e.g., diffusability) and, to this end, are characterizing the protein profiles for each protein.

**Summary**: The focus of the research program is shifting more towards analysis of one particular process functioning downstream of the dorsal-ventral axis GRN, the coordination of cell movements. Our working hypothesis is that transcriptional inputs help to coordinate groups of cells during collective cell movements. Therefore, we believe these two research directions, the analysis of *cis*-regulatory mechanism and the quantitative analysis of cell movements, will intersect. Our goal is to extend our imaging approaches to assay all the cells within a developing *Drosophila* embryo, in the context of defined genetic and molecular perturbation, in order to understand the movements of each and every cell as an output of the genomic regulatory code. In addition, we will strive to develop additional methods that will allow us to visualize transcription and the activation of signaling pathways in a live developing embryo. To describe development of an organism as a sequence of molecular events is our ultimate goal.

**371. Design flexibility in *cis*-regulatory control of gene expression**

*Louisa Liberman, Angelike Stathopoulos*

In early *Drosophila melanogaster* embryos, the maternal transcription factor Dorsal is present in a nuclear gradient that controls distinct patterns of gene expression and cell fate specification along the dorsal-ventral axis. How gene expression is controlled within the broad lateral domain of the presumptive neurogenic ectoderm is not understood. To investigate transcriptional control during neurogenic ectoderm specification, we examined the divergence and function of an embryonic *cis*-regulatory element controlling the gene *short gastrulation (sog).* Endogenous *sog* expression is conserved between *D. melanogaster* and the other *Drosophilid* species we examined. However, incomplete binding site conservation was revealed when we conducted a comparative analysis of these orthologous *sog cis*-regulatory sequences. Transgenic regulatory element reporters in *D. melanogaster* are expressed in a similar pattern to *sog*, confirming that these sequences are *bona fide* regulatory elements, and suggesting flexible regulatory architecture. Mutational analysis of conserved putative transcription factor binding sites reveals that sites for Dorsal and Zelda, a ubiquitous maternal transcription factor, are required for proper expression of *sog*. Synthetic regulatory element analysis demonstrates that sites for either Zelda or STAT, another ubiquitous maternal activator, direct broad lateral expression when paired with Dorsal binding sites. By searching for such sites in the genomic sequence flanking other genes expressed in a similar domain, we identified a novel enhancer for the gene *Neu3*. Our results demonstrate flexibility in regulatory element architecture with conserved regulatory logic and developmental output.

**372. The correlation between *in vivo* binding of transcription factors to DNA and the regulation of *Drosophila* early embryonic gene expression**

*Anil Ozdemir, Leslie Dunipace, Angelike Stathopoulos*

Classical genetic screens, expression-profiling experiments, and computational methods have identified many key targets of the Dorsal-Twist-Snail transcription factor gene network. These target genes interact to pattern the dorsal-ventral axis of *Drosophila* embryos. The significant amount of data available, however, is still not sufficient to explain the underlying signaling networks that control patterning, as well as cell movements. Therefore, to comprehensively elucidate the *in vivo* target genes and *cis*-regulatory elements, we performed ChIP-chip (Dorsal,Twist, and Snail) and ChIP-Seq (Twist), comparing both datasets to test whether the newly developed ChIP-Seq method would yield comparable or better-resolution binding data. Both approaches successfully identified genes that are known targets of Dorsal-Twist-Snail gene network, and, surprisingly, several novel enhancers of known target genes were also identified. Furthermore, ChIP-Chip revealed ~100 novel targets of which approximately half were also identified via ChIP-Seq. To test whether newly identified regions bound by Dorsal, Twist and/or Snail function as *cis*-regulatory sequences, we analyzed the ability of this DNA to support the reporter gene expression. About 80% of the enhancers tested, to date, direct expression along the dorsal-ventral axis, as expected. This result demonstrates these methods are highly effective in identifying functional transcription factor–DNA interactions. The false positive rate with ChIP-Seq was lower than for ChIP-chip.

To test the contribution of the individual enhancers to spatial and temporal expression of these targets, we are using a recently developed method that facilitates transgenesis of constructs of large size. This method allows insertion of DNA sequences into fly genome, and thereby makes it possible to study the structure of gene regulatory elements *in vivo.* Using recombineering techniques, we intend to manipulate or delete a specific *cis*-regulatory sequence from genomic sequence flanking a target gene of interest and to monitor changes in gene expression pattern by using reporters integrated into the gene locus contained within the transgene.

Finally, to gain insight into the function of the novel target genes during early *Drosophila* morphogenesis, we will knockdown expression of a representative set of target genes by RNAi. *In vivo* live imaging of such embryos will help us understand the function of the newly identified targets during dorsal-ventral patterning or the regulation of gastrulation cell movements. Overall, this project addresses fundamental questions in developmental biology and contributes to the strong interest in understanding the gene regulatory networks during animal development.

**373. The role of dorsally functioning repressors in dorsal-ventral patterning**

*Mayra Garcia, Angelike Stathopoulos*

The dorsal ventral axis of the *Drosophila* embryo is patterned by a nuclear gradient of the Dorsal transcription factor, which establishes the mesoderm, neurogenic ectoderm, and dorsal ectoderm. Targets in the neurogenic ectoderm have high affinity Dorsal binding sites and can be activated by both high and intermediate levels of dorsal but are not expressed in the mesoderm due to repression by the Snail transcription factor, a mesoderm specific gene. Likewise, the ventral most neurogenic ectoderm genes repress the dorsal most genes, to further subdivide the dorsal-ventral axis. Currently, there are no known repressors in the dorsal ectoderm that delineate the dorsal borders of neurogenic ectoderm specific genes. We would like to identify these repressors and integrate them into the current dorsal-ventral patterning network.

To identify theses repressors we analyzed the *cis*-regulatory regions controlling early embryonic expression of the *intermediate neuroblast defective (ind)* and *ventral nervous system defective (vnd)* genes. We isolated a 12 base pair *cis*-regulatory element (A-box) from the *ind* enhancer that is sufficient to cause dorsal repression. We are currently trying to identify the transcription factor that binds this element using a biochemical approach. Specifically, we are using stage-specific embryo extracts to isolate the candidate proteins using affinity chromatography and mass spectrometry. We have identified several candidates, predicted transcription factors that bind specifically to the A-box columns, through mass spectroscopy. We are also taking a bioinformatics approach to search for candidates that have known binding sites similar to the A-box element. Currently, we have seen some overlap in the bioinformatics candidates and the biochemical candidates. However, further analysis is still needed to separate positives from false positives in order to definitively identify the transcription factor that functions in dorsal regions to delineate the dorsal border of *ind.*

**374. Dynamic interpretation of the Hedgehog morphogen gradient in the *Drosophila* wing**

*Marcos Nahmad[*], John Doyle[*], Angelike Stathopoulos*

Morphogens are signaling molecules that organize developmental patterning by acting at a distance in a concentration-dependent manner. Despite much recent discussion about the formation and interpretation of morphogenetic signals, the interpretation of an extracellular gradient into different domains of gene expression is not well understood. The Hedgehog (Hh) protein belongs to a family of secreted proteins that act as morphogens throughout the course of animal development. To provide insights into the mechanism that supports different outputs of Hh-dependent gene expression, we first formulated a mathematical model of Hh signaling in the *Drosophila* wing imaginal disc. A steady-state analysis of the model reveals that the Hh extracellular gradient is translated into a step-like profile of the activated signaling transducer protein Smoothened (Smo). This result suggests that, at steady state, Hh responding cells may distinguish reliably between ON and OFF states of the signaling pathway, but cannot explain how the Hh steady-state gradient is able to induce more than two different spatial domains of gene expression. Computer simulations reveal that during the formation of the Hh gradient there is a spatial "overshoot" of the gradient, that is, a transient gradient with a longer range than the Hh steady-state gradient. We provide evidence that this transient overshoot of the Hh gradient depends on the gene network architecture and demonstrate experimentally that it is required for establishing different domains of gene expression in response to Hh signaling. Given the evolutionary conservation of Hh signaling and the existence of similar gene network architectures in metazoan development, our results propose a potentially general mechanism for morphogen interpretation and support the idea that gradient dynamics play a fundamental role in developmental patterning.

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**375. Spatial regulation of NF-κB signaling in early *Drosophila* embryogenesis**

*Marcos Nahmad, Greg Reeves, Scott E. Fraser[*], Angela Stathopoulos*

The ability of developing systems to regenerate and preserve proportions with respect to size has fascinated several generations of embryologists and naturalists. Despite the increasing knowledge on morphogenetic pattern formation, the molecular relationships between gradients and size remain elusive. We ask how patterns accommodate natural or artificial variation in the size of a developmental field. We investigate the patterning of the embryonic dorsoventral (DV) axis with respect to feedback mechanisms that ensure its proper scaling despite large variations in the size of the DV domain. The *Drosophila* NF-κB homologue, Dorsal (dl), is the only known maternal source of DV information in the embryo. As such, the ability to generate a well-proportioned insect initially depends on whether or not patterning by dl – and factors downstream of it – scales with respect to the size of the DV axis.

We quantify gene expression data of known dl target genes – such as *sna*, *sog*, *vnd*, and *ind* (see Fig. 1) - in both wild-type and mutant genetic backgrounds. We have presently seen appreciable scaling with respect to natural variations in the size of the embryo for all genes investigated. We also employ a system in which the dl gradient is altered to run along the anteroposterior (AP) axis (Fig. 2). Since the AP axis is 2-fold larger than the DV axis, we use this artificial system to study mechanisms of size-dependent scaling. Remarkably, we see scaling in this artificial system as well. Previous models have

suggested opposing gradients may provide size-dependent positional information. If such an opposing gradient exists in this system, it should depend – at least initially – on dl itself. We are using molecular genetics and mathematical modeling to ask whether it is possible to establish size-dependent positional information in a system with a single independent gradient.
*Professor, Division of Biology, Caltech

Figure 1:

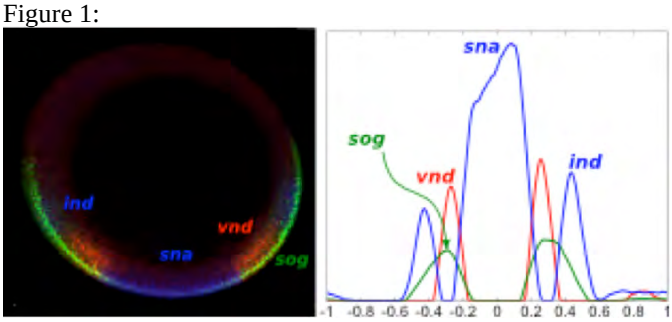
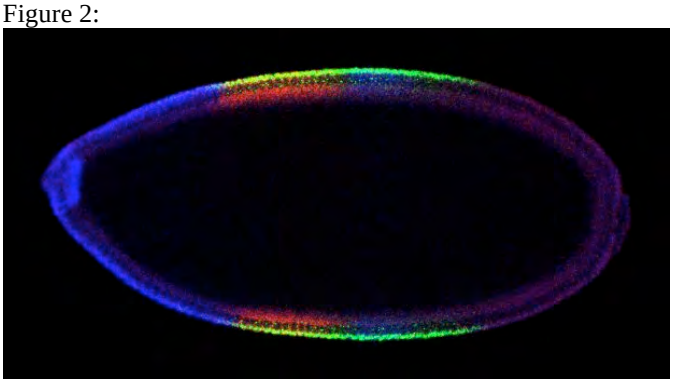



Figure 2:



**376. Dynamic analyses of *Drosophila* gastrulation reveal spatial organization of cell behaviors**

*Amy McMahon, Willy Supatto, Scott E. Fraser*, Angelike Stathopoulos*

The collective movement of distinct groups of cells contributes to morphogenesis during development, yet it remains unclear how the coordinated movement of many cells is accomplished. We define an imaging approach and quantitative methods that now make it possible to obtain and analyze the trajectories of hundreds of cells deep within *Drosophila* embryos during gastrulation. We find evidence for coordinated movements of mesoderm and ectoderm cells. In addition, we define autonomous movements of the mesoderm cells that are directed and exhibit high-level spatial organization. Such dynamic imaging studies provide insights into the mechanisms guiding collective cell movement and provides a framework that allows us to interpret mutant phenotypes dynamically. Thus, we are able to determine that FGF signaling is required during mesoderm spreading for collapse of the invaginated mesoderm and provide evidence that a distinct signal must promote the directed movement of mesoderm cells within *Drosophila* embryos. We contend that decomposing complex cell movements is required to dissect the behavior of cell cohorts to obtain mechanistic insights into the underlying biology of collective cell migration.
**Professor, Division of Biology, Caltech*

**377. Investigating the spatial organization of dividing cells in the migrating mesoderm**

*Nathanie Trisnadi, Amy McMahon, Angelike Stathopoulos*

Development of the mesoderm in *Drosophila* embryos involves major morphogenetic processes: invagination to form the ventral furrow, collapse of the furrow, and migration of cells into a monolayer. Cells also pause twice during the migration to undergo cell division. We have shown these processes are highly coordinated both spatially and temporally, but it is currently unknown what synchronizes these cellular behaviors. We are interested in what establishes the spatial organization and how this organization plays a role in directing mesodermal migration. Tracking data from our lab suggests that daughter cells stay together during migration and that, if they are separated, the two cells come back together. Mitosis is also thought to propagate within the mesoderm in traveling waves. If so, spatial organization may be crucial in synchronizing divisions while retaining information to resume coordinated migration. We propose that differential gene expression is responsible for this spatial organization and may be established even prior to the invagination.

We are currently screening candidate genes based on their expression profile to determine how the spatial organization of the mesoderm is created and maintained. We are developing tools to examine these phenomena using the photoswitchable Dendra fluorescent protein and reporter lines. With this system, we are able to track individual cells starting from the invagination until the migration is completed. Further investigation will reveal how spatial organization is important in coordinating cell divisions with movements during mesoderm migration.

