Cellular Imaging at the Nanoscale: Poster Abstract Booklet

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Wavelength and pH Dependent Detection of Homocysteine

Aabha Barve, Mark Lowry, Robert M. Strongin
Portland State University. E-mail: aabha@pdx.edu

Elevated levels of homocysteine in human plasma have been associated with neural tube defects cardiovascular and Alzheimer diseases. Whereas, research has shown that the deficiency of cysteine is associated with conditions such as slow growth in children, hair depigmentation, liver damage, muscles and fat loss. Therefore, it is important to detect the physiological concentrations of cysteine and homocysteine in human plasma. In the past, many techniques such as spectrophotometry, fluorimetry and electrochemical methods have been used to detect these biomolecules, but despite being sensitive, cysteine and homocysteine responses interfere with each other. Previously, we have reported that the fluorescence quenching of fluorescein dialdehyde at pH(9.5) in response to cysteine and homocysteine due to the formation of 5 and 6 membered heterocycles respectively. Herein, we are trying to optimize the system by evaluating several pH values and excitation wavelengths. We show that the response of fluorescein dialdehyde to thiols is dependent on pH, excitation wavelengths and time. In our future work, we aim to improve the detection limit of homocysteine and cysteine and understand the mechanism of reaction of these aldehyde bearing chromophores with amino thiols.
Synthesis and Characterization of Photoswitchable Fluorophores for Multispectral Super Resolution Microscopy

Amy M. Bittel, Andrew Nickerson, Li-Jung Lin, Xiaolin Nan, Summer L. Gibbs

Department of Biomedical Engineering, Oregon Health and Science University. E-mail: bittel@ohsu.edu

Super resolution microscopy (SRM) has overcome the historic spatial resolution limit of light microscopy, enabling visualization and investigation of cellular structures and multi-protein complexes at the nanometer scale with fluorescence. Using single-molecule localization microscopy the precise location of a stochastically activated population of photoswitchable fluorophores is determined during the collection of many images to form a single image with resolution on the order of 10-20 nm, an order of magnitude improvement over conventional fluorescence microscopy. However, single-molecule SRM is currently limited to a maximum of 4 color imaging in a single sample due to suboptimal photoswitchable fluorescent probes and conventional bandpass filtering for emission detection. In the current work, libraries of novel fluorophores are synthesized using peptide nucleic acid (PNA) encoded combinatorial solid phase synthesis as the chemical platform to create optimized photoswitchable fluorophores. Libraries are synthesized using the split and mix technique on solid supports followed by separation and identification via PNA hybridization with complimentary oligonucleotide microarrays enabling high-throughput screening to characterize optical and photoswitching properties of the fluorophore libraries. Using this method, a set of photoswitchable fluorophores, that can be excited by 4-6 laser lines but emit throughout the spectral range (450-850nm), will be created for multispectral super-resolution microscopy (MSSRM). MSSRM will revolutionize fluorescence microscopy for the scientific community as 20 color nanometer resolution images of a single sample will be possible. This will have profound effects on biological research, including the study of cell signaling pathways, bacterial and viral infections, disease mechanisms, and other cell pathology.
Ligand Deployment and Sensing in a Large, 3-D Extracellular Space

Michael Danilchik & Elizabeth Brown

Integrative Biosciences and Cell and Developmental Biology, Oregon Health & Science University
E-mail: danilchi@ohsu.edu

In Xenopus embryos, dorsal-ventral axis specification occurs prior to the onset of zygotic transcription at the midblastula transition (MBT), and thus depends on localization or localized activation of maternal components. Activation of the canonical Wnt pathway in dorsal lineages depends on deployment of a maternal Wnt ligand, probably Xwnt11b, modulated by secreted extracellular factors, including Xwnt5a, Xdkk1, HSPGs (e.g., Xglyp-4) and glycosyltransferases (e.g., X.ext1). Spatial information about the extracellular environment in which these factors and their receptors are expressed and interact during the early cleavage stages is lacking; hence this confocal, SEM, and TEM exploration of the Xenopus blastocoel. Three findings emerge from these studies. First, large, spherical extracellular vesicles extruded from the roof and floor of the blastocoel undergo fragmentation, releasing micron-scale microvesicles containing multiple layers of membrane surrounding a core of dense, branched proteoglycan aggregates. Second, the free basolateral surfaces of early embryos display a high degree of protrusive activity, with numerous short filopodia flailing about in the blastocoel, potentially dispersing secreted materials over long distances. Third, nonadjacent blastomeres maintain long-term contacts with each other during early development via thousands of extremely long, stable membrane nanotubes. These structures actively traffic both membrane and cytoplasm, exhibit retrograde actin flow, and are endocytotically active. Engulfed blastocoelic material is rapidly transferred to an acidified endosomal compartment, suggesting the nanotubular structures play a role in long-range detection of secreted Wnt ligands in the blastocoel. Consistent with this idea, pharmacological blockage of endosome acidification results in dorsal-ventral axis defects. We conclude that cell-signaling ligands are secreted during early development into a spatially complex extracellular compartment whose membrane protrusive dynamics plays roles in the dispersal and detection of morphogenic factors.
Multi-photon Excitation and Characterization of Novel Fluorophores for Cellular Imaging

Fredrick M. DeArmond¹, Robert M. Strongin², Allan Kachelmeier³, Peter Steyger³, Erik J. Sánchez¹,⁴

¹Department of Physics, Portland State University, ²Department of Chemistry, Portland State University, ³Oregon Hearing Research Center, Oregon Health & Science University, ⁴Department of Electrical and Computer Engineering, Portland State University. E-mail: fmd@pdx.edu

Aminoglycoside-induced ototoxicity and nephrotoxicity are well-documented, yet the mechanisms required for cellular uptake and clearance of aminoglycosides, particularly gentamicin, are not yet fully understood. The lack of precise understanding of these mechanisms has proven to be a problem faced by researchers who ultimately hope to develop ways of mitigating the debilitating effects of these life-saving drugs – deafness and vestibular deficits. The work presented here represents progress on two parallel projects intended to address this issue from two different angles. An increase in resolution over traditional far field fluorescence microscopy is necessary in order to track gentamicin directly. This increase in resolution is especially important for tracking gentamicin through highly heterogeneous cyto-architectural structures, such as the stria vascularis within the mammalian cochlea. To assist in these efforts, novel fluorophores are being characterized on a single molecule level to identify potential candidates for use in super-resolution systems which will provide the necessary resolution.

