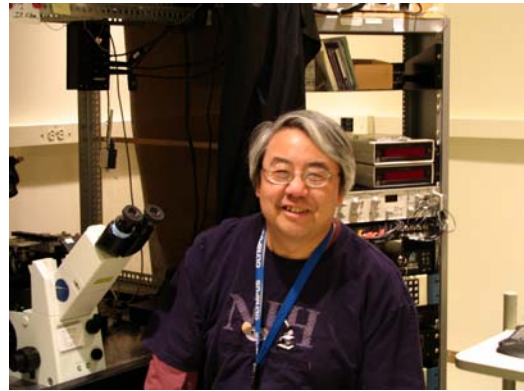

Laboratory of Molecular Physiology

Stephen R. Ikeda, MD, PhD, *Chief*

The goals of the Laboratory are to explore molecular mechanisms contributing to synaptic transmission and neuronal excitability at the molecular, cellular and organismic level. An emphasis is placed on developing new technologies to explore neuronal cellular and subcellular signaling pathways based on advanced optical techniques.



The *Section on Transmitter Signaling*, headed by Dr. Stephen R. Ikeda, focuses primarily on determining the molecular mechanisms underlying G-protein coupled receptor (GPCR) modulation of neuronal high-threshold Ca^{2+} channels in neuronal systems using electrophysiological and molecular techniques. A second goal is the development of optical sensors based on fluorescence resonance energy transfer (FRET) and reporter protein complementation to examine GPCR activity in living cells. Techniques in the laboratory include whole-cell voltage-clamp, heterologous expression in neurons by microinjection and viral techniques, wide-field FRET imaging, and total internal reflectance fluorescence microscopy. GPCRs (e.g., CB1, CRF, mGluR, NPY, and nociceptin receptors) comprise major cellular targets for pharmaceuticals used in the treatment of alcoholism and other addictive disorders. Additionally, N-type Ca^{2+} channels and heterotrimeric G-protein signaling pathways utilizing $\text{G}\beta\gamma$ have been directly implicated in ethanol reward and consumption mechanisms. Thus, increasing our knowledge of the basic mechanisms underlying GPCR modulation of Ca^{2+} and other ion channels should help clarify molecular mechanisms underlying the therapeutic efficacy of agents used for the treatment of alcoholism and addiction.

The *Section on Cellular Biophotonics*, headed by Dr. Steven S. Vogel, is focused on understanding how cells integrate exocytosis and endocytosis to regulate the abundance and assembly of membrane proteins on the cell surface. Toward this end his section has been developing advanced imaging techniques for the determination of protein-protein interaction and protein complex stoichiometry in living cells. Techniques available in the section include single- and two-photon laser scanning confocal microscopy, FRET imaging, spectral imaging, fluorescence lifetime imaging (FLIM), and time-resolved and steady state anisotropy. Model systems used in the laboratory include: Developing sea urchin eggs, transfected cell lines, and primary hippocampal neurons in culture. One project in the Section is investigating a form of endocytosis that occurs at cleavage furrows and appears to be required for cell division. The second project involves using FRET imaging to study protein-protein interactions in synapses.

The *Section on Model Synaptic Systems*, headed by Dr. Fumihito Ono, focuses on the cellular and molecular mechanisms underlying synaptic transmission in the vertebrate model organism *Danio rerio* (zebrafish). Zebrafish are genetically malleable, optically transparent at early stages of development, and amenable to higher throughput methodologies. Extensive regions of synteny between zebrafish and human genomes make *Danio* an ideal model for translational research.

The transparent nature of the early zebrafish embryo meshes well with the advanced imaging and electrophysiological expertise within the Laboratory.

The integration of the three Sections is designed to bring together investigators with common interests in molecules involved in neuronal excitability and synaptic transmission yet expertise in diverse but complementary techniques. The emphasis on model systems facilitates the application, from the cellular to organismic level, of new technologies in an efficient and cost-effective manner.