**378. FGF ligands in *Drosophila* have distinct activities required to support cell migration and differentiation**

*Snehalata Kadam, Amy McMahon, Phoebe Tzou, Sarah Payne, Angelike Stathopoulos*

Fibroblast growth factor (FGF) signaling controls a vast array of biological processes including cell differentiation and migration, as well as wound healing and malignancy. In vertebrates, FGF signaling is quite complex with over a hundred FGF ligand-receptor combinations predicted. The fly, *Drosophila melanogaster*, presents a simplified model system to study FGF signaling, with only three ligands and two FGF receptors (FGFRs) identified within the genome. Here we analyze the specificity of FGFR activation [Heartless (Htl) and Breathless] by each of the FGF ligand-encoding genes [Pyramus (Pyr), Thisbe (Ths), and Branchless] in *Drosophila*. We find evidence that Pyr and Ths can

activate the Htl FGFR, and that only Branchless can activate the Breathless FGFR. To examine the role of each ligand in supporting activation of the Htl FGFR, we utilize genetic approaches focusing on the earliest stages of embryonic development. When Pyr and Ths genes are equivalently expressed using the Gal4 system, we find that these ligands support qualitatively different FGFR signaling responses suggesting that the processes of cell migration versus differentiation have different ligand requirements. Both Pyr and Ths ligands function in a non-autonomous fashion to support mesoderm spreading during gastrulation, but surprisingly Pyr exhibits a longer range of action. Examination of *pyr* and *ths* single mutants demonstrates that both exhibit defects in mesoderm spreading during gastrulation, yet only *pyr* mutants exhibit severe defects in dorsal mesoderm specification. Our findings demonstrate that these FGF ligands are not regulated solely by differential expression, but instead that the sequences of these linked genes have evolved to serve different functions. We conclude that the *Drosophila* FGF proteins have different activities, in part due to their differences in range of action, which are required to support specific FGF-dependent processes.

**379. Characterization of Pyramus and Thisbe proteins, *Drosophila* FGF ligands**

*Sarah Payne, Angelike Stathopoulos*

Fibroblast Growth Factors (FGFs) are involved in important developmental processes including mesoderm induction and patterning, organ formation, and neuronal differentiation. A pressing question in the FGF field is how specificity is achieved and distinct cellular responses accomplished with so many ligands compared to receptors. The FGF family of ligands has two relatively new members in *Drosophila*, *pyramus* (*pyr*) and *thisbe* (*ths*), both of which activate the *heartless* (*htl*) FGFR. This 2:1 ratio of *pyr* and *ths* ligands to their receptor *htl* provides a simplified model of the situation in vertebrates. We use this model to investigate how signaling by *pyr* versus *ths* could give rise to different cellular responses. We made epitope-tagged versions of Pyr and Ths proteins and have shown these proteins to be functional in the embryo. These epitope tags have allowed us to visualize the proteins by western and immunofluorescence in the embryo and in transiently transfected S2 cells, which are derived from *Drosophila* embryonic cells. We have learned that there are indeed differences in the Pyr and Ths proteins and how they appear to be processed that could impact range-of-action and regulated activation. We plan to continue the efforts in order to work out the mechanism for differential signaling.

**Publication**

McMahon, A., Supatto, W., Fraser, S.E. and Stathopoulos, A. (2008) Dynamic analyses of *Drosophila* gastrulation provide insights into collective cell migration. *Science*. In press.

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**Research and Laboratory Staff:** Mary Alvarez, Christopher Cronin, John DeModena, Shahla Gharib, Gladys Medina, Barbara Perry

**Support**: The work described in the following research reports has been supported by:
Helen Hay Whitney Foundation
Howard Hughes Medical Institute
Human Frontiers of Science
Moore Foundation
National Institutes of Health, USPHS

**Summary**: We seek to understand how the genome controls the development, behavior and physiology of *C. elegans*. We use molecular genetics to understand detailed mechanisms, and functional genomics to obtain global views of development and behavior. We take computational approaches to understand signal transduction, developmental pattern formation and behavioral circuits. We try to couple tightly computation and experimental data, in part to use computation to make experimental tests more efficient. Moreover, we study other genomes, genetics, and biology of other nematodes to help us comprehend *C. elegans*, to learn how development and behavior evolve, and to learn how to control parasitic and pestilent nematodes.

In the area of signal transduction, we continue to define pathway interactions and to understand the determinants of signaling specificity: how does the same pathway lead to distinct outcomes in different tissues? For these studies we analyze EGF-receptor signaling, WNT signaling, and G-protein-mediated signaling pathways. We found that the EGF-receptor acts in a single neuron, ALA, during this process, and acts via a phospholipase-C-gamma-mediated diacylglycerol-signaling pathway to control a sleep-like state, lethargus. We have found genes necessary for expression of the EGF-receptor in the ALA neuron.

Vulval development involves a remarkable series of intercellular signaling events that coordinate the patterning of the uterine and vulval epithelia and allow them to connect precisely. Specification of the anchor cell from the ventral uterine epithelium breaks the symmetry of the gonad. The anchor cell then produces the vulval-inducing signal, LIN-3, an epidermal growth factor-like protein that acts via *C. elegans* homologs of EGF-receptor, RAS and MAP kinase. Inductive signaling is regulated at the level of ligand production as well as the responsiveness of the receiving cells. LIN-3 is produced in a highly localized and regulator manner. After the anchor cell induces the vulva, a complex program of further pattern formation, cell type specification and morphogenesis follows. The primary (1°) vulval lineage generates an E-F-F-E pattern of cell types while the 2° vulval lineage generates an A-B-C-D pattern of cell types. We now have our hands on a number of receptor proteins, transcription factors and regulated genes; we are trying to define this regulatory network to understand how organogenesis is genetically programmed. For example, we found that the PAX2/5/8 type homeodomain protein EGL-38 regulates the F cell type while the tx-like protein COG-1 regulates the E cell type. Regulation by the EGF-receptor, WNT and HOM-C pathways impinge not only on vulval development but also the neuroectoblast P12 specification, male hook and spicule development. By comparing these examples with vulval development, we seek to understand the signaling specificity and signal integration. The relative contributions of EGF and WNT differ in each example. Multipotent cells with three fates utilize NOTCH signaling as well as EGF/WNT signaling.

Our efforts in genomics are experimental and computational. Our experimental genome annotation includes identifying *in vitro* binding sites for transcription factors, testing enhancer function in transgenic worms, and systematic inactivation of *C. elegans* transcription factors. We are investigating ways to compare the genomes of *Caenorhabditis* species. In particular, we are collaborating with the Genome Sequencing Center of Washington University to annotate three new nematode genomes. Our computational projects involve establishing pipelines for *cis*-regulatory computational analysis, new programs to use orthology and known binding sites or motifs, etc. We have successfully combined information from worms, flies and yeast to predict gene-gene interactions in *C. elegans*, and now *Drosophila*. This predicted network was used to interpret the relationship among genes expressed in a nematode that parasitizes sheep. We are part of the WormBase Consortium, which develops and maintains WormBase, a web-accessible comprehensive database of the genome, genetics and biology of *C. elegans* and close relatives (www.wormbase.org).

We have developed Textpresso (www.textpresso.org), an ontology-based search engine for full text of biological papers. Textpresso is used by *C. elegans* researchers, as well as the WormBase staff; we have made versions for *Neuroscience* (www.textpresso.org/neuroscience) as part of the Neuroscience Information Network and *Drosophila* (in collaboration with FlyBase (www.flybase.org). We are

also part of the Gene Ontology Consortium (www.geneontology.org), which seeks to annotate gene and protein function with a standardized, organized vocabulary.

Our behavioral studies focus on understanding male mating behavior as well as locomotion of both sexes. For specific projects we study egg laying, feeding, chemotaxis, osmotic avoidance, among other simple behaviors. Mating behavior, with its multiple steps, is arguably the most complex of *C. elegans* behaviors. Because it is not essential for reproduction, given the presence of internally self-fertilizing hermaphrodites, male mating is useful to elucidate how genes control behavior. We are studying several aspects of male mating behavior to understand the neuronal circuits that control the behavior and how they are genetically encoded. This year we discovered components of the mating pheromone made by hermaphrodites and sensed by males. Our comparative studies include both analyzing behavioral differences among species, and genetic analysis of *C. briggsae*, *Pristionchus pacificus* (a nematode species we discovered during the 1990s), and *Heterorhabditis bacteriophora* (an insect-killing nematode). We discovered that *H. bacteriophora* uses the same sensory neuron as *C. elegans* to respond to cues to exit the infective juvenile or dauer larval stage.

**380. Characterization of neuroendocrine cell specification and function in *C. elegans***

*Cheryl Van Buskirk*

We are studying how a single cell, the ALA neuron of *C. elegans*, becomes specified to express a specific battery of genes and how Epidermal Growth Factor (EGF) signal transduction within this cell leads to the induction of sleep-like behavior. We have identified two homeodomain transcription factors that are required for the expression of several genes within the ALA neuron, including *let-23*, encoding the EGF receptor. We have also identified a regulatory element that is capable of driving expression of GFP specifically within this cell. We are currently investigating the transcriptional regulatory network that governs ALA-specific gene expression. Part of this project involves the identification of the entire repertoire of ALA-expressed genes, through motif-driven searches and single cell gene expression profiling. Characterization of the ALA transcriptome will also aid in our ongoing identification of genes that play a role in EGF-dependent sleep, shedding light on the mechanism by which the ALA neuron controls behavior. EGF-dependent sleep-like states are conserved across species, and the study of this phenomenon in *C. elegans* provides not only a powerful genetic model for the dissection of sleep regulation, but also allows an integrative approach for the study of cell type specification, signal transduction and neuroendocrine function.

**381. Computational and transgenic *cis*-regulatory analysis of a *C. elegans* Hox cluster**

*Steven Gregory Kuntz*

Laborious *in vivo* promoter dissection is commonly necessary to identify animal *cis*-regulatory modules. Studies in the nematode *Caenorhabditis elegans* have used pairwise genomic comparisons with the closely related *C. briggsae* to highlight putative modules by conservation. Comparisons with additional genomes at appropriate evolutionary distances should improve the predictive power. The parameters necessary for ideal sensitivity and specificity of *cis*-regulatory predictions have not previously been quantified in nematodes. Due to genomic differences between the various animal phyla, it is expected that these parameters will differ from similar parameters in chordates or arthropods. By comparing five *Caenorhabditis* genomes (*C. elegans*, *C. briggsae*, *C. brenneri*, *C. remanei*, and *C.* sp. 3 PS1010) to identify candidate non-coding regulatory modules, we were able to empirically test various predictive parameters. For the species *C. brenneri* and *C.* sp. 3 PS1010, the entire genomic assembly was not available, so we sequenced ~0.5% of these genomes. The *ceh-13/lin-39* Hox clusters of these nematodes were computationally scanned for ungapped sequence similarity, and therefore conservation suppressed for insertions and deletions, using the MUSSA (Multi-Species Sequence Analysis) algorithm. Based on an initial computational analysis, the entire intergenic and intronic non-coding sequence was divided into 21 regions, including both regions exhibiting strong conservation and regions lacking notable conservation. Through iterative sequence analysis and transgenic assays of all these regions, we identified MUSSA parameters that yielded 100% specificity and a 75% recovery of all functional regions. In total, 12 regions drove detectible and reproducible expression, with the expression patterns matching the expected native expression patterns observed for *ceh-13* and *lin-39*. We are currently testing whether these parameters are similarly successful in identifying functional *cis*-regulatory sites in other loci of the nematode *C. elegans* that have been previously dissected through *in vivo* promoter dissection. This should provide significant insight into the utility of these computational predictions in nematodes.

**382. RNA sequence analysis of microdissected neurons in *C. elegans***

*Erich M. Schwarz, Ali Mortazavi, Miriam B. Goodman[1], Martin Chalfie[2], Paul W. Sternberg*

Ultrasequencing of cDNA has allowed unprecedented resolution of genome-wide transcription in mammals and other organisms. We have used the single-neuron RT-PCR protocol of Dulac and Axel to generate cDNA from defined individual neurons in *C. elegans*, which we have then sequenced to millions of Solexa reads and mapped to the *C. elegans* genome. We currently have data from 2-3 individual neurons of the following types: AFD (a primary thermoreceptor); ASER (a laterally

asymmetrical taste receptor); and PLML (a body wall mechanoreceptor). We find that our data from AFD are consistent with published expression data, but only loosely overlap with AFD-specific gene sets observed previously via microarrays against cultured neurons. We also find that, while neuron-to-neuron variability is quite high, consensus positives from 2-3 neurons can define reliable gene sets that, in PLML, include two new genes with a previously undescribed mechanosensory mutant phenotype. The greatest strength of this technology is likely to be in identifying transcriptional patterns from strictly postembryonic cells such as the anchor cell (inducing vulval development), the linker cell (guiding male gonad morphogenesis), and many male sex-specific neurons (enabling mating behavior).

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**383. Opposing Wnt pathways orient cell polarity during organogenesis**

*Jennifer L. Green, Takao Inoue*

The orientation of asymmetric cell division contributes to the organization of cells within a tissue or organ. For example, mirror-image symmetry of the *C. elegans* vulva is achieved by the opposite division orientation of the vulval precursor cells (VPCs) flanking the axis of symmetry. We characterized the molecular mechanisms contributing to this division pattern. Wnts MOM-2 and LIN-44 are expressed at the axis of symmetry and orient the VPCs toward the center. These Wnts act via Fz/LIN-17 and Ryk/LIN-18, which control beta-catenin localization and activate gene transcription. In addition, VPCs on both sides of the axis of symmetry possess a uniform underlying "ground" polarity, established by the instructive activity of Wnt/EGL-20. EGL-20 establishes ground polarity via a novel type of signaling involving the Ror receptor tyrosine kinase CAM-1 and the planar cell polarity component Van Gogh/VANG-1. Thus, tissue polarity is determined by the integration of multiple Wnt pathways.