A second approach to mitigating the effects of gentamicin in clinical applications is to identify the specific proteins responsible for regulating the cellular uptake and clearance of gentamicin. However, to achieve this goal we need to first understand the mechanisms of uncharacterized quenching behavior in a variety of lipid environments, including cell-free, fixed cells and live cells. To identify these quenching mechanisms, we intend to perform a series of fluorescence correlation spectroscopy (FCS) experiments in parallel with the characterization of individual fluorophores.

Both approaches utilize non-linear multi-photon excitation using an ultrafast mode-locked Ti:Sapphire laser to provide a high degree of flexibility when using fluorophores with unique absorption characteristics, as well as providing inherently good spatial resolution in z for inter- and intra-cellular imaging.
New Fluorescent Probes for Visualizing Autophagy

Nick J Dolman, Kevin Chambers, WenJun Zhou, Rachel Smith, Kyle Gee, Michael Janes

Molecular Probes Labelling and Detection Technologies, Life Technologies Corporation, Eugene, Oregon, USA. E-mail: Nicholas.Dolman@lifetech.com

Autophagy functions as a predominately pro survival mechanism removing damaged or aggregated cellular components from the cytosol. This is achieved by the encompassment of cargo, via an emerging family of receptors, by the isolation membrane that then fuses to form the double-membrane autophagosome. The autophagosome itself carries no degradative capability. To degrade these components, the autophagosome needs to deliver them to the lysosome thereby forming the autolysosome. Here we show imaging assays that allow discrimination of autophagosome to autolysosome progression through multi-color imaging using novel fluorescent lysosomal probes. By applying this approach we have quantified the kinetics of autophagosome formation, fusion with the lysosome and acidification. As stated above autophagy functions as a mechanism by which a cell can remove damaged organelles or aggregated proteins. We also describe novel imaging assays for specific forms of autophagy such as mitophagy or of aggregated proteins. For mitophagy we describe the use of environmentally sensitive and insensitive mitochondrial probes in conjunction with autophagy specific markers in multi-color time-lapse imaging. Through the use of metabolic labeling of proteins we show imaging assays to assess the formation, localization and clearance of protein aggregates. Data relevant to clearance of both short- and long-lived proteins by the Proteasomal or lysosomal pathways respectively will be shown.
Frequency Modulation for Non-Contact SPM Cellular Imaging

Rodolfo Fernandez, Elia Zegarra, Andres La Rosa

Department of Physics, Portland State University. E-mail: rfern@pdx.edu

The main challenges posed by Scanning Probe Microscopy (SPM) imaging of biological samples are the viability of the specimen throughout the imaging process and the unwanted modification of the cellular membrane due to molecular transfer or excessive interaction with the probe. Several groups have performed cellular and molecular SPM imaging in fixed and in vivo samples. Thus far the success has been limited to relative hard specimens. Currently, for certain soft biological samples the damage incurred by the tip-sample forces ultimately destroys the subject of investigation. Much of the effort is directed towards developing the ability to produce a signal (amplitude or phase) with enough Signal to Noise Ratio (SNR) for accurate feedback control, but without damaging interaction. Acoustic excitation and detection is a good candidate for such non-invasive probing. Sub-surface acoustic techniques have already been developed, using multi-frequency techniques. However, crucial to the reliability of these techniques for biological imaging is the determination of membrane modifications caused by the probe. Here we present a novel Quartz Tuning Fork SPM (QTF-SPM) scheme that employs acoustic sensing to measure the amplitude of oscillation of the QTF (without the phase distortions introduced by the electrical excitation) in addition to an acoustic sensor located beneath the specimen. Most importantly, the setup included a Frequency Modulation (FM) force mode that enables detection of the onset of conservative interactions. In order to determine the surface position, current measurements when in proximity to the substrate were simultaneously measured. Approach-retraction curves show the transfer of energy through phononic channels between the tip and the surface, with the probe still several nanometers away but within the confines of the adsorbed water layer. These results seem to validate the usage of acoustic techniques for SPM imaging of soft biological materials.
Synthesis and Characterization of Nerve-Specific Fluorophores for Image-Guided Surgery

Kayla Hackman, Theresa M. Koppie, Dusan Pavnik, Summer L. Gibbs

Department of Biomedical Engineering, Oregon Health & Science University. Email: hackman@ohsu.edu

Nerve damage during surgery causes significant morbidity for patients leaving them with chronic pain, loss of function, and a decreased quality of life following healing of the surgical site. Although neuroanatomy is well known, nerve visualization in the operating suite is challenging due to patient to patient variability and the nature of the small, translucent nerve tissue which is protect deep within muscle and adipose. Nerve visualization could be improved through administration of a Small molecule fluorophore with specificity for nerve tissue following local or systemic administration. However, few small molecule fluorophores are known that partition into nerve tissue following in vivo administration and none are currently optimized for clinical use. In the current work peptide nucleic acid (PNA) encoded combinatorial solid phase synthesis is utilized to synthesize libraries of novel fluorophores from known nerve-specific chemical scaffolds. escent probes have unique optical properties that are determined by the specific electronic structure within each molecule. Such molecules have been used across a wide range of fields, from high-resolution microscopy to illumination of cancerous tumor surgical margins. Each application has unique requirements for an 'ideal' fluorophore candidate. Presently, there is a great deal of interest in tuning optical properties through manipulation of electronic structure, which often requires synthesis of a large library of compounds that are screened one by one for the desired traits. While split and mix solid phase synthesis allows for the relatively quick creation of a large, high-purity library of compounds, the issue of identifying the structural makeup of individual members remains. Here we show Encoding the design and synthesis of a large library of fluorophores encoded with peptide nucleic acid (PNA) tags. We found that the tagging small molecule fluorophores with unique PNA sequences allows for spatial deconvolution of their synthetic history through utilization of DNA microarrays while maintaining the desired optical and biological properties. This method of tagging and subsequent identification can be applied to a wide variety of library sizes through manipulation of the PNA codons. The reported platform creates a method in which large small molecule organic fluorophore libraries with hundreds to thousands of members can be quickly efficiently synthesized created, screened and identified for further study. By expediting the identification process, a greater number of "hit" molecules can be identified in a shorter amount of time, while simultaneously decreasing time spent on molecules with less ideal traits. We anticipate that this platform can be utilized in conjunction with known fluorescent molecule scaffolds to tune an ideal biological small molecule candidate to any desired optical property through creation of large, diverse small molecule libraries An example library from the distyrylbenzene (DSB) fluorophore scaffold was synthesized and screened for nerve-specific properties. Utilizing the DSB library with >200 unique members enabled quantitative structure activity relationship (QSAR) modeling to elucidate the relationship between chemical structure and nerve partitioning. The QSAR is currently be utilized to direct library synthesis of a near infrared nerve-specific fluorophores for optimal in vivo imaging.
Simple and Selective Detection of Homocysteine

Lovemore Hakuna, Jorge O. Escobedo and Robert M. Strongin*

Department of Chemistry, Portland State University, Portland, OR 97201. E-mail: lhakun@pdx.edu

Elevated plasma homocysteine levels are associated with increased risk of myocardial infarction, stroke and venous thromboembolism. Homocysteine has also been linked to increased risk of Alzheimer's disease, neural tube defects and complications during pregnancy, inflammatory bowel disease and osteoporosis. There is a strong case for detecting and treating hyperhomocysteinemia in patients with a history of premature cardiovascular disease, stroke, or venous thromboembolism. Homocysteine monitoring is of interest for the screening of inborn errors of methionine metabolism.

