Combining DNA Nanotechnology and Fluorescence Polarization Microscopy to Determine the Orientation of DNA Bound Fluorophores

Deborah K. Fygenlos, Christoph Schneider, Hunter Banks.

We determine the orientation of the transition dipole moment of a fluorophore bound to dsDNA using fluorescence polarization microscopy and compare with published results. We use DNA nanotubes to present the dsDNA in a known orientation and query a variety of intercalating (e.g., YoYo-1, TOTO), groove-binding (e.g., DAPI) and covalently linked (e.g., Fluorescein, Cy3, Cy5) dyes. We use a de Sénarmont prism in front of the camera to record simultaneous images of fluorescence polarized perpendicular and parallel to the DNA nanotube axis, making the associated polarization ratios insensitive to photobleaching. Our results suggest this technique can be used to detect helical supertwist, and possibly other nanoscale structural features, of DNA nanostructures.

3D Visualization of Mitochondrial Network and Nucleoids of mtDNA in INS1E and HepG2 Cells at 30 Nm Resolution by Biplane FPALM Microscopy

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Three-dimensional (3D) super-resolution microscopy, using a biplane detection scheme, termed biplane photo-activated localization microscopy (Biplane FPALM), enables imaging of volumes as thick as whole cells and could reveal unknown details of cellular organization. Hence, we attempted to visualize mitochondrial reticulum via the matrix space loaded with mitochondria-addressed Eos, while transfecting cells by lentiviral expression. Our 3D images of single Eos molecules in the matrix space have proven the continuous character of mitochondrial reticulum, i.e., an existence of a highly interconnected major mitochondrial reticulum in insulinoma INS1E and oxidative-phosphorylation dependent glutaminolitic hepatoma HepG2 cells (Fig.1). Also, using Eos-conjugate of mitochondrial transcription factor-A (TFAM), we have imaged nucleoids of mitochondrial DNA (mtDNA) in which TFAM represents a major assessor protein. Using PA-CFP2-TFAM and mitochondria-addressed Eos, the first 3D two color super-resolution images were obtained for mitochondrial reticulum together with the distribution of mt nucleoids in it. In intact cells we have found mt nucleoids of a narrow size distribution.

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Photoactivatable Hemiycanine Chromophores as Fluorescent Lables

Na Liu, Stacy Wilson, Leslie M. Loew.

A series of photoactivatable hemicyanine chromophores containing azido group instead of the usual amino donor group has been designed and synthesized. Before photolysis, these compounds have negligible fluorescence. Upon photolysis, a molecule of nitrogen is lost from the azido group leaving behind a highly reactive nitrene moiety, which can react with and crosslink neighboring molecules to produce an amine. This fully restores the “push-pull” environmentally sensitive hemicyanine chromophore. If the molecule is in a cell membrane it will covalently crosslink lipid or protein molecules and remain relatively immobile. The photoactivity of the dyes was tested on neuroblastoma cells by selectively shining near UV or visible light on a small region of the cell and then observing the whole cell’s fluorescence using a confocal microscope. This experiment shows strong fluorescence primarily in the illuminated area, which proves that we can selectively and stably stain a portion of the cell membrane using these photoactivatable hemicyanine dyes. The photoreleased dyes are fluorescent in the membrane because the amino-chromophore is similar to our voltage sensitive ASP and ANEP chromophores. These compounds are promising as targetable sensors of membrane potential.

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Nuclear Receptor (PXR), Ligand and Co-Activator Interactions Measured by Total Internal Reflection Fluorescence Microscopy