**384. A blend of small molecules regulates both mating and development in *Caenorhabditis elegans***

*J. Srinivasan, F. Kaplan, R. Ajredini, C. Zachariah, H.T. Alborn, P.E. Teal, R.U. Malik, A.S. Edison, P.W. Sternberg, F.C. Schroeder*

In many organisms, population-density sensing and sexual attraction rely on small-molecule-based signalling systems. In the nematode *Caenorhabditis elegans*, population density is monitored through specific glycosides of the dideoxysugar ascarylose (the 'ascarosides') that promote entry into an alternative larval stage, the non-feeding and highly persistent dauer stage. In addition, adult *C. elegans* males are attracted to hermaphrodites by a previously unidentified small-molecule signal. Here we show, by means of combinatorial activity-guided fractionation of the *C. elegans* metabolome, that the mating signal consists of a synergistic blend of three dauer-inducing ascarosides, which we call ascr#2, ascr#3 and ascr#4. This blend of ascarosides acts as a potent male attractant at very low concentrations, whereas at the higher concentrations required for dauer formation the compounds no longer attract males and instead deter hermaphrodites. The ascarosides ascr#2 and ascr#3 carry different, but overlapping, information, as ascr#3 is more potent as a male attractant than ascr#2, whereas ascr#2 is slightly more potent than ascr#3 in promoting dauer formation. We demonstrate that ascr#2, ascr#3 and ascr#4 are strongly synergistic, and that two types of neuron, the amphid single-ciliated sensory neuron type K (ASK) and the male-specific cephalic companion neuron (CEM), are required for male attraction by ascr#3. On the basis of these results, male attraction and dauer formation in *C. elegans* appear as alternative behavioral responses to a common set of signaling molecules. The ascaroside signalling system thus connects reproductive and developmental pathways and represents a unique example of structure- and
concentration-dependent differential activity of signaling molecules. We are continuing to investigate the neuronal response of the ascarosides and their ability to affect behavior of other species.

**385. Acute carbon dioxide avoidance in *Caenorhabditis elegans***

*E.A Hallem*

Carbon dioxide is produced as a by-product of cellular respiration by all aerobic organisms and thus serves for many animals as an important indicator of food, mates, and predators. However, whether free-living terrestrial nematodes such as *Caenorhabditis elegans* respond to $CO_2$ was unclear. We have demonstrated that adult *C. elegans* display an acute avoidance response upon exposure to $CO_2$ that is characterized by the cessation of forward movement and the rapid initiation of backward movement. This response is mediated by a cGMP signaling pathway that includes the cGMP-gated heteromeric channel TAX-2/TAX-4. $CO_2$ avoidance is modulated by multiple signaling molecules, including the neuropeptide Y receptor NPR-1 and the calcineurin subunits TAX-6 and CNB-1. Nutritional status also modulates $CO_2$ responsiveness via the insulin and TGFbeta signaling pathways. $CO_2$ response is mediated by a neural circuit that includes the BAG neurons, a pair of sensory neurons of previously unknown function. TAX-2/TAX-4 function in the BAG neurons to mediate acute $CO_2$ avoidance. Our results demonstrate that *C. elegans* senses and responds to $CO_2$ using multiple signaling pathways and a neural network that includes the BAG neurons and that this response is modulated by the physiological state of the worm.

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**Support:** The work described in the following research reports has been supported by:
Beckman Institute
CalState CSUPERB
National Aeronautics and Space Administration (NASA)
National Institutes of Health/BIST
National Institutes of Health/NHGRI
Simons Foundation

**Summary**: Our group is interested in the composition, evolution and function of gene regulatory networks. We often use muscle development, degeneration, and regeneration as a model system. This network is one among a large class whose members govern how cell fates and states are specified, executed and maintained. The approaches we take to these problems make heavy use of genome-wide and proteome-wide assays to extract connectivity relationships and evolutionary relatedness. Evolution of networks is studied in collaboration with Paul Sternberg and his group to compare mammalian and worm networks, and with John Allman and his group to compare selected brain networks of primates and rodents.

A longstanding goal for us is to understand regulatory events that drive the progression of multipotential mesodermal precursor cells or adult stem cells to become determined unipotential muscle progenitors and, finally, fully differentiated cells. We currently study these cell states and transitions using functional genomics tools based on new ultra high throughput DNA sequencing methods coupled with comparative genomics. In mouse and other vertebrates, the muscle lineage arises from paraxial mesoderm to produce muscle (also bone, skin and fat, among other derivatives). Skeletal myogenesis is governed by both positive- and negative-acting regulatory factors. The MyoD family of four closely related, positive-acting transcription factors are key. Upon transfection, each can trigger nonmuscle recipient cells enter, and often to execute, the myogenic differentiation program. Our working model emphasizes that MyoD family factors are able to do this because they are highly connected and cross-talk within their group and with other collaborating factors (like MEF2 factors) and other regulators that now include microRNAs. This frames the problem in network terms. It is also clear that negative regulators of skeletal myogenesis are probably just as important for regulating the outcome as are the positive regulators. Multiple negative regulators of skeletal muscle are expressed in multipotential mesodermal precursors and in proliferating muscle precursors (myoblasts). It is generally believed that some of these are important for specifying and/or maintaining precursor cells in an undifferentiated state, though exactly how the system works is unknown. This focuses attention on defining the *in vivo* target repertoire for the repressors, and learning their relationship with the positive-acting regulators.

Because we wanted to develop the genome-wide input and output maps for multiple activating and repressing regulators, we were motivated to develop ChIPSeq and RNASeq methods. They are designed to make high resolution *in vivo* protein:DNA interaction maps in large mammalian genomes and to produce sensitive and quantitative RNA expression profiles (Johnson *et al*., 2007 Mortazavi, Williams et al. 2008 and below). By mapping *in vivo* factor occupancy maps for key regulators and learning how they change from one cell state to another, we hope to address some longstanding puzzles: What distinguishes sequence motifs in the DNA that are actively bound by their cognate regulators *in vivo* from other motif instances that are identical in sequence but are not similarly bound? How does the occupancy connectivity map predict the transcriptional output of target genes? What is the target promoter repertoire for a given bound regulator on the chromosome? Work by Brian Williams, Ali Mortazavi, Gordon Kwan, Tony Kirilusha and Katherine Fisher all contribute to an effort that aims to map all target sites of – ultimately - all transcription factors significantly expressed in muscle precursors and myocytes.

The MUSSA/MUSSAGL informatics tools (previous work of Tristan DeBuysscher, Diane Trout and Brandon King) have been used to find candidate conserved regulatory elements in both worm and mammalian phyla. This is part of an ongoing partnership with the Sternberg lab that involves several joint projects (see Steven Kuntz and Ali Mortazavi entries below and in the Sternberg lab Section). In addition to clarifying how many and which worm genomes give us the most leverage for identifying functionally important noncoding elements in the genome, we seek insights into the evolution ancient gene networks that drive myogenesis and cell cycle across the large phylogenetic distances separating vertebrates, worms, flies and for cell cycle, even plants and yeasts.

To define protein:protein complexes in the network more comprehensively, we developed a collaborative effort with the Deshaies lab and now with the new Beckman Proteome Exploration Lab. This enabled postdoctoral fellow Libera Berghella to identify a new repressor activity (encoded by the MSY3/4 gene) that acts on myogenin, the MyoD family member most directly responsible for execution of differentiation (Berghella et al., 2008). This repression activity operates through a very highly conserved cis-acting DNA sequence element in myogenin, which appears to have two distinct functions, one acting in early development to open the gene for transcription, and a second, repressive function that operates later on during muscle innervation and maturation.

During the past year we have also become part of the NIH Project ENCODE, which aims to functionally annotate the human genome. In the ENCODE project our work, performed in collaboration the Rick Myers group at

Stanford and Hudson Alpha Institute, focuses on developing and applying large scale measurements of transcription factor:genome interactions and transcriptome structure.

**386. ChIP-Seq**

*Ali Mortazavi, Ken McCue, Rick Myers*

*In vivo* protein-DNA interactions connect each transcription factor with its direct targets to form a gene network scaffold. Although much is known about transcription factor binding and action at specific genes, far less is known about the composition and function of entire factor:DNA interactomes, especially for organisms with large genomes. Direct physical interactions between transcription factors or cofactors and the chromosome can be detected by chromatin immunoprecipitation. In ChIP experiments, an immune reagent specific for a DNA-binding factor is used to enrich target DNA sites to which the factor was bound in the living cell. The enriched DNA sites are then identified and quantified.

For the gigabase-size genomes of vertebrates, it had been difficult to make ChIP measurements that combine high accuracy, whole-genome completeness, and high binding site resolution. These data quality and depth issues are not minor technical matters because they dictate whether candidate gene network structure can be inferred with reasonable certainty and comprehensiveness, and they determine how effectively the data can be used to discover binding site motifs by computational methods. A further challenge was to map factor-binding sites with high positional resolution.

We first used the NRSF repressor to work out the ChIPSeq method because we had previously generated a detailed genome-wide computational model for its target sites along with a large set of individually measured, validated true positive and true negative sites (Mortazavi *et al*., 2006). The resulting method was used to map *in vivo* binding of the neuronal restrictive silencing factor NRSF/REST, to 1,946 locations in the human genome. The data display sharp resolution of binding position (+/-50 bp), which facilitated motif finding and allowed us to identify new, noncanonical NRSF binding motifs (Johnson et al, 2007). This year the method was extended to mapping RNA Polymerase 2 occupancy across active genes, which discovered interesting patterns of Pol2 phophoisoform occupancy, to an evolutionary study of NRSF binding in dog, mouse, human and horse genomes.

Recent informatics work included development of more advanced methods for detecting ChIPSeq signals and for subdividing compound regions of factor occupancy into their individual contributing sites.

**387. RNASeq: Transcriptome profiling by direct DNA sequencing, mapping and quantifying the skeletal muscle transcriptome using RNASeq, an ultra high throughput sequence counting method**

*Ali Mortazavi, Brian Williams*

High throughput transcription profiling allows us to measure the global output of transcriptional regulatory networks. An ideal transcription profile has a low signal to noise ratio and is a comprehensive survey of the entire genome. We have developed methods and informatics for transcriptome profiling by high throughput DNA sequencing (called RNASeq) (Mortazavi et al., Nature Methods, 2008), because it overcomes the limitations of microarray systems. Using the current Solexa/ Illumina sequencing instrument, RNASeq produces short 35 nt sequence "reads" from a cDNA template of an RNA population, which are then aligned to a genome assembly for quantification and mapping. We have improved the uniformity of read coverage across transcripts by fragmentation of the mRNA template to reduce the effects of RNA secondary structure on reverse transcription. Our software package (ERANGE) counts and combines the aligned sequence reads within the known borders of transcription units for message quantitation. Sequence reads which cannot be assigned to a unique genomic address by alignment are assigned to a location based on the statistical weight provided by neighboring uniquely assigned reads. ERANGE also contains a subroutine (RNAFAR) for identifying novel transcriptionally active regions of the genome, and for extending the known borders of annotated transcription units. Using spiked-in control RNA sequences from *Arabidopsis* and the lambda phage genome, we have demonstrated a dynamic range of linear response over 5 orders of magnitude. Transcript quantitation is reported in RPKM units (reads pre kilobase per million mappable sequence reads) which indicates that RNASeq transcript quantitation is a function of both the molar concentration of the transcript in the sample and transcript length. The larger goal of this project is to correlate the comprehensive skeletal muscle transcriptome map with high throughput measures of skeletal-muscle-specific transcription factor site occupancy (ChIPSeq – see projects by Mortazavi). By mapping the physical connection points in the gene regulatory model with its transcriptional output, we hope to infer pertinent parts of the regulatory logic that drives differentiation of skeletal muscle.

**388. Skeletal muscle transcriptional regulatory network**

*Brian Williams, Anthony Kirilusha, Katherine Fisher, Gordon Kwan, Sandy Sharp, Ali Mortazavi*

Progression from undifferentiated myoblast precursor cells to differentiated cells with identifiable functional traits is regulated primarily at the level of gene transcription. Genome-scale experimental approaches allow us to assay all the changes in the transcriptome

during the differentiation step, and also to map the antecedent changes in transcription factor binding patterns on the genome and associated changes in chromatin structure. A first analysis of such data asks how changes in factor binding patterns are related to changes in the RNA population expressed. Transcription factor-site occupancy maps are being developed by immunoprecipitating chromatin preparations from the C2C12 skeletal muscle cell line using antibodies for transcription factors known to be key regulators of muscle differentiation along with more general regulators of transcription activation and repression. Shared analysis work in the project is to integrate occupancy maps and transcription output for factors that now include MyoD, myogenin, E47, Mef2, NRSF, SRF. We have been able to define the dominant sequence motif for MRF binding, which differed from that expected in older literature based on a few instances, showed that most enhancer of muscle transcription bind both MyoD and myogenin, but that an important class of sites are bound with high preference by myogenin or Myod. We also defined the class of MyoD target sites active in myoblasts. This definitively establishes that an original model in literature, in which MyoD was not active for DNA binding in myoblasts due to its interactions Id class HLH regulators and other modifications, is not correct. We used the binding datasets to identify a series motifs that are co-enriched in muscle enhancers. Some of these are expected from prior studies of individual enhancers, and some are novel. A global question arising form the study is this: Over half the high quality *in vivo* binding sites are not adjacent any gene regulated in a muscle specific manner. Are these sites acting on more distant genes? Are these due to evolution in motion – sites of no significance functionally that have emerged and have not yet deteriorated? Are they sites being held in check by nearby unrecognized repressors? Answering these and related questions with high throughput functional assays coupled with key factor perturbations will be a next step.