In addition to analytical HPLC with a derivatization step, gas chromatography/mass spectrometry (GC-MS) and commercial immunoassays are often used to monitor Hcy. GC-MS requires instrumentation that is not typically applied at point-of-care. The immunoassays require enzymes, antibodies and biomolecules that are relatively expensive and fragile; therefore, careful attention to storage conditions is required.

A protocol will be presented based on the kinetically favored formation of the alpha-amino carbon-centered radical of homocysteine, that allows for the selective reduction of methyl viologen dication to its corresponding radical cation in human blood plasma. This characteristic reduction mechanism has proven useful in promoting the selective development of chromogen solution color by homocysteine. Other commonly occurring thiols (such as cysteine and glutathione) as well as other amino acids do not produce interference. No washing steps, biological reagents or sample pre-processing steps apart from disulfide reduction are needed.
Investigating Thyroid Hormone Action in Myelination Disorders

Meredith D. Hartley and Thomas S. Scanlan

Oregon Health & Science University. E-mail: hartleme@ohsu.edu

X-linked adrenoleukodystrophy (X-ALD) is a rare genetic disease that affects the adrenal cortex and central nervous system. In the most severe clinical phenotype, which presents in 35-40% of patients, the disease manifests as inflammatory cerebral demyelination at age 5-8 and patients survive for only 1-2 years. X-ALD is caused by mutation of a single gene, ABCD1, which leads to an accumulation of very long chain fatty acids (VLCFAs) in patients. ABCD2 is close homolog of ABCD1 and upregulation of ABCD2 has been shown to compensate for defective ABCD1. It is known that ABCD2 is regulated by thyroid hormone, and our study seeks to further evaluate the ability of thyromimetics to upregulate ABCD2. Thyromimetics are tissue-selective thyroid hormone receptor agonists that avoid thyrotoxic effects.

In this study we have utilized the thyromimetic sobetirome, which was developed in the Scanlan laboratory and is currently in clinical trials. We evaluated the regulation of ABCD2 using quantitative PCR, and we measured the levels of VLCFAs with GC-MS. Our results demonstrate that both thyroid hormone and sobetirome can induce ABCD2 in vitro in mouse liver cells, cultured oligodendrocytes and microglia, and skin fibroblasts harvested from X-ALD patients. We also observed in vivo upregulation of ABCD2 in brain and liver tissue of mice treated systemically with thyroid hormone or sobetirome. In addition, mice treated with thyroid hormone or sobetirome show lower levels of VLCFAs in plasma and erythrocytes. Ongoing efforts involve imaging the effects of thyroid hormone and sobetirome on demyelination in disease models. These results represent our current strategy for validating the thyromimetic sobetirome as an efficacious treatment for X-ALD.
Life Without a Diamine Transporter: a *T. cruzi* Perspective

Marie-Pierre Hasne and Buddy Ullman

Department of Biochemistry and Molecular Biology, Oregon Health & Science University. E-mail: hasnem@ohsu.edu

Polyamines (putrescine, cadaverine, spermidine, spermine) are essential nutrients in all living organisms including *Trypanosoma cruzi*. This parasite, however, lacks the first enzymatic step of the canonical polyamine pathway and cannot synthesize putrescine de novo; *T. cruzi* polyamine transport, therefore, plays an indispensable nutritional function by supplying the parasite polyamine pool, and enabling the survival and proliferation of this pathogen. We have previously identified in *T. cruzi*, a high affinity putrescine-cadaverine transporter, TcPOT1, which accounts for the capacity of the parasite to transport these diamines. To assess the contribution of this transporter to the overall parasite diamine transport capability, as well as parasite growth and infectivity, a Δtcpot1 null mutant cell line was generated by targeted gene replacement. As anticipated, the Δtcpot1 mutant lacked high-affinity putrescine-cadaverine transport capability but was able to transport diamines by a non-saturable mechanism. Spermidine, and arginine transport was not compromised in the Δtcpot1 line confirming the TcPOT1 transporter specificity towards diamines. Although the ability to infect Vero cells was comparable between wild type and the Δtcpot1 parasites, the mutant exhibited a reduced capacity to proliferate; despite the presence of residual spermidine and spermine transporter, the loss of a high-affinity diamines import affects parasite proliferation. These results provide insight into intracellular polyamine availability and suggest a restricted polyamine environment.
**Single Cell Supersensitive Phosphoprotein Profiling Quantum Dot Assay Platform for Heterogeneous Drug Response Analysis**

Thomas Jacob¹, Anupriya Agarwal², Thomas O’Hare ², Damien Ramunno-Johnson⁵, Brian J. Druker², Tania Q. Vu¹

Department of Biomedical Engineering¹, Knight Cancer Institute², Oregon Health & Science University, Portland OR 97239. E-mail: jacobt@ohsu.edu

Quantification of cellular phosphoprotein (PP) is critical for understanding drug response sensitivity and PP target effects. We present a drug profiling platform that images single, intensely bright PP antibody-bound fluorescent quantum dot (QD) nanoparticles. The sensitivity of our PP-QD platform enables quantification of single activated PP complexes in single cells, thereby exceeding techniques (Western blot, FACS) currently limited by sensitivity or ensemble detection (PP levels in large cell populations). The medical value of this technique is shown by identification of single drug-resistant cells in leukemia patients.
Mammary Gland Biology and Breast Cancer: Integrated 3D imaging of cells and tissues

Danielle M. Jorgens, Wenting Tsai, Hildur Palsdottir, Jessie K. Lee, Melissa Perez, Kester Coutinho, Andrew Tauscher, Manfred Auer, in collaboration with Andrew Ewald, Zena Werb, Jamie Inman & Mina Bissell

UC Berkeley & Lawrence Berkeley National Laboratory. E-mail: nyxhadanielle@gmail.com