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Laboratory of Molecular Physiology

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Henry L. Puhl III, PhD, Staff Scientist

Damian Williams, PhD, Postdoctoral Fellow

Yu-Jin Won, PhD, Postdoctoral Fellow

Van B. Lu, PhD, Postdoctoral Fellow

Brandon Stauffer, BS, Postbac IRTA

Recent members

Juan Guo, MD, PhD, Postdoctoral Fellow

Huanmian Chen, MD, PhD, Postdoctoral Fellow



V Lu, B Stauffer, D Williams, H Puhl, S Ikeda, Y Won

Ion Channel Modulation by Second Messenger Systems

The *Section on Transmitter Signaling* focuses primarily on determining the molecular mechanisms underlying G-protein coupled receptor (GPCR) modulation of voltage-gated Ca^{2+} channels in neuronal systems using electrophysiological, optical, molecular, and biochemical techniques. A consequence of modulation, which usually manifests as a decrease in current flow through the channel, neuronal excitability and neurotransmitter release at synapses is modified. Although several signaling pathways have been identified, the best-studied is a direct inhibition of the ion channel by G-protein $\beta\gamma$ -subunits liberated from the G-protein heterotrimer following agonist-mediated receptor activation. This canonical pathway is shared among the high-voltage activated Ca^{2+} channels of the $\text{Ca}_v2.x$ family ($\text{Ca}_v2.1$ – 2.3 ; P/Q-, N-, and R-type, respectively) and represents one of the most widely studied and best understood mechanism of presynaptic inhibition. GPCRs (e.g., CB1, CRF, mGluR, NPY, and nociceptin) comprise major cellular targets for pharmaceuticals used in the treatment of alcoholism and other addictive disorders. Additionally, N-type Ca^{2+} channels and heterotrimeric G-protein signaling pathways utilizing $\text{G}\beta\gamma$ have been directly implicated in ethanol reward and consumption mechanisms.

Optical methods for quantifying protein-protein interactions in living cells

Chen, Puhl

We have previously demonstrated that Förster resonance energy transfer (FRET) efficiency and the relative concentration of donor and acceptor fluorophores can be determined in living cells using 3-cube wide-field fluorescence microscopy. In this manuscript, we extended the methodology to estimate the effective equilibrium dissociation constant (K_d) and the intrinsic FRET efficiency (E_{max}) of an interacting donor-acceptor pair. Assuming bimolecular interaction, the predicted FRET efficiency is a function of donor concentration, acceptor concentration, K_d , and E_{max} . We estimated K_d and E_{max} by minimizing the sum of the squared error (*SSE*) between the predicted and measured FRET efficiency. This was accomplished by examining the topology of *SSE* values for a matrix of hypothetical K_d and E_{max} values. Applying an *F*-test, the 95% confidence contour of K_d and E_{max} was calculated. We tested the method by expressing an

inducible FRET fusion pair consisting of FKBP12–Cerulean and Frb–Venus in HeLa cells. As the K_d for FKBP12-rapamycin and Frb has been analytically determined, the relative K_d (in fluorescence units) could be calibrated with a value based on protein concentration. The described methodology should be useful for comparing protein-protein interaction affinities in living cells.

Chen H, Puhl HL, Ikeda SR. Estimating protein-protein interaction affinity in living cells using quantitative FRET measurements.

J Biomed Optics 12:054011, 2007.

Calcium channel modulation via atypical cannabinoid-related GPCRs and endogenous ligands

Guo, Williams, Puhl

GPR35 is a G protein coupled receptor recently “de-orphanized” using high throughput intracellular calcium measurements in clonal cell lines expressing a chimeric G-protein α -subunit. From these screens, kynurenic acid, an endogenous metabolite of tryptophan, and zaprinast, a synthetic inhibitor of cyclic guanosine monophosphate specific phosphodiesterase, emerged as potential agonists for GPR35. To investigate the coupling of GPR35 to natively expressed neuronal signaling pathways and effectors, we heterologously expressed GPR35 in rat sympathetic neurons and examined the modulation of N-type ($\text{Ca}_v2.2$) calcium channels. In neurons expressing GPR35, calcium channels were inhibited in the absence of overt agonist indicating a tonic receptor activity. Application of kynurenic acid or zaprinast resulted in robust voltage-dependent calcium current inhibition characteristic of $\text{G}\beta\gamma$ -mediated modulation. Both agonist-independent and -dependent effects of GPR35 were blocked by *Bordetella pertussis* toxin pretreatment indicating the involvement of $\text{G}_{i/o}$ proteins. In neurons expressing GPR35a, a short splice variant of GPR35, zaprinast was more potent ($\text{EC}_{50} = 1 \mu\text{M}$) than kynurenic acid (58 μM), but had a similar efficacy (approximately 60% maximal calcium current inhibition). Expression of GPR35b, which has an additional 31 residues at the N-terminus, produced similar results but with much greater variability. Both GPR35a and GPR35b appeared to have similar expression patterns when fused to fluorescent proteins. These results suggest a potential role for GPR35 in regulating neuronal excitability and synaptic release.