**389. A new repressor mediates transcriptional down-regulation of myogenin during innervation**

*Libera Berghella, Shirley Pease*

Myogenin, one of the four-member MyoD family of bHLH myogenic transcription regulators, is a crucial regulator of myogenesis that is down-regulated at the RNA level during muscle fiber maturation as a consequence of innervation. Comparative genomic analysis of sequences flanking myogenin using an early version of MUSSA (see entry from Tristan De Buysscher below), highlighted a highly conserved 17bp element (myogHCE). We asked what its function is, first by using lentiviral-mediated transgenesis. This showed that it mediates and is essential for postnatal repression of myogenin in transgenic animals. Subsequent mass spec analysis of proteins enriched by binding to this motif identified a candidate-transacting factor from adult muscle that could be responsible for this regulatory action. It binds myogHCE *in vitro* and occupies the locus in adult skeletal muscle. Altering its levels experimentally shows that it can repress differentiation in C2C12 moblasts in the gain of function mode, and that it can also regulate multiple effects of innervation in adult muscle. It binds the myogHCE motif as part of a complex that includes the homeodomain protein Pbx. Genome-wide analysis identified a family of related conserved elements, one of which is located 35 Kb upstream of MyoD in mouse and also interacts with MSY-3 *in vivo*. Taken together, these results suggest MSY-3 is a new component of the muscle regulatory network, critical for proper innervation-dependent activity during development. This work is being published in *Genes & Development*, 2008.

**390. Genome-wide comparative analysis of the NRSF/REST target gene network**

*Ali Mortazavi*

We are investigating the role of a major transcriptional repressor in the evolution of the corresponding gene regulatory network (GRN). We are using a combination of computational prediction and direct experimentation to define the genome-wide set of direct targets of the well-known Neuron-Restrictive Silencer Factor (NRSF/REST). This repressor was originally identified as a global repressor of neuronal genes in non-neuronal tissues in work in the Anderson lab at Caltech in the late 80's. It has more recently been shown to repress neuronal genes in stem cells prior to their differentiation. Roles for it have also been suggested in pancreatic development, cardiac lineages, and lymphocyte lineages, although it is not clear whether these roles differ from repression of neuronal genes in non-neuronal tissues during embryogenesis. NRSF is an ideal model factor for defining a regulatory network from the trans-factor point of view, in part because it has a long (21 bp) and relatively well-defined binding motif. Basic network architecture questions include: What are all the *in vivo* targets of NRSF? In what cells are they occupied by the factor and with what consequences? How has this network evolved since its beginnings in the vertebrate lineage?

Our first approach to these questions used Cistematic (see Mortazavi and Aerni above) across human, mouse and dog genomes to leverage evolutionary conservation to refine the motif model and to locate instances and their adjacent genes. The major experimental tests of sites identified computationally, along with sites of the same size and similar base composition that are predicted not to bind NRSF, are ChIP assays (chromatin immunoprecipitation). Initially ~100 predicted sites from the group of ~700 were assayed by Q-PCR. These allowed us to evaluate the predictive success of our model of the human NRSF target repertoire in Jurkat cells (Mortazavi *et al*., 2006). We are finding similar site occupancy patterns in mouse muscle cells. The composition of the computational target gene set was also interesting because it includes multiple microRNAs and regulators of neuron specific splicing. Among these are microRNAs predicted by sequence to be plausible regulators of NRSF and its Co-Repressor, CoREST, suggesting a dynamic feedback. The perfect conservation of the entire zinc-finger set that comprises the NRSF DNA-binding domain throughout all sequenced vertebrates is striking. Coupled with the absence of any identifiable

NRSF transcription factor in all sequenced invertebrates, suggests the notion that the emergence of NRSF in the vertebrates may have been needed to permit "reuse" of neuronal genes in non-neuronal, vertebrate-specific regulatory networks.

To further test and refine the computationally-derived model of the NRSF interactome, we worked with Dave Johnson in the Myers lab to measure the entire global "NRSFome" experimentally by using microarray and very high throughput sample sequencing (e.g., Solexa) to make ChIP measurements (see first entry above). We are now using ChIPSeq to map NRSF interactomes in mouse, horse, and dog genomes, for comparison with the human version. The aims are to determine which target genes, and which detectably conserved binding sites, are used in all interactomes; how many new NRSF/target relationships emerge in each genome, and how many have disappeared. This work will also investigate how the NRSF interactome map changes from one cell type to another within the same specie. In the first NRSF ChIPSEq work, performed in human T cells, we found that NRSF binds to a family of previously unappreciated half-site motifs as well as to its canonical full site (Johnson *et al*., 2007). This suggested a working hypothesis for the evolution of strong full-length sites from a pool of less optimal half-sites separated by flexible spacing. Patterns of site evolution and use across the four species in the current work should begin to test this idea.

**391. Computational and transgenic cis-regulatory analysis of a *C. elegans* Hox cluster**

*Steven Gregory Kuntz*

Laborious *in vivo* promoter dissection is has been the most frequently used way to identify animal cis-regulatory modules. Previous studies in the nematode *Caenorhabditis elegans* took advantage of pairwise sequence comparison with the closely related *C. briggsae* genome to highlight putative modules by conservation. Comparisons with additional genomes at appropriate evolutionary distances would be expected to improve the predictive power. The parameters necessary for optimal sensitivity and specificity of cis-regulatory predictions have not previously been quantified in nematodes. Due to genome structure differences between the various animal phyla, it is expected that these parameters will differ from similar parameters in chordates or arthropods. By comparing five *Caenorhabditis* genomes (*C. elegans*, *C. briggsae*, *C. brenneri*, *C. remanei*, and *C*. sp. 3 PS1010) to identify candidate non-coding regulatory modules, we were able to empirically test various predictive parameters. For the species *C. brenneri* and *C.* sp. 3 PS1010, the entire genomic assembly was not available, so we sequenced ~0.5% of these genomes. The *ceh-13/lin-39* Hox clusters of these nematodes were computationally scanned for ungapped sequence similarity, and therefore conservation suppressed for insertions and deletions, using the MUSSA (Multi-Species Sequence Analysis) algorithm. Based on an initial computational analysis, the entire intergenic and intronic non-coding sequence was divided into 21 regions, including both regions exhibiting strong conservation and regions lacking notable conservation. Through iterative sequence analysis and transgenic assays of all these regions, we identified MUSSA parameters that yielded 100% specificity and a 77% recovery of all functional regions. In total, 12 regions drove detectible and reproducible expression, with the expression patterns matching the expected native expression patterns observed for *ceh-13* and *lin-39*. We are currently testing whether these parameters are similarly successful in identifying functional cis-regulatory sites in other loci of the nematode *C. elegans* that have been previously dissected through *in vivo* promoter dissection. This should provide significant insight into the utility of these computational predictions in nematodes.

**392. Muscle tissue differentiation factor identification by synthetic lethal analysis**

*Steven Gregory Kuntz*

Biological networks are sometimes remarkably resistant to insults, being able to achieve their end-point even when parts of the network are deleted or significantly changed in structure. By studying evolutionarily ancient networks that execute very similar functions over substantial distances, hope to gain insights into which network structural features are variable or dispensible and versus critical – and in what ways they are critical. Striated muscle differentiation is an excellent model for transcription network analysis, being an evolutionarily ancient system. This study focuses on the differentiation of bodywall muscle in the late embryo of the nematode *Caenorhabditis elegans*. From extensive genetic and molecular studies, primarily focused on individual components, numerous muscle differentiation factors are known. However, we speculated that additional components of the network exist and will be best revealed by screens that further disrupt an already compromised network. Therefore, we have conducted a synthetic lethal screen, using a transcription factor RNAi library to knock down genes that may interact with well-described muscle mutant backgrounds. Several candidate factors have been identified from the screen and informatically prioritized for further study based on existing GFP expression pattern, in situ hybridization, and microarray expression data. High-ranking results from the screen are being analyzed for further interactions with known components of the differentiation network, including *hlh-1*, *hnd-1*, and *unc-120*. A more detailed understanding of these interactions will be complimented by lineage-specific studies of differentiation to further determine if and how some genes in the network compensate for mutations in others. The identification and description of additional components will help us construct a draft map of the worm muscle differentiation network.

**393. Gene expression profiling of primate brain regions and cells that mediate social interaction**

*Brian Williams*, Nicole Tetreault*, Mingshun Liu, Virginie Goubert, Brandon King*

Von Economo neurons (VENs), are a recently evolved population of specialized neurons found in parts of layer 5 of the human cortex. They are believed to be involved in the perception of social emotions, and intuitive assessment of complex, uncertain circumstances. A working hypothesis is that the brain areas where VENs are concentrated [frontoinsular cortex (FI) and anterior cingulate cortex (ACC)] may be dysfunctional in autistic individuals. These relationships have been deduced from anatomical and pathological evidence, including selective VEN pathology in another disorder (fronto temporal dementia). But little is known about the molecular genetics of FI, ACC and the VENs. In this collaboration with the Allman lab, we are developing a deep transcriptional profile of mRNAs expressed in brain domains strongly associated with regulation of social behavior, and then within these areas, profiles of the VENs themselves. By RNA-Seq profiling we are attempting to discover a defining set of molecular markers for VENs and for other cells involved in mediating aspects of social behavior. We also seek characteristic genes and gene clusters to suggest hypotheses concerning the development and evolution of the neuronal circuitry that includes VENs. At the cellular level, one specific goal is to identify all ion channels and expressed in VENs to inform functional modeling efforts, and another is to seek clues to their unusual morphology. Initial RNA studies are focused on ACC and FI, plus nearby regions that lack VENs and mediate different functions. Since the VENs are only a small fraction of all the cells located in their native subregions of the brain, we are using an immunological labeling and *in situ* hybridization to test and validate candidate genes emerging from the profiling effort. Initial findings are showing both known and novel RNAs that are either up-regulated significantly or down-regulated significantly in autistic samples versus age-matched control brains. We now hope to expand the scope of this project to trace evolution of VENs and related cells, including possible antecedents in mice where genetic manipulations will be possible.

**394. *In vitro* degeneration system for skeletal muscle**

*Brian A. Williams, Gilberto Hernandez, Jr.*

Skeletal muscle is one of the most noticeably compromised tissues during normal human aging, and undergoes active degeneration (cachectic wasting) during neoplastic disease progression. We are attempting to understand the molecular regulation of this process by modeling skeletal muscle degeneration *in vitro* using the C2C12 skeletal muscle cell line. Our previous results indicate that members of the E3 ubiquitin ligase family, which have been shown to be upregulated during degeneration of skeletal muscle tissue, are substantially expressed during the normal differentiation of C2C12 cells when cultured under atmospheric oxygen conditions. Previously published work has indicated that generators of reactive oxygen species (ROS) such as hydrogen peroxide can cause upregulation of the E3 ubiquitin ligases. We are manipulating culture conditions in order to cause inducible expression of the E3 ligases, with the goal of identifying specific transcriptional regulatory effects on a genome-wide scale. We are also developing more sensitive and reliable quantitative real time RT-PCR assays for a small battery of genes known to play a role in degeneration and stress response. cDNAs for this battery have been cloned and expressed as RNA molecules, for use as "spike-in" controls in quantitative RT-PCR experiments.

**395. Transcriptional and post-transcriptional regulation of skeletal muscle atrophy**

*Gilberto Hernandez, Jr.*

My goal is to identify and then learn the function of components of the network controlling the change in regulatory state in skeletal muscle, from one of relative homeostasis to that which leads to the major proteolysis that characterizes muscle atrophy in many disease states. A major contributor to the proteolysis seen in many models of skeletal muscle atrophy is the ubiquitin proteosome pathway, and two key E3 ligases mediate much of this effect. They are Muscle Atrophy F-box (MAFbx) and Muscle RING Finger-1(MuRF-1). Current understanding of the signaling cascade(s) regulating muscle mass have already benefited from the use of MAFbx and MuRF-1 as primary regulatory targets and molecular effectors of atrophy. However, both cis and trans-acting components that control MAFb, MuRf-1 and other atrophy related genes and non-coding regulatory molecules, is only partly known. By working from functional cis-regulatory elements, identified partly by their conservation through evolution and partly by experiment, we are attempting to define additional components. We further hope to use these cis-acting regulatory regions, and sequence motifs within them, to identify other genes that share them and are part of the atrophy response gene network.

Sequence similarity analysis with emphasis on motifs shared among 3'UTR regions of atrophy-related genes, has led me to focus on specific elements as predicted target sites for transcription factors binding or for microRNA interaction. I am now using a denervation model in mouse, sciatic nerve transection, to induce atrophy in the muscles innervated by the sciatic nerve. Expression analysis and subsequent perturbation experiments of atrophy-related genes and the putative microRNAs that co-regulate them, will be used to assemble components of the gene regulatory network controlling the expression of muscle atrophy-related genes.

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**396. HTSWorkflow: High-throughput sequencing workflow software and analysis**

*Diane Trout, Brandon W. King, Rami Rauch*[*]*, Timothy E. Reddy*[*]*, Gavin Sherlock*[*]

High throughput sequencing technology (HTS) is now widely available, and is being used in many different assays, of genome function and status such as ChIP-Seq, RNA-Seq and Methyl-Seq, as well as in general DNA sequencing of genomes and BAC clone pools. The size of the data-sets generated in these experiments (currently, several gigabases per week per instrument) presents a significant challenge for efficiently managing sample tracking, acquiring and attaching all relevant covariate information and performing downstream data management, archiving and analysis in an integrated manner.