Among the fascinating questions in cancer biology are the parallels in cell behavior to developmental biology processes of tissue generation and remodeling. In collaboration with Andrew Ewald and Zena Werb we have studied mouse organoids grown in 3D Matrigel tissue culture using video time-lapse fluorescence microscopy and electron microscopy (multiscale) ultrastructural analysis. In particular we have imaged cell and tissue organization of organoids in culture undergoing branching morphogenesis, and found that upon branching the overall tissue organization and polarity stays intact while individual cells, depending on their precise position within the tissue undergo dramatic changes in cell morphology, polarity and behavior. In the regions between the luminal cells and the cells facing the ECM, cell are only loosely attached to one another and display extensive networks of membrane extensions, a hallmark of cancer tissues. We have used advanced FIB/SEM and SBF/SEM imaging to document the rather complex nature of these membrane extensions. To our surprise such characteristics have also been found as collaborations with Mina Bissel’s lab in S1 acini and T4 cell aggregates, suggesting that loss of cell polarity precedes loss of growth arrest and maybe the hallmark of both embryonic as well as precancerous and cancerous tissues. We have begun to map out the entire intermediate filament (IF) network in S1 acini and T4 cell aggregates and have found significant differences in IF organization between the premalignant and the malignant cell phenotype. Naturally we are currently examining other differences in ultrastructural organization between these two cell phenotypes.
Spatial Distribution of Single-Molecule AKT Signaling Complexes in Breast Cancer Using Quantum Dot Imaging

Lam, Wai Yan¹; Ramunno-Johnson, Damien¹; Chin, Koei²; Gray, Joe²; Vu, Tania³

¹Department of Biomedical Engineering, Oregon Health and Science University, ²OHSU Center for Spatial Systems Bioscience, Oregon Health and Science University. E-mail: lamw@ohsu.edu

The notion of temporal trafficking and spatial location of signaling molecules in the subcellular environment as a critical means by which cells conduct normal signaling is a concept that is becoming increasingly recognized in recent years. However, technological means to probe the location of single-molecules quantitatively, precisely, and more importantly, with context to distances and location of subcellular structures (e.g. plasma membrane, Golgi, nucleus) remain a new unaddressed challenge. The serine-threonine phosphoprotein, Akt, is a key PI3K pathway signaling molecule that is dysregulated in a majority of cancers and a major therapeutic target. Akt is thought to traffic from cytoplasmic locations to the plasma membrane, nucleus, and possibly Golgi to regulate various aspects of tumor progression. A compelling study (Badve et al, 2010) found that tumors with pAkt localized in the nucleus correlated with higher survival rates in breast cancer, suggesting the potential clinical importance of Akt localization as a prognostic indicator. However, current means to locate Akt are qualitative pathological assessments that are performed at low-resolution. Moreover, immunofluorescent studies using diffuse fluorescent dyes have yielded conflicting reports about Akt localization. Such methods overlook potentially key spatial information about the organization of individual Akt complexes and make no quantitative measurements of Akt in relation to important cellular structures. Using a combination of sensitive probes, novel high-resolution structured-illumination microscopy, and customized image-analysis algorithms, we make use of fluorescent nanocrystal quantum dots to localize and quantitatively compute the distance of hundreds of single pAkt complexes relative to the plasma membrane and nucleus in individual breast cancer cells. We validate and compare our results to standard immunofluorescence assays using common Alexa Fluor dye. We find that single pAkt complexes distribute from the membrane toward the nucleus upon increasing duration of growth factor heregulin stimulation, and that pAkt is heterogeneously distributed within and among single cells. These data demonstrate the new capability of our technology compared to current low-resolution methods and emphasize the important and necessary concept of localizing individual molecular signaling complexes to better understand signaling. In the future, we will extend our technology to looking at Akt distribution in other cancer models and correlate spatial similarities and differences of Akt localization between different cancers to further elucidate the function of Akt in the context of its individual cellular environment.
Engineering the Nanomaterials-Bio Interface

Lester Lampert, Andrew Barnum, Haiyan Li, and Jun Jiao

Mechanical and Materials Engineering Department & Department of Physics, Portland State University. Email: lester.lampert@pdx.edu

Recent advancements in bioengineering have revitalized the outlook for biosensing and what is now being termed as ‘electroceuticals’, electronic devices for health status monitoring and therapy. For example, the multiple electrode array has been in use for many years but has been limited to extracellular signals and observation of action potentials rather than sub-threshold graded potentials across a single cell. Therefore it is important to transition to the intracellular sensing regime to improve the signal to noise ratio and gain insight into single cell mechanics and bio-functions. To further the development of intracellular recording devices, it is essential to determine the viability and interaction of the cell membrane with the functional portions of any proposed device. One method for directly viewing the interaction of a cell with a particular substrate and/or material is by sectioning the sample via focused ion beam (FIB) milling. Through this process, one is able to selectively probe different regions of the cell, create lamellae for use with transmission electron microscopy (TEM), and potentially create a three-dimensional reconstruction of the internal cellular structures through cryoelectron tomography. Here, we report FIB milling of dendritic cells (DC 2.4) cultured onto a substrate of vertically aligned carbon nanotubes (VACNTs). Carbon nanotubes (CNTs) are a commonly chosen material for presenting various molecules into the cytosol of eukaryotic cells, whether it is used for intracellular recording or as a vector for delivering DNA, antigen, or other substances.
Visualizing Internalized Cargo Acidification in Live Cells with Novel pH-Sensing Fluorescent Dyes


Molecular Probes, part of Life Technologies, Eugene, Oregon, USA. E-mail: Chris.Langsdorf@lifetech.com

The basic cellular internalization processes of endocytosis and phagocytosis are important to many areas of cell biology including receptor internalization, pathogen response, and apoptotic cell clearance. However, the ability to study these processes has historically been limited by the lack of quality tools to directly monitor the internalization and acidification of cargo. Here we present an assortment of tools to enable research into cellular internalization pathways with particular relevance to immunology. Two fluorogenic, pH-sensitive probes, one green and one red, are presented. These dyes are non-fluorescent at the neutral pH typically found in the cytosol and extracellular environment, but become brightly fluorescent in acidic cellular compartments, permitting direct monitoring of internalization and acidification processes. We present experimental data in which these dyes were used to label microorganisms including yeast and gram-positive and -negative bacteria. Live cell imaging and flow cytometry were then used to monitor the phagocytosis and acidification of these labeled particles by cultured macrophages over time. We anticipate that these tools will enable additional research into the fields of receptor trafficking, drug delivery, host-pathogen response, and clearance of apoptotic cells.
RAS Dimerization-Mediated Cell Signaling Studied with Super Resolution Microscopy

Li-Jung Lin, Eric Collisson, Tanja Meyer, Andrew Nickerson, Tao Huang, Frank McCormick, Joe Gray, Steven Chu, and Xiaolin Nan