In another study, the effects of N-arachidonoyl L-serine (ARA-S), a recently discovered lipoamino acid found in the central nervous system, on N-type calcium channels of rat sympathetic ganglion neurons were determined using whole-cell patch-clamp. Application of ARA-S produced a rapid and reversible augmentation of calcium current that was voltage-dependent and resulted from a hyperpolarizing shift in the activation curve. ARA-S did not influence G-protein modulation of calcium channels and appeared to act independently of G-protein coupled receptors. These findings provide a foundation for investigating possible roles for ARA-S in nervous system function.

Publications 2008:

- Guo J, Williams, DJ, Puhl HL, Ikeda SR. Activation of GPR35, an orphan G protein coupled receptor, inhibits N-type Ca^{2+} channels in rat mammalian neurons.
J Pharmacol Exp Ther 324:342–51, 2008.
- Guo J, Williams, DJ, Ikeda SR. *N*-Arachidonyl L-serine, a putative endocannabinoid, alters the activation of N-type Ca^{2+} channels in sympathetic neurons.
J Neurophysiol 100:1147–51, 2008.

Sensory neuron-specific Na^+ channel ($\text{Na}_v1.8$) function and expression

Puhl; in collaboration with Schofield

The tetrodotoxin (TTX)-resistant Na^+ current arising from $\text{Na}_v1.8$ containing channels participates in nociceptive pathways but is difficult to functionally express in traditional heterologous systems. In this study, we showed that injection of cDNA encoding mouse $\text{Na}_v1.8$ into the nuclei of rat superior cervical ganglion (SCG) neurons resulted in TTX-resistant Na^+ currents with amplitudes equal to or exceeding the currents arising from natively expressing channels of mouse dorsal root ganglion (DRG) neurons. The activation and inactivation properties of the heterologously expressed $\text{Na}_v1.8$ Na^+ channels were similar but not identical to native TTX-resistant channels. Most notably, the half-activation potential of the heterologously expressed $\text{Na}_v1.8$ channels were shifted approximately 10 mV toward more depolarized potentials. Fusion of fluorescent proteins to the N- or C-termini of $\text{Na}_v1.8$ did not substantially affect functional expression in SCG neurons. Unexpectedly, fluorescence was not concentrated at the plasma membrane but found throughout the interior of the neuron in a granular pattern. A similar expression pattern was observed in nodose ganglion neurons expressing the tagged channels. In contrast, expression of tagged $\text{Na}_v1.8$ in HeLa cells revealed a fluorescence pattern consistent with sequestration in the endoplasmic reticulum thus providing a basis for poor functional expression in clonal cell lines. Our results establish SCG neurons as a favorable surrogate for the expression and study of molecularly defined $\text{Na}_v1.8$ -containing channels. The data also indicate that unidentified factors may be required for the efficient functional expression of $\text{Na}_v1.8$ with a biophysical phenotype identical to that found in sensory neurons.