We are currently using the Solexa/Illumina platform for ChIP-Seq, RNA-Seq and Methyl-Seq as part of the ENCODE project, and have developed a LIMSystem, HTS-Workflow, for addressing the initial capture of sample meta-data through the primary experiment analysis. Specifically, we use our software for: 1) experiment planning; 2) experiment tracking; and 3) data collecting and, 4) launching the initial data analysis. Our development goal is to provide genome center staff and clients with an intuitive web interface to track the progress of their samples and data through the process. The Web front-end connects with the data processing server, so that the user can define additional data analysis "tasks" and "projects" on the Web to be performed after the instrument specific analysis has finished. We have implemented and deployed HTS-Workflow using a python based Web development framework, Django.

[*]*Dept. of Genetics, Stanford University*

**397. A PCA-based way to mine large microarray datasets**

*Joe Roden, Chris Hart, Brandon King, Diane Trout*

In many instances where large-scale microarray analysis is part of a project, the biologically important goal is to identify relatively small sets of genes that share coherent expression across only some conditions, rather than all or most conditions, which is what traditional clustering algorithms find. The PCA (principle components analysis)-based tool developed in this project performs a complementary kind of datamining that helps one identify groups of genes that are highly upregulated and/or down-regulated similarly, across only a subset of conditions. Equally important is the need to learn which conditions are the decisive ones in forming such gene sets of interest, and how they relate to diverse conditional covariates, such as disease diagnosis or prognosis. This is a nontraditional use of PCA, and the software tools for doing it will be accessible from the group website. The method is described in a recent paper (Roden *et al.*, 2006) and the software is openly available at the Wold group website.

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# Facilities

Flow Cytometry and Cell Sorting Facility
Genetically Engineered Mouse Services
Millard and Muriel Jacobs Genetics and Genomics Laboratory
Monoclonal Antibody Facility
Nucleic Acid and Protein Sequence Analysis Computing Facility
Protein Expression Center
Protein Microanalytical Laboratory

**Flow Cytometry and Cell Sorting Facility**
**Facility Manager**: Rochelle Diamond
**Faculty Supervisor**: Ellen V. Rothenberg
**Sorting Operator**: Diana Perez
**Optics and Maintenance Specialist**: Patrick Koen

The Caltech Flow Cytometry and Cell Sorting Facility are located in 020 and 026 Kerckhoff Biology Building. This is a multi-user facility which provides expert assistance and advanced instrumentation to researchers for analyzing and separating various types of cells and micro-organisms according to their measurable properties of light scatter and fluorescence.

The facility is equipped with two research grade flow cytometer cell sorters and one analyzer:

- BD FACSAria, capable of analyzing at least nine colors utilizing three lasers (407 nm, 488 nm, and 633 nm), and of carrying out 4-way sorting up to 10,000 cells per second with reliable efficiency and recovery, or 1-way sorting into various cell culture plate configurations, such as for single-cell cloning. Up to 11 colors could be analyzed on this instrument if additional filters were purchased.

- BD FACSVantage SE, capable of analyzing five colors using two lasers, and of 2-way sorting up to 3,000 cells per second or 1-way sorting into various cell culture plate configurations.

- BD FACSCalibur, a four-color analyzer, together with an offline workstation, which are available to researchers for self-service analysis provided that they demonstrate competence to use the instrument or take training provided by the facility.

In addition, the facility makes Treestar's FlowJo off-line analysis program available to its clients through a network license. The facility also provides consultation services to all researchers on issues relating to flow cytometry, cell sorting, and cell separation techniques.

This past year the facility provided service to 20 laboratories from the Divisions of Biology, Chemistry and Chemical Engineering, as well as a laboratory from USC for groups for diverse applications. In all, the projects of 57 individual users were serviced. Additional new projects are in design for the Division of Geology and Planetary Science, at Caltech.

This is a representative sample of the projects that are under way: Jongmin Nam and Eric Davidson are developing a new experimental method for characterizing *cis*-regulatory modules during sea urchin embryogenesis. The key idea is that a *cis*-regulatory module of interest driving GFP reporter is injected into sea urchin embryos and cells expressing GFP proteins are enriched using the cell sorter. By comparing expression profiles of many endogenous indicator genes in GFP- positive cells and in control cells, they hope to infer the spatial activity of a *cis*-regulatory module and also the average regulatory state of cells where the module is active. The cell sorting step is one of the key steps in this project which is still in progress.

Carey Hsu and Peter Dervan have been working to quantitate the concentration of Py-Im polyamide-fluorescein conjugates in live cells by fluorescence methods including flow cytometry. The BD FACSCalibur System flow cytometer was utilized to measure the fluorescence of cells that had been dosed with these compounds and compare these values with those of known amounts of fluorophore.

Eric Haseltine and the Frances Arnold Group are studying how cell-cell communication coordinates population behavior using synthetic gene circuits. Specifically, they seek to understand how the structure of the signaling regulatory network affects such coordination in the presence of cell-to-cell heterogeneity. Flow cytometry was used to analyze the population responses, revealing significant bi-modality in one network architecture but not another (bi-modality implies less effective coordination).

The Elowitz group is focused on understanding how the juxtacrine signaling proteins Notch and Delta are regulated during development. They are using fluorescent reporters as a read-out for Notch activation by a plate-bound Delta protein. They have used the cell sorting facility to sort single cells to create stable cell lines of CHO and MDCK cells expressing the fluorescent reporters, mCherry, CFP, and YFP.

The *Arabidopsis* sepal epidermis contains cells with a wide distribution of sizes. Adrienne H.K. Roeder and Elliot M. Meyerowitz used flow cytometry to determine whether the cell size range of *Arabidopsis* sepal cells correlates with a range of DNA contents. The principle that cell size is generally correlated with ploidy level, or the number of copies of the chromosomes, was first described as the karyoplasmic ratio 80 years ago. Since then, the constancy of the ratio between DNA content and cytoplasmic volume has been demonstrated in nearly every organism from bacteria to *Arabidopsis*, and mammals. Adrienne found that the mature wild-type sepal contains 32% 2C diploid cells, 47% 4C, 6% 8C, and 0.6% 16C cells, which correlates with the size distribution. Mutants that perturb the pattern of cell sizes have corresponding effects on the ploidy distribution. These results suggest that cell size in the sepal epidermis is controlled by endoreduplication, a specialized cell cycle in which the cell grows and replicates its DNA but fails to divide.

The Barton laboratory in Chemistry has developed octahedral rhodium complexes that selectively bind to mismatched DNA with high specificity. DNA mismatches are a target of interest due to the correlation between mismatch repair deficiency and cancer. They have previously demonstrated the potential of these rhodium metalloinsertors to act as anti-cancer agents by selectively targeting cell lines with genetic mismatch repair deficiencies. Russell Ernst is interested in

further characterizing this response and elucidating the underlying mechanism. The Flow Cytometry facility is a central part of ongoing research to this end. Current assays include the use of propidium iodide protocols to quantify DNA content and examine cell-cycle distribution in conjunction with CFSE protocols to examine overall cellular proliferation. Future experiments are planned with cell death characterization protocols to identify whether the rhodium complexes induce an apoptotic response. Ultimately, insight gained from these investigations will be used to develop derivative compounds that display increased biological activity.

Josh Michener and Christina Smolke are using the cell sorter for the *in vivo* directed evolution of a P450 monooxygenase in *Saccharomyces cerevisiae*. An enzyme library is co-expressed with a riboswitch that responds to the enzymatic product. The riboswitch controls GFP expression, so better enzymes produce higher fluorescence. The library is then screened in high throughput at the single cell level using the FACSAria.

Stephanie Culler of the Smolke Group is using targeted gene expression in mammalian cells to select for *cis*-acting intronic regulators of alternative splicing. She has developed an *in vivo* selection strategy for cell-specific intronic regulators of alternative splicing, and is using it to screen a library of random 15 nucleotide inserts for functional activity within the introns of a three-exon, two-intron spinal motor neuron (SMN) mini-gene. GFP/SMN reporter constructs have been created such that functional intronic splicing silencers and enhancers can be selected using FACS through multiple rounds of selection in a mammalian cell line.

Another member of the Smolke Group, Yvonne Chen, is investigating the development and implementation of synthetic RNA-based regulatory systems that control the proliferation of T-cells in tumor environments. In the course of her research she has had to generate and sort for purity several stable cell lines expressing various marker proteins, including EGFP and CD19. The Caltech FACS facility is instrumental in her ability to complete this process.

Agnes Lukaszewicz of the David Anderson Group is studying embryonic motor neuron development. In particular, various preliminary observations led her to consider Cyclin Ds as makers of different populations in the ventricular zone of the embryonic spinal cord: Cyclin D1 expression is characteristic of neurogenic precursors, whereas Cyclin D2 expression would mark the neural stem cell/gliogenic population. But Cyclin Ds, known as positive cell cycle regulators, have recently been shown to modulate transcription factor activity. She has collected significant evidence that demonstrate that Cyclin D1 plays a role in promoting neuronal specification, whereas Cyclin D2 may be necessary for neural stem cell maintenance. In order to directly test whether modulations of Cyclin D1 and D2 level of can actually control the differentiation potential of spinal cord neural progenitors (i.e., neural stem cell vs. neuronal precursors), she adapted the single lentiviral platform developed by Iain Fraser in Mel Simon's lab at Caltech (Shin '06 *PNAS*), a system designed to allow a precise control of expression of genes of interest and microRNAs. After lentiviral infection of E13.5 CD15/MMA$^{+}$ FACS-sorted spinal cord precursors, Agnes is analyzing the impact of Cyclin D modulations on neurosphere formation and their developmental potential.

Mary Yui of the Rothenberg Group is studying early T cell-development in a mouse model of Type 1 diabetes to determine the genetic, molecular and cellular basis of specific T cell defects that may lead to autoimmune disease in these animals. She utilizes the single cell sorting and multiple fluorescence capabilities of the FACSAria sorter to highly purify numerically rare early T cell populations for two main purposes: (1) to study the developmental potential and lineage choices of these precursor cells in a cell co-culture system; and (2) to determine gene expression patterns of these specific early T cell populations in the context of gene regulatory networks in T cell development.

Jonathan Moore of the Ellen Rothenberg group is investigating which transcription factors regulate the variable lymphocyte receptor gene of a jawless vertebrate to see if these factors are shared with those known to be involved in jawed vertebrate immune gene regulation. The flow cytometry facility provides a way to highly purify lamprey cells expressing the variable lymphocyte receptor, as well as negative control cells. These cells are used to create nuclear extracts for gel shift experiments, RNA for qRT-PCR analysis, and DNA-protein complexes for chromatin immunoprecipitation experiments.

Deirdre Scripture Adams of the Rothenberg group is investigating the role of GATA-3 in early T cell-development. GATA-3 is known to be essential for the development of the earliest T lineage precursors, but its stage-specific role during the process of specification and commitment to the T lineage has not been defined. She is using retroviral expression of GATA-3 specific shRNA hairpins to knock down GATA-3 levels in DN-stage thymocytes and fetal-liver precursors, and then analyzing their ability to develop in an *in vitro* culture system. In order to clearly see the impact of loss of GATA-3 on developmental progression, pure populations of starting cells are essential. She uses the FACSAria for six-color sorting of fetal thymocytes, and the FACSVantage for five-color sorting of fetal liver precursors. Her project also requires the ability to follow the fate of a single cell after GATA-3 knockdown, and for this she uses the FACAria to sort single cells into individual wells of 96 well plates, and to assess their developmental potential *in vitro*.

Martin Budd of the Judy Campbell laboratory is examining the generation of single-stranded DNA at telomeres in *dna2 mre11* mutants as a function of cell cycle position. The hypothesis is the double *dna2 mre11* mutant will be defective in appearance of single-stranded DNA in S phase. Flow cytometry is used to assay the position in the cell cycle as a correlation with the single-stranded DNA.

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**Genetically Engineered Mouse Services**
**Director and Member of the Professional Staff:**
Shirley Pease
**Cryopreservation and Microinjection**: Juan Silva
**Embryonic Stem Cell Culture**: Simon Webster
**Mouse Colony Manager**: Jennifer Alex

In June 2005, the Genetically Altered Mouse Core and the Office of Laboratory Animal Resources (OLAR) combined to form the Caltech Laboratory Animal Services (CLAS). CLAS consists of two subdivisions, OLAR, which is headed by Dr. Janet Baer and Genetically Engineered Mouse Services (GEMs) that is headed by Shirley Pease. The purpose of the merger was to refine, streamline and standardize procedures for laboratory animal care and use on campus. GEMs continues to provide microinjection, cryopreservation, re-derivation and tissue culture services. In addition, we offer services in the form of rodent colony management and use, where required, in all animal Facilities.

Gene addition in the mammalian system is accomplished by injecting DNA into the pronucleus of a fertilized egg (Gordon *et al.*, 1980). This is a non-targeted event. Targeted disruption of specific genes, however, requires the manipulation of pluripotent embryonic stem (ES) cells *in vitro* and their subsequent return to the embryonic environment for incorporation into the developing embryo (Zijlstra *et al.*, 1989). The resulting chimeric mouse born is useful for two purposes: 1) it is comprised of tissue from two sources, the host embryo and the manipulated stem cells. More importantly, 2) it can be mated to produce descendants that are entirely transgenic, resulting from the ES cell contribution to the germline of the chimeric mouse. (The Nobel Prize in Physiology or Medicine was awarded this year to the pioneers of this technology, Mario Capecchi, Martin Evans and Oliver Smithies). The facility, in collaboration with Anderson, Baltimore, Kennedy, Lester, Patterson, Rothenberg, Simon, Varshavsky and Wold laboratories, has generated multiple transgenic, knockout and knockin mouse strains, amounting to nearly 170 mouse strains. The Facility together with the Baltimore lab, participated in the development of a new method for the introduction of DNA into early-stage embryos (Lois *et al.*, 2002). This method makes use of non-recombinant lentivirus as a vector for the introduction of DNA into one-cell embryos. The method has proven to be highly efficient and promises to be useful for studies in mice and rats, where large numbers of constructs need to be tested. This new methodology also makes feasible the generation of transgenic animals in species that were hitherto impractical to work with, due to the very low numbers of embryos available for use. Since the lentiviral vector method was established, 79 transient or established mouse models have been generated by this means, together with one Tg rat model. Facility staff has performed all embryo manipulation involved in the production of these new lines.