Biomedical Engineering, OHSU Center for Spatial Systems Biomedicine, Oregon Health & Science University. E-mail: linlij@ohsu.edu

RAS proteins are membrane-bound small GTPases that mediate growth factor receptor signaling and regulate cell proliferation, survival, and differentiation through an array of downstream pathways such as PI3K-Akt-mTOR and RAF-MEK-Erk (aka MAPK). Activating mutations in RAS genes are common genetic lesions in human cancer. However, it remains unclear how RAS operates on the membrane to activate its effectors, leaving it an intractable drug target. We have used photoactivated localization microscopy (PALM) to examine the nanoscale spatial organization of RAS proteins on the cell membrane. PALM is a recent super-resolution microscopy technique that enables cellular imaging at ~10 nm spatial resolution and protein counting with single-molecule precision. With PALM, we have previously discovered that RAS dimerization is both necessary and sufficient to activate RAF-MAPK. We now present preliminary evidence that RAS dimerization also activates PI3K-Akt. Specifically, mutant RAS expressed at levels significantly lower than that of endogenous RAS exist as monomers and does not activate PI3K/Akt. At higher expression levels when mutant RAS starts to form dimers, PI3K/Akt becomes activated. These results are analogous to our previous observations on RAS-dimer mediated MAPK activation. Future studies include a) to use an artificial dimerization system to validate the role of RAS dimerization in activating PI3K, and b) to use multicolor super-resolution microscopy to investigate how RAF and PI3K activation events from the same RAS dimers are spatially coordinated. Our studies will yield critical insight into the role of dimerization in RAS signaling and tumorigenesis, and provide the molecular basis for targeted therapy against mutant RAS in human cancer.
Investigating Protein Kinase A Dynamics in Neurons Using Single Molecule Tracking PhotoActivation Localization Microscopy

Brian R. Long and Haining Zhong

Vollum Institute, Oregon Health & Science University. E-mail: longbr@ohsu.edu

Protein Kinase A (PKA) is a key second messenger involved in many neuronal functions, including synaptic transmission and plasticity, neuronal excitability and protein trafficking and translation. PKA’s specificity is thought to be achieved in part by PKA’s preferential phosphorylation of substrates within its molecular vicinity. This anchoring specificity is mediated established by A Kinase Anchoring Proteins (AKAPs), which localize the PKA regulatory subunit (PKAr) and specific PKA substrates. However, when PKA it is activated by elevated cAMP concentrations, the PKA catalytic subunit (PKAc), dissociates from the anchored regulatory subunit, and it is unclear how PKA signaling specificity is maintained while PKAc is free to diffuse. Because much of the critical organization and dynamics of PKA take place below the diffraction limit of conventional microscopy (~200nm), we use single molecule tracking PhotoActivation Localization Microscopy (PALM), a recently-developed technique that can reconstruct protein distributions and dynamics from single-molecule trajectories at a resolution far surpassing conventional microscopy. In preliminary efforts, individual PKAc trajectories allow catalytically active PKAc vs inactive PKAc to be distinguished by their mobilities. The mobilities of PKAc trajectories in cultured neurons show a clear response to PKA activation, creating a high resolution reporter of PKA activity derived from single molecule dynamics.
Using the Helios NanoLab 650 DualBeam™ for Life Science Applications at OHSU

Claudia S. López,¹ Jessica L. Riesterer,² and Eric Barklis¹

¹Department of Molecular Microbiology and Immunology, Oregon Health & Science University, ²FEI Company. E-mail: lopezcl@ohsu.edu

Several unique key features of the FEI Helios NanoLab 650 DualBeam™ microscope, part of the OHSU/FEI Living Lab, enable successful imaging of biological materials in both 2- and 3-dimensions. Low accelerating voltage (350 eV – 2 keV) and moderate beam current (50 – 200 pA) coupled with the through-lens (TLD) and dedicated backscatter (DBS) detectors of the Helios enable collection of crisp images from properly stained samples. Combining SmartSCAN™ features with beam deceleration allows high-quality charge-free imaging of cells and tissue. SmartSCAN, embedded within the FEI microscope user interface, includes line integration, scan interlacing, frame averaging, and drift corrected frame integration (DCFI). This unique suite of features allows reduction of noise in images, drift mitigation, and charge mitigation all of which are common in Life Science samples.

Conductive coatings are often needed to prevent sample charging during scanning electron microscopy (SEM). However, coatings that are too thick can obscure small critical features and immuno-gold markers, and also adds time to sample processing. Using the Helios, cells can be imaged as-grown on glass, silicon, aluminum, and paper substrates in 2D without the need for conductive coatings. Using conditions specific for each substrate material, uncoated cellular bodies, multivesicular exosomes, and cellular protrusions can be studied unaltered. The majority of cells need not be stained prior to viewing.

The Helios’ focused ion beam (FIB) column is able to mill slices of resin-embedded tissue or cells as small as 3 nm thick. When combined with the SEM column’s imaging capability, FEI’s Auto Slice & View™ G3 software package fully automates the serial sectioning and data collection process. When samples are embedded, fixed, and highly stained, volumes can be collected over several days using userdefined conditions, and then, reconstructed and segmented using the Amira ResolveRT™ software package from VSG (an FEI company) to create 3D-volume representations and movies.
Cell-based Analysis of Oxidative Stress, Lipid Peroxidation and Lipid Peroxidation-Derived Protein Modifications Using Fluorescence Microscopy


Life Technologies. E-mail: Bhaskar.Mandavilli@lifetech.com

Oxidative stress plays an important role in the progression of several diseases including inflammation, atherosclerosis, aging and age-related degenerative disorders. Reactive oxygen species damage membrane bound lipids resulting in lipid peroxidation-derived protein modifications. Cell-based measurements of oxidative stress, lipid peroxidation and protein carbonylation by traditional fluorescence microscopy provide a powerful platform to quantitate oxidative stress and lipid peroxidation. Here, we used three different approaches to measure oxidative stress and lipid peroxidation in cells by fluorescence microscopy. 1) Two new fluorogenic probes, CellROX™ Orange and CellROX™ Green Reagents to measure oxidative stress in cells, 2) Image-iT® Lipid Peroxidation Kit for a ratiometric determination of lipid peroxidation in live cells 3) Click-iT® Lipid Peroxidation Imaging Kit, a click chemistry-based approach which utilizes incorporation of an alkyne-modified unsaturated fatty acid analog, linoleamide, into the cellular membranes. The resulting oxidation products, like 9, 12-dioxo-10(E) dodecenoic acid (DODE) can readily modify proteins and these modifications were readily detected in fixed cells by the copper-catalyzed click reaction using fluorescent azides. Using these approaches, we measured oxidative stress and lipid peroxidation caused by several oxidants in cells. Increases in oxidative stress, lipid peroxidation, and protein modifications were assessed by high content imaging and analysis as well as traditional fluorescence microscopy. In the models tested, at least 2-3 fold increases were observed compared to controls and responses were successfully inhibited by antioxidants. The three strategies described here provide powerful new tools for the assessment of oxidative stress in cells and convey distinct advantages over existing cell-based methods.
A Role for Adenine Nucleotides in the Sensing Mechanism to Purine Starvation in *Leishmania donovani*.