In addition, we identified the promoter for the tetrodotoxin insensitive sodium channel, $\text{Na}_v1.8$, which is encoded by the *Scn10a* gene. $\text{Na}_v1.8$ expression is restricted to small and medium diameter nociceptive sensory neurons of the dorsal root (DRG) and cranial sensory ganglia. In order to understand the stringent transcriptional regulation of the *Scn10a* gene, the sensory neuron specific promoter was functionally identified. While identifying the mRNA 5' end, alternative splicing within the 5' UTR was observed to create heterogeneity in the RNA transcript. Four kilobases of upstream genomic DNA was cloned and the presence of tissue specific promoter activity was tested by microinjection and adenoviral infection of fluorescent protein reporter constructs into primary mouse and rat neurons, and cell lines. The region contained many putative transcription factor binding sites and strong homology with the predicted rat ortholog. Homology to the predicted human ortholog was limited to the proximal end and several conserved *cis* elements were noted. Two regulatory modules were identified by microinjection of reporter constructs into DRG and superior cervical ganglia neurons: a neuron specific proximal promoter region between -1.6 and -0.2kb of the transcription start site cluster,

and a distal sensory neuron switch region beyond -1.6kb that restricted fluorescent protein expression to a subset of primary sensory neurons.

Publications 2008:

Schofield GG, Puhl HL, Ikeda SR. Properties of wild-type and fluorescent protein-tagged mouse tetrodotoxin-resistant sodium channel (Nav1.8) heterologously expressed in rat sympathetic neurons.

J Neurophysiol 99:1917–27, 2008.

Puhl HL, Ikeda SR. Identification of the sensory neuron specific regulatory region for the mouse gene encoding the voltage-gated sodium channel Nav1.8.

J Neurochem 106:1209–24, 2008.

Heterotrimeric G-protein interaction with GoLoco motif containing proteins.

Guo; in collaboration Willard, Zheng, Digby, Kimple, Johnston, Bosch, Willard, Lambert, Du, Siderovski

Heterotrimeric G-protein $G\alpha$ subunits and GoLoco motif proteins are key members of a conserved set of regulatory proteins that influence invertebrate asymmetric cell division and vertebrate neuroepithelium and epithelial progenitor differentiation. GoLoco motif proteins bind selectively to the inhibitory subclass ($G\alpha_i$) of $G\alpha$ subunits, and thus it is assumed that a $G\alpha_i$ /GoLoco motif protein complex plays a direct functional role in microtubule dynamics underlying spindle orientation and metaphase chromosomal segregation during cell division. To address this hypothesis directly, we rationally identified a point mutation to $G\alpha_i$ subunits that renders a selective loss-of-function for GoLoco motif binding: namely, an asparagine-to-isoleucine substitution in the α D- α E loop of the $G\alpha$ helical domain. This GoLoco-insensitivity (“GLi”) mutation prevented $G\alpha_{i1}$ association with all human GoLoco motif proteins and abrogated interaction between the *C. elegans* $G\alpha$ subunit GOA-1 and the GPR-1 GoLoco motif. In contrast, the GLi mutation did not perturb any other biochemical or signaling properties of $G\alpha$ subunits, including nucleotide binding, intrinsic and RGS protein-accelerated GTP hydrolysis, and interactions with $G\beta\gamma$ dimers, adenylyl cyclase and seven transmembrane-domain receptors. GoLoco-insensitivity rendered $G\alpha_i$ subunits unable to recruit GoLoco motif proteins such as GPSM2/LGN and GPSM3 to the plasma membrane, and abrogated the exaggerated mitotic spindle rocking normally seen upon ectopic expression of wild type $G\alpha_i$ subunits in kidney epithelial cells. This GLi mutation should prove valuable in establishing the physiological roles of $G\alpha_i$ /GoLoco motif protein complexes in microtubule dynamics and spindle function during cell division as well as delineate potential roles for GoLoco motifs in receptor-mediated signal transduction.

Publication 2008:

Willard FS, Zheng Z, Guo J, Digby GJ, Kimple AJ, Johnston CA, Bosch D, Willard MD, Lambert NA, Ikeda SR, Du Q, Siderovski DP. A point mutation to $G\alpha_i$ selectively blocks GoLoco motif binding: Direct evidence for $G\alpha$ /GoLoco complexes in mitotic spindle dynamics.

J Biol Chem 283:36698–710, 2008.