Microinjection equipment has been set up within the mouse facility, which operates on restricted access as part of a barrier system designed to safeguard the microbiological status of the animals. A room outside the facility has been allocated by the Division to be used primarily for teaching graduate students, technicians and postdocs the techniques involved in transgenic mouse production. This room has been operating since July 1996. Investigators have the option of using this room to perform their own microinjection of embryos, rather than using the full technical service available from the Genetically Engineered Mouse services.

At the merger, a new position was created, that of Colony Manager. An experienced technician has been appointed to the post and is available now to assist investigators with all colony management questions, primarily assisting investigators in making sure their experimental needs are met as economically as possible. GEMs Facility staff are currently working with IMSS to develop software that will assist technicians and investigators in the management of their mice. Amongst its features, this inter-relational system will track the breeding history of each strain and have the ability to generate family trees. The system will also report on production levels for each strain. Users will access the system to enter genotype results and work requests. An electronic signal will be sent to CLAS staff when work requests are made, helping us to manage work requests in a timely manner. The system will be basic but easy to use. We anticipate this will be a very useful animal management tool.

In tissue culture and the use of embryonic stem (ES) cells, the Facility has, in the past, participated in the derivation of new ES cell lines derived from genetically altered mice (see Simon laboratory Annual Report, 2001). This year, the Facility generated over forty new and as yet untested, embryonic stem cell lines, the majority of which are from C57BL/6 mice. This was a by-product of our wish to determine the most efficient approach to deriving such cell lines, since we anticipate that investigators may wish to use ES cells derived from their own genetically altered strains of mouse. Indeed, five such new ES cell lines have been derived from for the Rothenberg lab. Several investigators are using these pluripotent cells in research that involves pushing the cells down specific developmental pathways, and also to investigate the incorporation of extraordinarily large pieces of DNA into the mouse genome. The Facility is able to offer investigators a choice between working with ES cells on a 129 background, a C57BL/6 background or an F1 background, which is a mix between these two strains. We are able to manipulate and obtain germline transmission from all these ES cell types. C57BL/6 ES cells provide a significant advantage in that the mutation will be established initially on this well understood genetic background, instead of undertaking a two-year breeding program to reach the same point, having initially established the mutation on a sub-optimal genetic background. Hybrid ES cells have been reported to be useful for their vigor. Unlike ES cells from an inbred background, (e.g., C57BL/6 and 129), it is possible to

derive from hybrid ES cells live pups that are wholly of ES cell origin. Tetraploid embryos, (embryos with twice the normal number of chromosomes), are able to develop and contribute to extra-embryonic membrane cell lineages, but **not** to the development of any fetal tissues. Thus, a tetraploid embryo at blastocyst stage, injected with hybrid ES cells, will result in the production of an animal that is wholly of ES cell origin. ES cells from inbred strains such as C57BL/6 or 129 require a contribution to the developing fetus from the injected host blastocyst itself, for the production of viable pups. We recently established the production and use of tetraploid embryos at Caltech and have our first pups born from their use in combination with hybrid ES cells.

Once a new mouse model has been characterized, it may be cryopreserved, or sent to the Mutant Mouse Resource Center, to be made available to the research community in general. We currently have 93 mouse models cryopreserved. For each line, between 200 and 500 embryos at eight-cell stage have been preserved in liquid nitrogen. There are currently 27,419 embryos frozen in total. We shall continue to preserve embryos from mouse strains. The advantages of such a resource are many. Unique and valuable mouse strains that are currently not in use may be stored economically. In the event that genetic drift should affect any strain, over time, then the option to return to the original documented genetic material is available. Lastly, in the event of a microbiological or genetic contamination occurring within the mouse facility, we have the resources to set up clean and genetically reliable mouse stocks in an alternative location.

During 2006, Facility staff received training on the culture of human embryonic stem cells. We are about to establish the culture of H1 and H9 hES cells at Caltech. Initially, our goal will be to expand the cells and to cryopreserve stocks that may be made available to Caltech investigators at a later date. There are already a few investigators in the Biology Division who will wish to use the cells as soon as they can be made available.

Presently, thirteen principal investigators and their postdoctoral fellows or graduate students use GEMs services. In addition to the maintenance of nearly 100 different targeted and non-targeted strains, we also maintain colonies of inbred and outbred animals, which are used to support the development of new lines, by investigators at Caltech. We also have many mouse models on both an inbred and an outbred background, plus intercrosses between two or three different, but related, mouse models. In total, we currently maintain nearly 200 separate strains of mouse. Some of these strains are immune-deficient and require specialized care to protect them from bacteria commonly present in immune-competent animals. In immune-deficient animals, these hitherto harmless organisms can cause a problem. This may interfere with the well being of the animal and investigator ability to obtain reliable experimental results.

Listed below are the names of the eleven principal investigators and their postdoctoral fellows or graduate students who are presently using the transgenic facility.

***David Anderson***
Ben Deneen, Wulf Haubensak, Christian Hochstim, Walter Lerchner, Li Ching Lo, Agnes Lukaszewic, Sophia Vrontu

***David Baltimore***
Mark Boldin, Shengli Hao, Lili Yang

***Mark Davis - (Chemistry and Chemical Engineering)***
Derek Bartlett, Eric Kowel

***Ray Deshaies***
Narimon Harnapour

***Michael Elowitz***
Fred Tan

***Scott Fraser***
David Koos, Carol Readhead, Nicholas Plachta

***Mary Kennedy***
Eduardo Marcora, Andrew Medina-Marino, Leslie Schenker, Laurie Washburn

***Henry Lester***
Purnima Deshpande, Princess Imoukhuede, Herwig Just, Raad Nashmi

***Paul Patterson***
Ben Deverman, Ali Koshnan, Natalia Malkova, Limin Shi, Stephen Smith

***Ellen Rothenberg***
Deirdre Scripture-Adams, Chase Tydell, Mary Yui, Mark Zarnegar

***Melvin Simon***
Valeria Mancino

***Alexander Varshavsky***
Christopher Brower, Jun Sheng

***Barbara Wold***
Brian Williams

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**Millard and Muriel Jacobs Genetics and Genomics Laboratory**
**Director:** José Luis Riechmann
**Staff:** Brandon King, Vijaya Kumar, Tara Mastro, Lorian Schaeffer

**Support:** The work at the Laboratory has been supported by:
Millard and Muriel Jacobs Family Foundation
National Science Foundation

**Summary:** The goal of the Millard and Muriel Jacobs Genetics and Genomics Laboratory, in the Division of Biology, is to provide a suite of cutting edge genomic research tools to all interested Caltech scientists, with an emphasis on large-scale gene expression profiling and ultra-high throughput sequencing. The Laboratory performs gene expression analyses using DNA microarray technology, and is equipped with the necessary experimental and bioinformatics infrastructure that is needed to generate, store, and analyze large-scale datasets from a variety of microarray technological platforms. During the period of this report, the Laboratory has continued to provide support for genomics research to the Caltech community at large, including groups from the Division of Biology, the Division of Chemistry and Chemical Engineering, and the Division of Engineering and Applied Science.

In addition to the broad mission of the Laboratory, we are interested in the analysis of regulatory networks in *Arabidopsis* using genomic technologies, in particular those networks that are related to flower development. An important class of regulatory molecules in animals and plants are microRNAs (miRNAs): small non-coding RNAs (~20-24 nt in length) that regulate gene expression in a sequence-specific manner by targeting mRNAs for cleavage or translational repression. We are using genomic technologies (such as DNA microarrays) to characterize the *Arabidopsis* complement of microRNAs and its participation in floral development processes. In addition, we are using similar technologies in an effort to characterize novel coding short open reading frames present in the *Arabidopsis* genome and to study their possible involvement in flower development.

**Research Support**
*Division of Biology*
The Laboratory has worked with the groups of Professors David Anderson, David Baltimore, Seymour Benzer, Eric Davidson, Elliot Meyerowitz, Angela Stathopoulos, Paul Sternberg, and Barbara Wold. Microarray experiments have been performed using various technologies (Affymetrix, Nimblegen, Agilent, in-house manufactured microarrays), on various organisms (human, mice, *Drosophila*, *C. elegans*, *Arabidopsis*, sea urchin), and for a wide variety of research projects. These include: Transcription factors controlling mammalian neural stem cell development (Anderson); Signal transduction, transcriptional regulation, and microRNAs in the immune system (Baltimore); Flower development in *Arabidopsis* (Meyerowitz); Interactions between the nervous and immune systems (Patterson); Molecular genetics of nematode development and behavior (Sternberg), among others.

In addition, ultra-high-throughput sequencing, based on the Solexa platform, has been performed for the groups of Wold, Sternberg, Davidson, and Stathopoulos. This technology has been used for ChIP-Seq, RNA-Seq, and BAC sequencing experiments.

*Division of Chemistry and Chemical Engineering*
The Laboratory has worked with the groups of Professors Linda Hsieh-Wilson, Peter Dervan, and William A. Goddard. The Laboratory has manufactured carbohydrate microarrays that the Hsieh-Wilson group has used in its research, and performed gene expression profiling experiments using the Affymetrix technology for Professor Peter Dervan's laboratory. Together with W.A. Goddard and M. Diallo, the laboratory has used the Affymetrix technology in studies of toxicity of nanomaterials.

*Division of Engineering and Applied Science*
The Laboratory has worked together with the group of Professor Babak Hassibi to develop a real-time microarray technology that allows for the detection of interactions between probes and targets as the reaction takes place. The technology offers improvements in sensitivity, accuracy, and dynamic range over the existing microarray platforms.

**Infrastructure and capabilities:**
Since inception, the Laboratory has been well equipped to manufacture microarrays and to perform gene expression analyses using various microarray platforms, including Affymetrix GeneChips. Available equipment in the laboratory includes a MicroGrid II arrayer (BioRobotics); a GenePix 4200A scanner (Molecular Devices/Axon Instruments); a BioAnalyzer (Agilent Technologies); a MAUI hybridization system (Biomicrosystems); a QIAGEN 3000 liquid handling robot; an Affymetrix GeneArray 3000 7G scanner and fluidics station; and a high-throughput real-time PCR system (LightCycler 480, from Roche). A more comprehensive description of the laboratory infrastructure is available at: http://mmjggl.caltech.edu/.

The MicroGrid II arrayer allows us to produce large numbers of microarray slides in a cost effective manner, and to produce customized microarrays for which there is no equivalent commercial counterpart available. For example, we produce whole genome *Arabidopsis* microarrays using 70-mer oligonucleotide sets from Operon, which are used in various research projects related to flower development (work performed together with Professor E.M. Meyerowitz's group). Custom carbohydrate microarrays are used to study glycosaminoglycan-protein interactions (Professor Linda Hsieh-Wilson, Division of Chemistry). MicroRNA microarrays have been produced for mouse (Professor

David Baltimore) and *Arabidopsi*s (see below). Other customized microarrays include microarrays printed with Cy3-labeled probes, which are used to study the dynamics of DNA hybridization in real time (Professor Babak Hassibi, Division of Engineering and Applied Science).

The laboratory uses Resolver (from Rosetta Biosoftware) as its primary gene expression data analysis system. Resolver is a robust, enterprise-scale, gene expression system that combines a high capacity database and advanced analysis software in a high-performance server framework. The system is accessible through client stations using a web-based interface. We also have at our disposal additional microarray software tools and analysis packages, both public and commercial. The hardware infrastructure of the laboratory currently includes a Sun Fire V880 server (from Sun Microsystems), that we use for the Resolver database (Oracle) and analysis system.

To expand capabilities, two new instruments were deployed to the Laboratory this past year. The first was a next-generation sequencer, the Illumina (Solexa) Genome Analyzer. The Genome Analyzer uses a massively parallel sequencing approach to generate short sequence reads with ultra-high throughput (more than one billion bases of data in a single run) and reduced cost. We also recently added a new microarray scanner, from Agilent Technologies. This allows the laboratory to efficiently and effectively use Agilent microarrays, including the newer generation, higher-density arrays.

**398. Genomic analyses of *Arabidopsis* miRNAs: Their roles in flower development**

*Brandon King, Tara Mastro, Vijaya Kumar, Lorian Schaeffer, Yuling Jiao[1], José Luis Riechmann*

The focus of this project is to characterize the functions of the *Arabidopsis* complement of microRNAs (miRNAs), and to identify the gene regulatory networks in which they may participate –in particular during flower development–, by using microarray analysis of miRNA expression and other genomic approaches. MicroRNAs are small non-coding RNAs that regulate gene expression in a sequence-specific manner, and they have emerged as a very important class of regulatory molecules in plants and in metazoans. In *Arabidopsis*, more than 100 miRNAs have already been detected and/or predicted, and the *Arabidopsis* genome may in fact contain several hundred distinct miRNA loci - a number that demands the development of high-throughput methodologies for their study.

We have *Arabidopsis* miRNA microarrays that we manufacture using a custom collection of 2382 probes, encompassing: all known *Arabidopsis* miRNAs, a filtered selection of computationally predicted miRNAs, and a filtered selection of small RNA sequences derived from deep-sequencing projects or from various small-scale projects. We are using the microarray platform to analyze miRNA expression during flower development, as well as in response to a series of biotic stresses. We also developed a novel method to isolate RNA that is specifically enriched for decapped/cleaved and 5' phosphorylated mRNAs, which is then analyzed using standard gene expression microarrays.