Jessica L. Martin¹, Phillip A. Yates¹, Maria Belen Cassera², Jan M. Boitz¹, Audrey L. Fulwiler¹, Buddy Ullman¹, Nicola S. Carter¹

¹ Dept. Biochemistry & Molecular Biology, Oregon Health & Science University, ² Dept. of Biochemistry, Virginia Polytechnic Institute and State University. E-mail: majessic@ohsu.edu

The salvage of purines by *Leishmania* is an obligatory process that impacts both cell viability and growth. Our previous studies demonstrated that purine starvation provokes significant metabolic changes, including the upregulation of permeases and salvage enzymes involved in purine acquisition and interconversion. To understand how *Leishmania* sense and adapt to changes in their purine environment, we have exploited purine pathway mutants. While wild type parasites are able to transport and convert any single purine nucleobase or nucleoside to fulfill their adenylate and guanylate nucleotide requirement, these purine pathway mutants have restricted purine requirements for growth. Thus, these mutants can be maintained in high levels of an extracellular purine that is non-permissive for sustained parasite replication. By culturing these purine pathway mutants in purines permissive or non-permissive for growth and subsequently monitoring for the upregulation of specific purine transporters and salvage enzymes — hallmarks for the adaptive response to purine starvation — we have determined that the adaptation to purine starvation arises from the surveillance of intracellular purine pools rather than from the direct sensing of the extracellular purine environment. Furthermore, our data suggest that the response to purine starvation is more profound when perturbations within the adenylate nucleotide pool are elicited rather than within the guanylate nucleotide pool, suggesting that adenylate nucleotide imbalances may be a primary trigger for the response to purine stress. Purine starvation is also accompanied by striking morphological changes that appear to be accompanied by motility changes. Studies are currently underway to investigate these changes and determine if they represent an early step in the differentiation of these parasites to the infective metacyclic form that is observed in the sandfly vector.
**Improved N-glycan Labeling of TRPV5 with Cyclooctynes in the SPAAC Reaction**

Mandy H.L. Meuleners, Liz Leunissen, Jorge M.M. Verkade, Prof. Dr. J.G.J. Hoenderop, Prof Dr. R.J.M. Bindels, Prof. Dr. F.P.J.T. Rutjes; Associate professor Dr. F.L. van Delft

Institute for Molecules and Materials, Department of Organic Chemistry, Radboud University Nijmegen in collaboration with UMC St. Radboud, NCMLS, Department of Physiology. E-mail: mandy.meuleners@gmail.com

Transient receptor potential cation channel subtype 5 (TRPV5) is a highly selective Ca\(^{2+}\) channel present in the kidney with an extracellular, complex tetra-antennary N-glycan. In the field of glycobiology, new chemical tools are needed to investigate the regulation of glycosylated proteins in living systems. In this study, glycans were labeled by the introduction of an azide functionality. Azides react covalently and bioorthogonal with cyclooctynes in a strain-promoted [3+2] azide-alkyne cycloaddition (SPAAC) reaction. The SPAAC reaction is optimal for live cell surface labeling under physiological conditions, although new cyclooctyne probes with improved characteristics are desirable. Four different Bicyclo[6.1.0]nonyne (BCN) derivates were investigated for the specific labeling of the membrane protein TRPV5. The newly synthesized BCN probes contain either an additional hydrophilic group, an amine that gives a positive charge or a carboxylic acid that gives a negative charge. The cell permeability, localization, cell reaction kinetics and specific labeling of the membrane protein TRPV5 were investigated for these four cyclooctynes. Our data demonstrate that the newly synthesized cyclooctynes show improved cell membrane labeling characteristics compared to protein labeling with traditional BCN.
Photoactivated Localization Microscopy with Bimolecular Fluorescence Complementation (BiFC-PALM): Imaging Protein-Protein Interactions in a Cell with Nanometer Resolution

Andrew Nickerson, Li-Jung Lin, Tao Huang, and Xiaolin Nan

Department of Biomedical Engineering, Knight Cancer Institute, and Oregon Center for Spatial Systems Biology, Oregon Health and Science University.

Protein-protein interactions (PPIs) are integral to biology. Among existing methods for detection of PPIs, bimolecular fluorescence complementation (BiFC) has been widely used because interactions can be visually detected and spatially mapped in cells as fluorescence. Like all conventional light microscopy, however, the spatial resolution in BiFC has been diffraction limited at ~250 nm, providing little information of PPIs at the molecular scale. Here, we combine BiFC with photoactivated localization microscopy (PALM), a recent super-resolution imaging technique, to visualize PPIs at nanometer spatial resolution. PALM uses photoactivatable fluorescent proteins (PA-FPs) and operates by stochastic activation and sub-diffractive localization of individual PA-FP molecules. In BiFC-PALM, a photoactivatable mCherry (i.e., PAmCherry1, a commonly used PALM probe) is split into two non-fluorescent fragments, each of which is genetically fused to a target protein. When the two target proteins interact, the two fragments are brought into proximity to re-generate a PAmCherry1 molecule. Using a model PPI system, we found that the re-generated PAmCherry1 molecules retain photoactivation, single-molecule brightness, and other properties of the original PAmCherry1, which allows for PALM imaging at ~18 nm resolution. With BiFC-PALM, we obtained super-resolution maps of interactions between the small GTPase Ras and its downstream protein kinase Raf in mammalian cells. The images clearly showed that Ras/Raf complexes form nanoscale clusters on the cell membrane, a previously hypothetical attribute of Ras/Raf interaction. Our study demonstrated the feasibility and power of BiFC-PALM for studying PPIs at the single-molecule or nanometer scale, which can be readily extended to other PA-FPs and biological systems.
Quantification of S. Mutans Biofilm: Insights in Biofilm Morphology Using Confocal Microscopy and Image Analysis Software

Nicole Paterson¹, Samyia Chaudhry¹, Curt Machida¹, Kirsten Lampi¹, Michael Danilchik¹ and Jack Ferracane².