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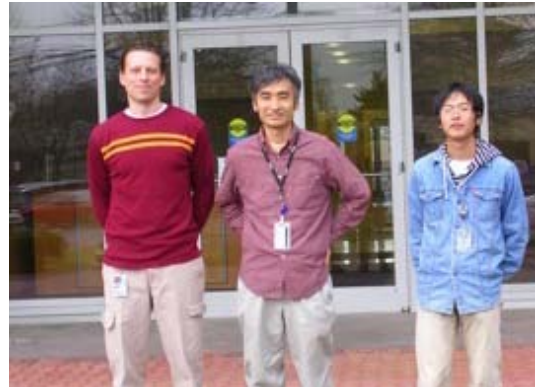
Section on Model Synaptic Systems

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J Urban, F Ono, T Ikenaga

Zebrafish as a model to study nervous system

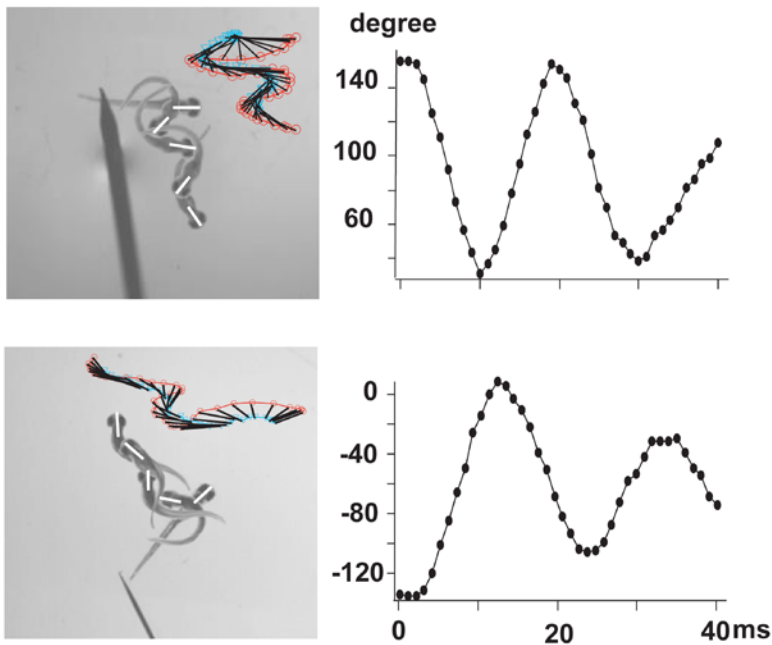
The section on model synaptic systems started in May 2007, when Fumihito Ono moved to NIH/NIAAA from the University of Florida. Though projects have undergone some shifts after moving to NIAAA, the research in our lab has always focused on the nervous system. In particular, we are interested in how neural functions lead to various behaviors of animals. We use a model system, zebrafish, to address this question. Zebrafish offer unique advantages that complement other commonly-used model systems such as mice.

We are currently studying the nervous system at several levels. An experimental paradigm we have used heavily for the past several years is the neuromuscular junction (NMJ). NMJ is a synapse between a motor neuron and a muscle cell. Not only is this synapse directly linked to various diseases arising from genetic defects, it also offers an exceptional accessibility for an array of experimental techniques. As a result, NMJ is the best-studied synapse in vertebrate biology. We have recently broadened our field and are now studying neural networks in the central nervous system and its response to ethanol.

Projects using neuromuscular junction

Our projects on NMJ center around locomotory mutants we discovered to have defects in two key molecules of the neuromuscular synapse. One mutant lacks acetylcholine receptors (AChR) in the muscle. As a result, the fish cannot mount a movement when the motor neuron releases ACh. Another mutant has a dysfunctional rapsyn. Rapsyn is a post-synaptic protein that brings AChRs together. In this fish, AChRs do not make clusters at the synapse and are diffusely distributed over the muscle cell surface. From the AChR-less mutant, we found that AChR plays an active role, directing rapsyn molecules to synapse. In rapsyn mutant fish, we found that AChRs not only fail to form clusters at synapse, but also their functions are altered. When motor neurons fire at a high frequency, the amplitude of AChR current remains constant in wild type, whereas in rapsyn-mutant fish the response shows a marked attenuation with repeated firing of motor neurons. Several projects aim to figure out the mechanisms underlying these unexpected functions of AChR and rapsyn.