[1]*Postdoctoral Scholar, Meyerowitz laboratory, Caltech*

**399. Genomic analyses of novel coding short open reading frames in *Arabidopsis*: Involvement in flower development**

*Tara Mastro, Vijaya Kumar, Yuling Jiao[1], José Luis Riechmann*

Transcriptome sequencing and whole genome tiling array studies have revealed significant levels of expression from numerous intergenic regions in animals and plants, suggesting the presence of genic sequences in un-annotated "intergenic" regions. In *Arabidopsis*, computational studies have identified many coding short ORFs (sORFs) with the potential to constitute novel coding genes (for example, Hanada *et al*., 2007, *Genome Res.* **17**:632). We have used custom-designed DNA microarrays to study the expression of ~6,000 sORFs in *Arabidopsis* floral tissues and during the process of flower development, using either the floral organ identity mutants (*ap1*, *ap2*, *ap3*, and *ag*), or an AP1-GR-based floral induction system (described in Wellmer *et al*., 2006, *PLoS Gen*. **2**:e117). Many of the sORFs are expressed in floral tissues, and ~400 show either predominant expression in a given tissue or differential expression throughout development. Further computational studies indicate that many of the expressed sORFs may indeed correspond to novel genes.

[1]*Postdoctoral Scholar, Meyerowitz laboratory, Caltech*

**Publications**

Alves-Ferreira, M., Wellmer, F., Banhara, A., Kumar, V., Riechmann, J.L. and Meyerowitz, E.M. (2007) Global expression profiling applied to the analysis of *Arabidopsis* stamen development. *Plant Physiol.* **145**(3):747-762.

Hassibi, A., Vikalo, H., Riechmann, J.L. and Hassibi, B. Real-time microarray detection. In preparation.

Kannangara, R., Branigan, C., Liu, Y., Penfield, T., Rao, V., Mouille, G., Höfte, H., Pauly, M., Riechmann, J.L. and Broun, P. (2007) Transcription factor WIN1/SHN1 regulates cutin biosynthesis in *Arabidopsis*. *Plant Cell* **19**(3):1278-1294.

***Publications acknowledging the laboratory:***

O'Connell, R.M., Taganov, K.D., Boldin, M.P., Cheng, G. and Baltimore, D. (2007) MicroRNA-155 is induced during the macrophage inflammatory response. *Proc. Natl. Acad. Sci. USA* **104**(5):1604-1609.

O'Connell, R.M., Rao, D.S., Chaudhuri, A.A., Boldin, M.P., Taganov, K.D., Nicoll, J., Paquette, R.L. and Baltimore, D. (2008) Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J. Exp. Med.* **205**(3):585-594.

Shipp, E.L. and Hsieh-Wilson, L.C. (2007) Profiling the sulfation specificities of glycosaminoglycan interactions with growth factors and chemotactic proteins using microarrays. *Chem. Biol.* **14**(2):195-208.

Wang, L., Dankert, H., Perona, P. and Anderson, D.J. (2008) A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **105**(15):5657-5663.

**Monoclonal Antibody Facility**
**Supervisor:** Paul H. Patterson
**Director:** Susan Ker-hwa Ou
**Staff:** Shi-Ying Kou

The Monoclonal Antibody Facility provides assistance to researchers wishing to generate monoclonal antibodies (mAbs), ascites fluid or other related tissue culture services. We also produce polyclonal ascites Abs by immunizing mice with antigen and then inducing the mice with sarcoma cells to obtain high titer, polyclonal ascites fluid. This method can provide 10-18 ml polyclonal ascites fluid per mouse by using small amounts of antigen. In addition to these service functions, the Facility also conducts research on the development of novel immunological techniques.

In its service capacity, the Facility produced Abs for the following groups during the past year. The Zinn lab obtained polyclonal ascites fluid against a gene encoding a zona pellucida domain that is expressed in various epithelial tissues during *Drosophila* embryogenesis, as well as polyclonal ascites fluid against polyA-binding protein (PABP), and polyclonal ascites fluid against Nunos (a translation repression factor). The Zinn lab also obtained mAbs against *Drosophila* translational protein NQ1NC. The Strauss lab obtained polyclonal ascites fluid against the Helicase, NS-3, NS-1 Dimer, and NS-1 Dodecamer proteins from Dengue 2 virus. The Schuman lab obtained polyclonal ascites fluid against POMP (proteasome maturation protein). The Ou lab (USC) obtained mAbs against Hepatitis C virus F protein. The Patterson lab obtained polyclonal ascites fluid against mutant huntingtin exon 1. The Zipursky lab (UCLA) obtained mAbs against cell-adhesion protein from Aplysia.

We are currently working with the following groups: Hsieh-Wilson lab is trying to obtain mAbs against CS-A polysaccharide. The Dealwis lab (Case Western University) is trying to obtain mAbs against N-terminus of amyloid-beta peptide. The Transmembrane Bioscience Company is trying to obtain mAbs and polyclonal ascites against recombinant protein expressed through the proprietary membrane protein overexpression system and the refolded surface membrane protein from *Rickettsia Prowazekii*. They are also trying to obtain mAbs and polyclonal ascites against the Rhesus protein. The Patterson Lab is trying to obtain mAbs against wild-type huntingtin exon 1.

**Publications that were based on the work done by the Facility:**

Biaron, D.M., Kabutatu, Z.P. and Hill, K.L. (2007) Stuck in reverse: Loss of LC1 in *trypanosome brucei* disrupts outer dynein arms and leads to reverse flagellar blat and backward movement. *J. Cell. Sci.* **120**:1513-1520.

Hollenstein, K., Frei, D.C. and Locher, K.P. (2007) Structure of an ABC transporter in complex with its binding protein. *Nature* **446**:213-216.

Maslov, D.A., Spremulli, L.L., Sharma, M.R., Bhargava, K., Grasso, D., Falick, A.M., Agrawal, R.K., Parker, C.E. and Simpson, L. (2007) Proteomics and electron microscopic characterization of the unusual mitochondrial ribosome-related 45S complex in *Leishmania tarentolae*. *Molec. Biochem. Parasitol*. **152**:203-212.

Navaratnaraj, C.K. and Kuhn, R.J. (2007) Functional characterization of the Sindbis virus E2 glycoprotein by transposon linker-insertion mutagenesis. *Virology* **363**:134-147.

Pinkett, H.W., Lee, A.T., Lum, P., Locher, K.P. and Rees, D.C. (2007) An inward-facing conformation of a putative metal-chelate-type ABC transporter. *Science* **315**:373-377.

Sotogaku, N., Tully, S.E., Gama, C.I., Higashi, H., Tanaka, M., Hsieh-Wilson, L.C. and Nishi, A. (2007) Activation of phospholipase C pathways by a synthetic chondroitin sulfate-E tetrasaccharide promotes neurite outgrowth of dopaminergic neurons. *J. Neurochem*. **103**:749-760.

**Nucleic Acid and Protein Sequence Analysis Computing Facility**
**Manager**: David R. Mathog
**Supervisor**: Stephen L. Mayo

The Sequence Analysis Facility (SAF) provides software, computers, and support for the analysis of nucleic acid and protein sequences. Current SAF hardware consists of a Sun Netra running Solaris, a small 20 node Beowulf cluster, a file server, a 26 ppm duplexing laser printer, and a 16 ppm duplexing color laser printer. The PCs that comprise the "structure analysis facility" are also located in our facility.

Most common programs for sequence analysis are available on the primary server http://saf.bio.caltech.edu/. These include the GCG and EMBOSS Packages, PRIMER3, Phred, Phrap, Cross_Match, Phylip, and HMMER. Many of these may be accessed through the W2H or Pise web interfaces. Other programs, custom written programs, or special databases are available on request. The PCs support hardware stereo under both Linux and Windows. Under Linux the programs Coot, O, PyMol, Molscript, CCP4, and Delphi are available. Under WindowsWinCoot, Swiss PDB Viewer, O, PyMol, POVray, and various drawing and animation programs may be used. The searchable documentation for these programs is available on the SAF web server. The lecture notes and homework from the introductory course "Fundamentals of Sequence Analysis" are also available on the SAF web server. Web pages have been provided for running common compute intensive jobs locally on the SAF Beowulf cluster. BLAST executes in a parallel mode so that searches complete faster than they do at the NCBI server. An enhanced parallel HHMER server offers the full set of HMMER programs plus the unique ability to search any of the installed BLAST databases with an HMM. Personal BLAST sequence databases up to 50Mb may be uploaded and searched. The multiple sequence alignment programs T-COFFEE, POA, Probcons, MAFFT, and Muscle are also available. Traces from any DNA sequencing facility may be uploaded and analyzed. The SAF also distributes these site licensed programs for PCs and Macs: DNASTAR, Gene Construction Kit, ChemSketch, and X-Win.

**Protein Expression Center**
**Director**: Jost Vielmetter
**Staff**: Michael Anaya, Chris Foglesong, Inderjit Nangiana
**Supervisor**: Barbara J. Wold
**Faculty Advisors**: Pamela J. Bjorkman, Mary B. Kennedy

The Protein Expression Center (PEC) provides protein expression and purification services mostly for Caltech researchers, but also for outside clients.

The expression systems currently used in the Center are as follows: (1) Bacterial expression of soluble and insoluble proteins; (2) Expression using the insect cell based baculovirus system; and, (3) Transient expression using mammalian (HEK293) cells in suspension culture. In addition, the PEC is equipped with a Biacore T100 instrument and offers support including hands-on training in the application of Surface Plasmon Resonance (SPR) based measurements of bio-molecular interactions on the Biacore Instrument.

Among the highlights of the past period was the successful production of active human antiviral antibodies and engineered antibody derivatives and viral glycoprotein antigens (influenza and HIV) in the context of the Engineering Immunity project (Pamela Bjorkman) and a DARPA funded project (Steven Mayo). Suspension cultures (30 ml to several liters) utilizing shake flasks on shakers allow us to be flexible in scale. We can even now grow mammalian suspension cultures in small volumes in multi-well plates that are compatible with high throughput techniques. This newly acquired technique opens the door for expression of whole protein libraries or multiple parallel tests allowing us to screen for optimal expression conditions.

We were able to maintain and improve the production of bacterially-expressed proteins significantly. Examples are plant proteins involved in morphogenesis and transcription factor fragments serving as antigens for antibody production to support Chromatin Immuno Precipitation (ChIP) technology. In addition, we were able to develop and improve a variety of our purification techniques with the help of our newly acquired AKTA Explorer chromatography system.

Protein expression optimization experiments have become a routine to ensure optimal and efficient expression in the baculovirus expression system. This is critical in order to ensure high and consistent protein yields and as a result, we have increased our rate of success with the expression projects coming to our center.

Another highlight of the Protein Expression Center is the continued implementation of the Biacore T100 instrument. The interest and use of this instrument has steadily increased since its acquisition and has become a very valued asset in the Caltech research community for the study of bio-molecular interactions.

**Protein Microanalytical facility (PPMAL)**
**Director**: Jie Zhou
**Faculty Advisor**: Professor Mary Kennedy
**Associate Biologist**: Felicia Rusnak

**ACTIVITY**
Mass spectrometry of large biomolecules and small organic molecules
Proteomics (In-gel enzymatic protein digestion; LC/MS/MS and data base search)
Protein (Edman) chemical sequencing
*De novo* peptide sequencing by mass spectrometry

**EQUIPMENT**
Quadrupole time-of-flight mass spectrometer (ABI QstarXL)
Triple quadrupole mass spectrometer (MDS Sciex API 365)
MALDI-TOF mass spectrometer (ABI Voyager de.str)
Capillary Protein sequencer (Procise cLC, ABI 492)
HPLC nanoflow, 2D (Eksigent)
HPLC (ABI microbore 140D pump, PE UV monitor)
MASCOT server

**NEW APPLICATIONS**
Song-Gil Lee from Professor Hsieh-Wilson's group had a project of the synthesis of a modified carbohydrate involving about 38 steps. We used ESI mass spectrometry to analyze those middle and final products. Extensive communications and reliable analyses of products after each step were crucial for the final synthesis of his compound. Dr. Tsui-Fen Chou from Professor Deshaies' group needed to identify an inhibitor binding to a specific Cys site in a protein extracted from cells. We used LC/MS to help her to locate the modification site in a specific protein. We also helped Chethana Kulkarni from Professor Tirrell's group to identify N-terminal modified and unmodified proteins from mixture and give quantitative measurement with LC/MS.

**THROUGHPUT AND INTERACTIONS**
During the first seven months of fiscal 2008, PPMAL interacted with 23 laboratories. Samples were analyzed from the Division of Biology, and Chemistry and Chemical Engineering (see list). A total of 2067 samples were analyzed. In addition to our work for campus faculty and staff, work was also performed for Nanogen, Insert Therapeutics (M. Davis), Neurion (D. Dougherty) and Harvard Children's Hospital (160 samples analyzed).

**MASS SPECTROMETRY**
In seven months, 2,008 proteins, peptides, oligonucleotides, and carbohydrates, small organic compounds have been analyzed. This extrapolates to an annual throughput of over 3,440, similar to the level for last fiscal year. Our off-campus activity recorded 160 samples.

**PROTEOMICS**
For the period covering this report, 45 digests had been analyzed. This extrapolates to an annual throughput of about 80 samples.