Departments of ¹Integrative Biosciences and ²Biomaterials and Biomechanics, School of Dentistry, Oregon Health & Science University. E-mail: pattersni@ohsu.edu

Confocal scanning laser microscopy (CSLM) is an imaging technique with the unique ability to interpret excited fluorophore emissions into 3D images that are useful for characterizing microbial populations. Two of the primary pathogens associated with dental caries are Streptococcus mutans and S. sobrinus. The purpose of this project is to assess the viability of cariogenic streptococci using CSLM images analyzed by Adobe® software, in conjunction with Invitrogen Live/Dead bacterial viability stain. CSLM was used to assess the viability and density of the S. mutans biofilm over a 5-day growth period, using daily replenishment with brain heart infusion medium. Metabolic activity was also assessed with other indicator assays, including the use of ATP-driven bioluminescence, which is a direct measure of ATP concentration in living cells. Living cells in the S. mutans biofilm are found at the apical surface in larger numbers, and most surprisingly, biofilm formation undergoes periods of fluctuating growth and death depending on cell density. The CSLM method allows for the quantification of biofilm density and viability, and has the potential to streamline further studies for therapeutic treatments. An understanding of biofilm indicators can lead to better therapies for biofilm-related diseases, including dental caries.
Using Super-Resolution Quantum Dot Detection and Automated Cell Segmentation to Study Subcellular Processes

Damien Ramunno-Johnson, Wai Yan Lam, Joe Gray, and Tania Vu

Department of Biomedical Engineering, School of Medicine, OHSU. E-mail: ramunnoj@ohsu.edu

The introduction of CCD cameras to microscopy changed the field and made it possible to introduce more quantitative imaging methods. One such method is localization microscopy, which gathers high precision position information for sub-diffraction-limited probes or markers. However, this location information becomes more useful if it can be put into context with other cellular structures, e.g., the plasma membrane, endosomes, and the nucleus. To relate the position of protein complexes to the plasma membrane, we developed image analysis software that quickly determines the cell membrane and proteins labeled with quantum dot probes (QD) in 3D. With this software, the processing time is down to minutes per cell. This opens up the possibility of collecting data on 100’s of cells, making it possible to perform detailed single cell measurements, as well as extracting large-scale global behavior from ensemble sets of cells. The figure shows a hippocampal cell where the serotonin receptors have been tagged with single fluorescent quantum dot (QD) probes to examine receptor internalization and receptor dynamics. The membrane and QD detection software is run producing a 3D data set containing the membrane and QD location data. Quantitative analysis, such as the distribution of receptor-plasma membrane distribution can then be computed.
3D imaging of the Early Embryonic Chicken Heart with Focused Ion Beam Scanning Electron Microscopy

Monique Y. Rennie¹, Claudia S. Lopez², Kent L. Thornburg¹,³, Sandra Rugonyi⁴

¹Knight Cardiovascular Institute, and Departments of ²Molecular Microbiology and Immunology, ³Medicine (Cardiology), and ⁴Biomedical Engineering, Oregon Health and Science University. E-mail: rennie@ohsu.edu

Focused ion beam scanning electron microscopy (FIB-SEM) tomography, or “slice and view”, is a novel technique for 3D sub-cellular imaging of tissue. A sample can be repeatedly milled with the FIB in small increments (down to 4 nm) and each block face imaged with the SEM at nanometer resolutions. Though widely applied in material sciences, FIB-SEM systems have been little used for biological purposes and never used to image heart tissue, thus optimal fixation and staining are not known. In the current study, we used FIB-SEM to image cardiac tissue in the outflow tract of the chicken embryo during a period of rapid tissue growth and remodeling. We aimed to slice and view regions from each of the three layers of outflow tract tissue: contractile myocardium, cardiac jelly (mostly extracellular matrix), and endocardial cells, allowing for comparison of cellular and organelle volume fractions between these regions. Further, high-resolution 3D images would enable segmentation of structures as fine as the ribosomes, primitive collagen fibrils and myofibrils (~20 nm in diameter), for example. Myofibrils are the basic functional unit of the cardiac myocyte. Myofibrillar alignment in the myocardium is critical for proper heart development and contraction, but its study has been severely hampered by a lack of 3D EM data.

To ensure rapid fixation the day 4 chicken embryo heart was placed in fixative (2% paraformaldehyde + 2% glutaraldehyde) during outflow tract dissection, which preserved organelle membrane structure. Post fixation, staining with potassium ferrocyanide, osmium tetroxide and uranyl acetate provided excellent tissue contrast on EM images. Three regions (22 x 17 μm) from the sample were scanned on an FEI Helios Nanolab 650 Dualbeam scanning electron microscope. FEI’s Auto Slice & View™ G3 software package fully automates the serial sectioning and imaging of samples. Between 4-15 nm of Epon resin was removed per slice with the FIB, depending on heart region. Each fresh block face was imaged with the SEM at a voltage of 2kV in backscatter mode with inverse contrast (8 frame integrations with drift correction). Up to 1100 slices were obtained per heart region. To obtain a tomographic dataset, image stacks were aligned using Amira ResolveRT™ software package (FEI), creating volumes 800-1500 μm³ in size.

Preliminary data reveal numerous advantages of FIB-SEM over traditional EM for imaging the heart wall. Segmentation of 3D data allows for measurement of cellular and organelle volume fractions from which we aim to compare between the regions of the outflow tract wall. Thus, we have demonstrated that FEI-SEM is feasible in the chicken embryo and that it could provide a method for understanding improper embryonic development of contractile heart muscle.
Correlating Photoactivated Localization Microscopy and Electron Microscopy

Danielle C. Robinson¹,², Brian R. Long², and Haining Zhong²

¹Neuroscience Graduate Program, ²Vollum Institute OHSU. E-mail: robidani@ohsu.edu

Understanding the structural basis of synaptic function requires quantitative assessment of protein organization within tiny synaptic compartments. Photoactivated Light Microscopy (PALM) is a superresolution technique that uses photoswitchable fluorophores to reveal protein organization at a high enough resolution to explore the synapse. While PALM can provide precise protein localization with high labeling density, it does not provide the context of the surrounding tissue or details of dendritic spine ultrastructure that can be observed with Electron Microscopy (EM). We are developing a method to correlate PALM images with the underlying fine structure of the synapse visible via EM. A current challenge is the optimization of sample processing to preserve both the signal of fluorescent proteins for PALM in conditions that yield meaningful ultrastructural information for EM analysis. Preliminary work with the photoswitchable fluorophore mEos shows that preservation of the fluorophore is possible. We anticipate that the correlation of PALM with EM will contribute a uniquely detailed view of the functionally significant protein architecture at the synapse.
The Design and Synthesis of Enzyme-Activated Fluorophores for Biomarker Discovery

Tallman, Katie R.; Lopez, Nicholas A.; Beatty, Kimberly E.