AChR-less fish has an analogous disease in humans, which is called Fetal Akinesia Deformation Sequences (FADS). Human embryos that harbor mutation in one of the AChR genes suffer premature death in the first trimester. We introduced a modified AChR gene into the mutant fish. The introduced gene expressed in all muscle cells, which led to a successful rescue of the mutant fish. The rescued fish survives well beyond sexual maturation, and they can mate normally, producing offspring. To the best of our knowledge, this is the first case of a mutant animal corresponding to first trimester lethality in human that has been rescued by a transgene and survived to adulthood. This result is reported in a paper now in press for the Journal of Neuroscience (Epley et al., 2008).



Movement of embryos at 3 days post fertilization in response to a tail stimulus. An embryo rescued by a transgene is shown in the upper panel and a wild type embryo in the lower panel. Insets represent the swimming as a series of rostral midlines. Head angles plotted against time are shown on the right. (Epley et al., 2008)

The AChR-less mutant and the rapsyn mutant also provide a unique opportunity to study functions of AChRs expressed in the central nervous system (CNS) which play important roles in various neural disorders including alcoholism. In the neuromuscular synapse of AChR-less mutant, we showed that pre-synaptic machinery releasing ACh develops normally. These AChR-less mutants therefore allow us to study brain-type AChRs in a real synaptic context, when the receptors are ectopically expressed in the muscle cells. We are using this "model synapse" to study functions of brain type AChR receptors, $\alpha 4\beta 2$ and $\alpha 7$, which are difficult to study in their native environment.

Projects on the central nervous system

One of the major advantages of zebrafish is that it is amenable to large-scale forward genetics. We have performed several forward genetic screenings on zebrafish. A transgenic zebrafish isolated from one such screening expresses red fluorescent protein (RFP) in a sub-population of neurons. In these neurons, the expression of a transcription factor *pax8*, whose gene was "trapped" by the RFP gene, is replaced by RFP. In the spinal cord, the labeling of *pax8* expressing cells *in vivo* enabled us to identify a population of inter-neurons, which was not previously described. We are now characterizing these inter-neurons in terms of their connections to other spinal neurons, and studying the effect of deleting *pax8* and related genes for the neural network in the spinal cord. Use of this and other lines of fish will allow us to study functions of developmental genes in living animals

Implications for ethanol-related diseases

The link of on-going projects in the lab to ethanol can be found at several levels. The AChRs we are expressing in the NMJ determine the sensitivity of brain to nicotine, and it is implicated that alcohol and nicotine exert reciprocal interactions in the human brain, as suggested by the comorbidity of alcoholism and nicotine addiction.

We are also performing experiments to study the direct effects of ethanol on the zebrafish CNS, using an array of techniques we have developed over the years. We are studying the intoxicated embryos at the level of genomics, cellular physiology, anatomy and locomotion. Embryos grown in Ethanol in these experiments are comparable to fetuses with Fetal Alcohol Syndrome (FAS) in human. Insights gained from these experiments will help to clarify the mechanisms of FAS.

Publications 2007-2008:

Ono F. An emerging picture of synapse formation: A balance of two opposing pathways.

Science Signaling 1: pe3, 2008.

Epley K, Urban J, Ikenaga T, Ono F. A modified acetylcholine receptor d-subunit enables a null mutant to survive beyond sexual maturation.

J Neurosci In press, 2008.

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Section on Cellular Biophotonics

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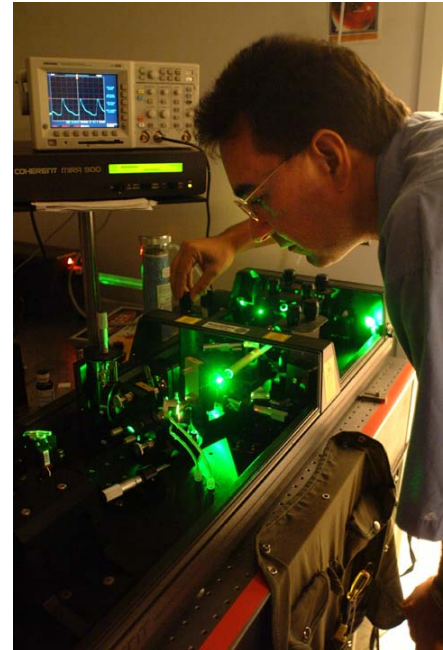
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FRET imaging of protein-protein interactions in living cells