**PROTEIN AND PEPTIDE SEQUENCE ANALYSIS**
The lab has sequenced proteins and peptides for 147 cycles. The average number of residues per sample was about nine.

| PROTEIN MICROANALYTICAL FACILITY (PPMAL)<br>OCTOBER, 2007 – APRIL, 2008<br>LIST OF FACILITY USERS AND ACTIVITY | | | | | |
|---|---|---|---|---|---|
| **On-Campus** | | | | | |
| **On Campus** | Number of Samples | Number of Mass Spectrometry | Number of Proteomics | Number of Sequences | Number of Sequenced Cycles |
| Barton | 1084 | 1082 | | 2 | 24 |
| Bjorkman | 7 | 2 | | 5 | 65 |
| Davis | 6 | 6 | | | |
| Dervan | 14 | 14 | | | |
| Deshaies | 5 | | 5 | | |
| Dougherty | 26 | 26 | | | |
| Fraser | 80 | 80 | | | |
| Gray | 208 | 205 | 3 | | |
| Heath | 335 | 335 | | | |
| Hsieh-Wilson | 223 | 223 | | | |
| Jensen | 4 | | 4 | | |
| Kennedy | 7 | | 7 | | |
| Mayo | 16 | 16 | | | |
| Rees | 8 | | 8 | | |
| Shan | 5 | 1 | 2 | 2 | 10 |
| Simon | 5 | | 5 | | |
| Tirrell | 7 | 7 | | | |
| Varshavsky | 16 | | 11 | 5 | 24 |
| Winkler | 11 | 11 | | | |
| **TOTALS** | **2067** | **2008** | **45** | **14** | **123** |
| **Off-Campus** | | | | | |
| Harvard Med School | 3 | | | 3 | 24 |
| Nanogen | 149 | 149 | | | |
| Insert Therapeutics (M. Davis) | 1 | 1 | | | |
| Neurion (D. Dougherty) | 7 | 7 | | | |
| **TOTALS** | **160** | **157** | | | |

# Graduates

## DOCTOR OF PHILOSOPHY - 2008
## DIVISION OF BIOLOGY

**STIJN CASSENAER, PH.D.**
B.S., University of California, San Diego, 1999
Thesis: Spike-Timing Dependent Plasticity and Synchronous Oscillations in an Invertebrate Olfactory System.

**ROBERT SIDNEY COX III, PH.D.**
B.S., New College of University of San Francisco, 2001
Thesis: Transcriptional Regulation and Combinatorial Genetic Logic in Synthetic Bacterial Circuits.

**JENNIFER LEIGH GREEN, PH.D.**
A.A., Los Angeles Pierce College, 1999; B.S., University of California, Los Angeles, 2001
Thesis: The *C. elegans* ROR Receptor Tyrosine Kinase, CAM-1, Regulates Wnt Signaling by Two Distinct Mechanisms.

**ASHA MUTHURAMAN IYER, PH.D.**
B.S., Stanford University, 1998
Thesis: FMRI Correlates of Planning Goal-Directed Actions.

**ALI MORTAZAVI, PH.D.**
B.S., California Institute of Technology, 1993; M.S., California State University, Los Angeles, 2004
Thesis: Structure and Evolution of Mammalian Gene Networks.

**GRANT HAVERSTOCK MULLIKEN, PH.D. - (COMPUTATION AND NEURAL SYSTEMS)**
B.S., The Colorado School of Mines, 1998; M.S., The Johns Hopkins University, 2002
Thesis: Continuous Sensorimotor Control Mechanisms in Posterior Parietal Cortex: Forward Model Encoding and Trajectory Decoding.

**UELI RUTISHAUSER, PH.D. - (COMPUTATION AND NEURAL SYSTEMS)**
B.S., University of Applied Sciences, Rapperswil, 2003
Thesis: Learning and Representation of Declarative Memories by Single Neurons in the Human Brain.

**ANNA MARIA SALAZAR, PH.D.**
B.S., California Institute of Technology, 2007
Thesis: A Pumilio Domain that Forms Heritable Amyloid Aggregates in Yeast Can Regulate Pumilio-Mediated Translational Repression in *Drosophila*.

**STEPHEN EDWARD PAUCHA SMITH, PH.D.**
B.A., Occidental College, 2002
Thesis: Maternal Immune Activation and Abnormal Behavior in the Adult Offspring: Towards a Mechanism.

**LUIGI ANDREA WARREN, PH.D.**
B.Sc., University College London, 1982; B.S., Columbia University, 2001
Thesis: Single-Cell Gene-Expression Analysis by Quantitative RT-PCR.

**BRIAN MATTHEW ZID, PH.D.**
B.S., Truman State University, 2000
Thesis: Translational Control Mediates Lifespan Extension Due to Dietary Restriction in *Drosophila*.

### BACHELOR OF SCIENCE, BIOLOGY - 2008

Angela Weijane Chang*, Biology; Vamsidhar Chavalula*, Biology and Electrical Engineering; Molly L. Davis, Biology; René Michele Davis, Biology; Joseph Clarence Donovan*, Biology and Business Economics and Management; Leighland John Feinman, Biology and History; Csilla Nani Felsen*, Biology and English; Ludi Feng, Biology; Sarah Anthony Ferguson, Engineering and Applied Science (CNS); Elizabeh Gilliam, Biology; Zhiyun Guan*, Biology; Jennifer An Hawley, Biology; Harold Wenjing Hsu*, Biology and English; Tsung-Pai James Huang*, Biology; Deborah Jiang, Biology and History (minor); Richard Hayden Jones, Biology; Lindsey Marie King, Biology; Victoria Hunzu Pon*, Biology; Daniel Yuenheen Poon, Biology; Alexander Rigel Siegel*, Biology and Chemistry; Damien Zadour Soghoian, Biology; Pakpoom Subsoontorn*, Biology and Computer Science; Sarah Elizabeth Wadsworth, Biology; Mary Elizabeth Wahl*, Biology; Wei Eileen Xie, Biology; Yingding Xu*, Biology; Hanwen Yan, Biology and Business Economics and Management; Jessica Mary Yano*, Biology; Ziqing Zhao*, Chemistry and Biology.

*Students whose names are followed by an asterisk are being graduated with honor in accordance with a vote of the faculty.

# Financial Support

The financial support available for the work of the Division of Biology comes from many sources: The Institute's General Budget and Endowment and special endowment funds; from gifts, grants or contracts from individuals, corporations, foundations, associations, and U.S. government agencies.

Agouron Institute
Air Force Office of Scientific Research
Alfred P. Sloan Foundation
(Sloan Center for Theoretical Neuroscience)
Alzheimer's Association
American Cancer Society
American Federation for Aging Research
American Foundation for Alzheimer's Research
American Heart Association
Amgen, Inc.
Andy Lou and Hugh Colvin Postdoctoral Fellowship
Anonymous and personal donations also made
Army Research Office (Institute for Collaborative Biotechnologies)
Arnold and Mabel Beckman Fellowship Program
Arnold and Mabel Beckman Foundation
Arrowhead Research
Arrowhead Grubstake Fund
Atazoa Ltd.
Autism Foundation
Autism Speaks Foundation

BAE Systems/ARL
Balzan Foundation
Beard Charitable Foundation
Beckman Fellowship
Beckman Institute
Beschorman Memorial Fund
Anne P. and Benjamin F. Biaggini Professorship of Biology
Bill and Melinda Gates Foundation
Bing Professorship in Behavioral Biology
Bing Professorship Research Pool
Biological Sciences Initiative
Ethel Wilson and Robert Bowles Professor of Biological Sciences
Brown University
James G. Boswell Foundation
James G. Boswell Professorship of Neuroscience
Donald Bren Foundation
Bren Professorship of Biology
Eli and Edythe Broad Foundations
Brown University/NIH
Burroughs Wellcome Fund

California Institute of Regenerative Medicine (CIRM)
Callie McGrath Charitable Trust
CalState CSUPERB
Caltech Brain Imaging Center Discovery Grant
Caltech Center for Biological Circuits Design
Caltech President's Fund
Camilla Chandler Frost Fellowship
Cancer Research Fund of the Walter Winchell-Damon Runyon Foundation
Carl Zeiss Jena
Norman Chandler Professorship in Cell Biology
Charles B. Corser Fund
Cline Neuroscience Discovery Grant
Colvin Fund for Research Initiatives in Biomedical Sciences
Crohn's and Colitis Foundation of America
Croucher Foundation
Albert and Kate Page Crutcher
Cure Autism Now Foundation

Damon Runyon Cancer Research Fund
Damon Runyon-Walter Winchell Fund
Dana Foundation
David and Lucile Packard Foundation
Allen and Lenabelle Davis Professorship of Biology
Defense Advanced Research Projects Agency (DARPA)
Della Martin Fellowship
Della Martin Foundation
Department of Energy (DOE)
DNA Sequencer Patent Royalty Funds
Donald E. and Delia B. Baxter Foundation

Edelman Discovery Fund
Edelman Foundation
Edelman Trust
Elizabeth Ross Fellowship
Ellison Medical Foundation
Engineering Research Center for Neuromorphic Systems (ERC-NSF)
ERATO
European Molecular Biology Organization (EMBO)
Evelyn Sharp Fellowship

John and Ellamae Fehrer Endowed Biomedical Discovery Fund
Ferguson Fellowship
Ferguson Fund for Biology
Lawrence L. and Audrey W. Ferguson
Fletcher Jones Foundation
G. Louis Fletcher
Fling Charitable Trust
Fondazione International Balzan
Foundation Blanceflor Boncopagni-Lu Dovisi
Foundation for NIH Research/Bill and Melinda Gates Foundation
Mrs. Laura Frlan
Frank P. Hixon Fund

French Association for Cancer Research
John Douglas French Alzheimer's Foundation
Ms. Laura Frlan
Mrs. Camilla C. Frost

Louis A. Garfinkle Memorial Lab Fund
Gates Grubstake Fund
German Academy of Natural Scientists Leopoldina
Georgia Institute of Technology
William T. Gimbel Discovery Fund in Neuroscience
Ginger and Ted Jenkins
Gordon and Betty Moore Fellowship
Gordon and Betty Moore Foundation
Gordon Ross Fellowship Program
Gordon Ross Medical Foundation
Gosney Fellowship Fund
Griffuel Prize in Cancer Research
Grubstake Presidents Fund

William D. Hacker Trust
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Lawrence A. Hanson, Jr. Professorship of Biology
The Helen Hay Whitney Foundation
Hereditary Disease Foundation
Hicks Fund for Alzheimer Research
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House Ear Institute
Howard Hughes Medical Institute
Human Frontiers Science
Human Frontiers of Science
Huntington Hospital Research Institute

James S. McDonnell Foundation
Jane Coffin Childs Memorial Fund
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Joyce Funds
Juvenile Diabetes Research Foundation

Keck Discovery Fund
W.M. Keck Foundation
Kenneth T. and Eileen L. Norris Foundation
Kroc Foundation

Lawrence A. Hanson Jr. Professorship of Biology
Leonard B. Edelman Discovery Fund
Leukemia and Lymphoma Society
Life Sciences Research Foundation
Life Sciences Research Foundation Fellowship supported by the Howard Hughes Medical Institute
Lund University

March of Dimes
Lucille P. Markey Charitable Trust
Margaret E. Early Medical Research Trust
The G. Harold & Leila Y. Mathers Charitable Foundation
Max Planck Research Award for International Cooperation
Helen and Arthur McCallum Foundation
McGrath Foundation
McKnight Endowment Fund for Neuroscience
McKnight Foundation
McKnight Neuroscience of Brain Disorders Award
Merck & Co., Inc.
Mettler Fund for Autism
Michael J. Fox Foundation
MicroPropulsion Corporation/DARPA
The Millard and Muriel Jacobs Family Foundation
Mind Science Foundation
Moore Center for Biological Circuit Design
Moore Foundation

NARSAD
NASA/Ames
National Aeronautics and Space Administration (NASA)
National Alliance for Research on Schizophrenia and Depression
National Cancer Institute
National Eye Institute
National Geospatial Intelligence Agency
National Heart Lung and Blood Institute
National Human Genome Research Institute
National Institute for Biomedical Imaging and Bioengineering
National Institute for Neurological Diseases and Stroke
National Institute of Allergy and Infectious Diseases
National Institute of Child Health and Human Development
National Institute of Diabetes and Digestive and Kidney Diseases
National Institute of General Medical Science
National Institute of Mental Health, NRSA, USPHS
National Institute of Neurological Disorders and Stroke
National Institute on Aging
National Institute on Alcohol Abuse and Alcoholism
National Institute on Deafness and Other Communication Disorders
National Institute on Drug Abuse
National Institutes of Health, BIST, DE, NHGRI, NIDCR, NINDS, USPHS, NIGMS
National Research Service Award, National Institutes of Health
National Science Foundation (NSF)
Neurion Pharmaceuticals
Norman & Annemarie Davidson Fund for Research in Biology
Norman Chandler Professorship in Cell Biology
Norman Davidson Lectureship
Norman W. Church Fund

Office of Naval Research

The Packard Foundation
Ralph M. Parsons Foundation
Passano Foundation
Pediatric Dengue Vaccine Initiative
Gustavus and Louise Pfeiffer Foundation
Pharmaceutical Research and Manufacturers of American Foundation (PhRMA)
Phillip Morris External Research Program
Plum Foundation
Priztker Neurogenesis Research Consortium

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# Index of Names

3D reconstruction of 24 hpf zebrafish embryos using *in toto* imaging. Embryos were labeled with RNA encoding for membrane cherry and H2B-GFP at the one-cell stage. 24 hpf embryos were placed in 30% Daniau/1X tricaine solution and into a lateral agarose embryo-array mold. Imaging was performed on a LSM Pascal confocal, 40X 1.1NA objective, 2μ m z-step. Megacapture program was used to tile and capture over the whole embryo, and 2D z-sections were reconstructed with Megamontage. The final 3D view was obtained using Imaris software.

See abstract 273 by Frederique M. Ruf-Zamojski and Sean Megason, who are in the Scott E. Fraser lab.