Department of Physiology and Pharmacology, Oregon Health and Science University. E-mail: tallman@ohsu.edu

Tuberculosis (TB) is an infectious disease caused by several species of bacteria, including Mycobacterium tuberculosis. With proper diagnosis and treatment, the majority of cases are curable. However, TB ultimately kills more than half of those with active infections, leading to approximately 1.5 million deaths a year. Unfortunately, inadequate and outdated diagnostic tests leave many cases of TB undiagnosed. The long-term goal of our research is to identify new biomarkers for TB, create enzyme-activatable probes, and then translate these probes into diagnostic assays for low resource settings. Our current efforts are focused on synthesizing new fluorogenic probes that are activated by mycobacterial sulfatases and esterases. Fluorogenic probes undergo an enzyme-mediated chemical change that alters the molecule’s fluorescence emission or quantum yield. This change in fluorescence can be used to detect mycobacterial enzyme activity and to distinguish different strains and species of mycobacteria. We expect that these “molecular fingerprints” can be exploited for the rapid identification of pathogens for point of care TB diagnostics.
Cytoplasmic Trafficking and Recycling of BDNF-TrkB Receptor Complexes by Real-Time Single Quantum Dot Tracking in Sensory and CNS Neurons

Anke Vermehren-Schmaedick¹, Thomas Jacob¹, Wesley Krueger², Damien Rammuno-Johnson¹, Agnieszka Balkowiec³, Keith Lidke², Tania Q Vu³

¹Department of Biomedical Engineering, Center for Spatial Systems Biology, Oregon Health and Science University, ²Department of Physics & Astronomy, University of New Mexico, ³Department of Integrative Biosciences, Oregon Health and Science University. E-mail: vermehre@ohsu.edu

Growth factor, such as brain-derived neurotrophic factor (BDNF), are highly expressed in the brain where they play key roles activating downstream signaling cascades that result in brain development, learning and memory. Despite the clinical importance of BDNF, the precise molecular mechanisms underlying BDNF signaling such as the spatiotemporal dynamics of ligand-receptor trafficking, and protein-protein transport in cytoplasmic compartments are elusive and largely inaccessible to investigation due to the lack of technologies for visualizing individual ligand-activated receptor complexes in single living cells. To study the subcellular localization and movement of discrete BDNF-TrkB complexes in time and space, we synthesized and carefully validated the use of quantum dot-conjugated BDNF (QD-BDNF) to activate and tag TrkB receptors in rat hippocampal and primary sensory neurons from the nodose ganglion. Our results show that we have a successful and robust means for synthesizing QD-BDNF probes that: 1) bind with high molecular level of specificity to the target TrkB receptors, both, in vitro and in live neuron-based assays, 2) initiate downstream signaling to support survival of BDNF-dependent embryonic sensory neurons in culture, 3) lead to activation of downstream signaling molecules in the kinase pathway, and 4) supersede the detection of Alexa Flou-BDNF probes, allowing us to study QD-BDNF-TrkB trafficking at physiological concentrations that are at least 100 times lower than previously possible (e.g. 50pM BDNF). In BDNF-TrkB trafficking studies using live neurons, we show for the first time that, once BDNF-TrkB complexes are internalized, they traverse intricate curvilinear paths within the neuronal cytoplasm, abruptly switching between periods of mobility and immobility. Of note, we provide new real-time observations that BDNF-TrkB complexes recycle back to the membrane and that recycling occurs both near the cytosolic face of the plasma membrane as well as from BDNF-TrkB complexes that traverse toward the membrane from compartments located deeper within the cell cytoplasm. In summary, our extensive molecular-level validations show that our QD-BDNF probes can be synthesized and applied in a robust manner for studying important BDNF-TrkB trafficking phenomena, such as receptor recycling and trafficking within the cytoplasm of live neurons.
Direct Observation in vivo Identifies Novel Wnt Signaling Mechanism

Misha Naiman 1,2, Daniel Lybrand 1,2, Gregory Scott 1, Tara Johnson 1,3, Naz Erdeniz 1, Elizabeth Swanson 1, Mark Peifer 4, Marcel Wehrli 1.

1Cell & Developmental Biology, Oregon Health & Science University, 2Reed College, 3St. Mary Academy, 4Biology, University of North Carolina.

Wnt/β-catenin signaling is critically important in development, yet at least a dozen different models have been postulated to explain β-catenin regulation. One reason for this uncertainty is that components have additional functions in other pathways, for which these components are abundant; this severely complicates the analysis. We opted for an approach using physiological expression levels and apply Bimolecular Fluorescence Complementation (BiFC) to provide molecular resolution in vivo, thus positively identifying protein-protein interactions. The Drosophila wing disc epithelium is exposed to a gradient of Wnt signal, where the transduced signal provides a continuum of inhibition of the key regulator, the destruction complex. It is assembled around the scaffold protein Axin and targets β-catenin for degradation. The active destruction complex has never been visualized in vivo but must contain both Axin and Shaggy (GSK3). We directly detect the assembled Axin-Sgg BiFC complex, which reveals a gradient of active destruction complex, complementary to the Wnt gradient. This finding pinpoints loss of Shaggy kinase from the Axin complex as the critical event in induction of the intracellular Wnt/β-catenin signal. This novel mechanism is complemented by our visualization of the inactive destruction complex, using Axin-APC2 BiFC. This complex lacks Shaggy but binds β-catenin and is present in a gradient co-linear with Wnt. For the first time, we can directly observe the conversion of the active to the inactive destruction complex, evident at de novo Axin-APC2 BiFC, upon pulsed Wnt signaling in vivo. We establish a series of dynamically changing interactions that provide a framework to discriminate between competing pathway models.
Neutral Atom Microscopy

Philip Witham, Erik Sánchez

Physics, Portland State University Physics. E-mail: pjw@pdx.edu

Neutral Atom Microscopy (NAM) is an Imaging technique that uses a low-energy beam of non-charged atoms or molecules. At under 0.1 eV beam energy, there is very little effect on any sample that can withstand a vacuum. Images are formed by the scattering of thermal energy Helium atoms from the top atomic surface of samples, with no penetration. Because of the non-invasive character of the beam, freedom from tip-sample interactions or tip aspect ratio limitations, and atomic surface sensitivity, researchers have pursued this idea for some time. The “pinhole” NAM invented at PSU is the first generating reflection mode images, and holds the record for spatial resolution in NAM. With further development, this technique will reach nano-scale resolution. From molecular beam experiments, it is known that surface composition information will appear, such as by atomic diffraction from crystal surfaces. The latest images and current work are presented.