The Section on Cellular Biophotonics uses imaging techniques, such as two-photon microscopy, spectral imaging, fluorescence lifetime microscopy, and fluorescence anisotropy analysis to study how protein complexes regulate synaptic function in living cells. Recently, we have concentrated our efforts on utilizing Förster's Resonance Energy Transfer (FRET) to monitor protein-protein interactions. This method has great potential for studying protein interactions because it is sensitive to changes in the distance separating two fluorophores on the 1-10 nm scale. FRET imaging in conjunction with the development of spectral variants of Green Fluorescent Protein (GFP) provides the opportunity to genetically tag synaptic proteins of interest and monitor their interactions with other labeled proteins in real time.

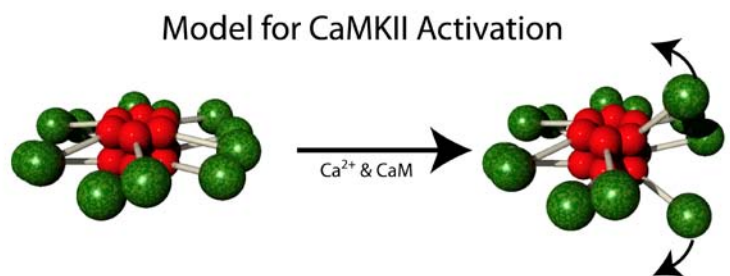


Our Section's initial efforts concentrated on 1. Building and testing a laser scanning microscope specifically designed for studying protein-protein interactions in living cells, 2. Developing new methods for measuring FRET, and 3. Overcoming some of the practical limitations of FRET imaging. The microscope we have constructed is a fully functional laser scanning two-photon microscope, with the additional capabilities of measuring fluorescent emission spectra (spectral imaging), fluorescent lifetime decays (FLIM), and fluorescent anisotropy lifetime decays (rFLIM). These added capabilities make it specifically useful for monitoring FRET between either dissimilar (Hetero-FRET) or similar (Homo-FRET) fluorophores.

Currently we are working on 2 major projects:

Our first project uses time-resolved fluorescence anisotropy decay analysis to monitor changes in the multimeric structure of Cam kinase-II. This abundant post-synaptic enzyme has been shown to play a pivotal role in learning and memory. It is believed that long lived structural changes in this

protein complex might be the embodiment of some forms of memory. Preliminary results indicate that structural changes associated with Cam kinase-II activation can be detected with FRET imaging. We are currently interested in determining how the CaMKII holoenzyme is



assembled from α and β subunits. The specific combination of these subunits is thought to impact CaMKII's ability to regulate synaptic efficacy. We are also interested in exploiting a FRET-based assay for CaMKII activation that we have recently developed to determine what factors regulate the time-course of kinase activation in dendritic spines.

The second project, is investigating the role of Dysferlin in wound repair, and has direct relevance to understanding the molecular basis of Limb Girdle and Miyoshi Muscular Dystrophy. Both of these syndromes are caused by mutations in the protein Dysferlin, but the function of Dysferlin itself is not known. Our working hypothesis is that Dysferlin mediates calcium triggered membrane fusion utilized by plasma membrane wound repair mechanisms. This project combines our laboratory's long-held interest and expertise in using live cell imaging to study the mechanism of calcium triggered exocytosis in sea urchin eggs, with our more recent work using laser wounding and two-photon imaging to examine plasma membrane repair mechanisms. The Dysferlin project is funded, in part, by a gift from the Jain Foundation.

Publications 2008:

Covian-Nares JF, Smith RM, Vogel SS. Two independent forms of compensatory endocytosis maintain embryonic cell surface homeostasis during early development.

Dev Biol 316: 135-48, 2008.

Koushik SV, Vogel SS. Energy migration alters the fluorescence lifetime of Cerulean: Implications for FLIM-FRET measurements.

J Biomed Optics 13:031204, 2008.

Key prior publication:

Vogel SS, Thaler C, Koushik SV. Fanciful FRET.

Science STKE 2006(331)re2.

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