The pregnane X receptor (PXR) is a member of the nuclear receptor family. PXR acts in a ligand-dependent manner, in concert with its dimerization partner, the retinoid X receptor, and co-regulators, to modulate the expression of proteins that metabolize endogenous and exogenous compounds. Here, we report the use of total internal reflection fluorescence microscopy (TIRFM) for examining the interactions between the ligand-binding domain of PXR (PXR-LBD), its ligands, and a peptide derived from a co-activator. TIRFM is a surface-specific technique that enables us to study the behavior of fluorescent species close to or at interfaces. Our experimental system consists of biotinylated PXR-LBD immobilized on a fused silica substrate coated with NeutrAvidin and ovalbumin, a ligand (e.g., the antibiotic rifampicin) and a fluorescently labeled, 25 amino acid fragment of the steroid receptor co-activator (F-SRC-1). Using TIRFM, we measured the surface-associated fluorescence as a function of the F-SRC-1 concentration at fixed ligand concentrations. These curves were fit to a model of single-site binding to obtain apparent equilibrium constants. The apparent equilibrium constants as a function of ligand concentration were fit to an appropriate model to obtain binding constants for the ligand/PXR-LBD interaction, F-SRC-1 binding to apo and ligand-bound PXR-LBD and ligand binding to F-SRC-1-bound PXR-LBD. The approach yielded four, previously unmeasured, binding constants. These values indicate that the increase in PXR’s affinity for its co-activator peptide upon binding an activating ligand is modest. This observation implies that for gene expression to be significantly upregulated, the signal arising from the activation of PXR must be amplified downstream - possibly when co-activators like SRC-1 start recruiting the cell’s transcription machinery. Kinetic data obtained by combining TIRF illumination and fluorescence recovery after photobleaching and/or fluorescence correlation spectroscopy may also be discussed.

Measuring Surface Binding Thermodynamics and Kinetics by using Total Internal Reflection with Fluorescence Correlation Spectroscopy: Practical Considerations

Xiang Wang, Punya Navaratnarajah, Nancy L. Thompson.

The combination of total internal reflection illumination and fluorescence correlation spectroscopy (TIR-FCS) is an emerging method useful for, among a number of things, measuring the thermodynamic and kinetic parameters describing the reversible association of fluorescently labeled ligands in solution with immobilized, nonfluorescent surface binding sites. However, there are many parameters (both instrumental and intrinsic to the interaction of interest) that determine the nature of the acquired fluorescence fluctuation autocorrelation functions. In this work, we define criteria necessary for successful measurements, and then systematically explore the parameter space to define conditions that meet the criteria. The work is intended to serve as a guide for experimental design; in other words, to provide a methodology to identify experimental conditions that will yield reliable values of the thermodynamic and kinetic parameters for a given interaction. In vitro experiments to verify the theoretical predictions will be also provided.

Hyperspectral Raman and Fluorescence Microscopy of Individual Algal Cells for Biochemical Analysis


Green algae are photosynthetic microorganisms, similar in many ways to plants, that fuel cellular processes with biochemical energy derived from sunlight. Algae are known to produce and accumulate triacylglycerol (TAG) and lipid under certain stress conditions. Subsequently, algae have garnered significant research interest as candidates to produce the next-generation of advanced biofuels because lipids are convertible into biodiesel. Therefore, there is significant interest to develop methodologies and analytical tools to characterize and understand lipid biosynthesis in living algal cells.

Raman spectroscopy offers excellent chemical and spatial resolution of the carotenoid, TAG and TAG precursor compounds, while fluorescence spectral information provides complimentary and confirmatory information about the photosynthetic pigments. The combination of Raman mapping and hyperspectral fluorescence imaging can provide critical insights into the spatial biochemical makeup of algal cells. Additionally, since each algal species has its own unique set of carotenoids, the carotenoid signatures present an opportunity for identifying algae at the taxonomic group and possibly even at the species level. Here, we present a novel use of confocal Raman mapping and hyperspectral confocal fluorescence microscopy with multivariate curve resolution tools to investigate carotenoid and lipid spectral signatures in living algal strains. We will 1) Identify algal species from mixed samples based on carotenoid spectroscopic signatures 2) localize, identify and quantify carotenoid distribution in individual algal cells and 3) determine the lipid content of individual cells from several algal species. This novel approach will demonstrate the feasibility of visualizing biochemical processes in live algal cells using spectroscopic imaging techniques.

Hyperspectral Raman and Fluorescence Microscopy of Individual Algal Cells for Biochemical Analysis
To demonstrate the speed and resolution of the IMS approach, we monitored spectral emission range of the common FP-FRET biosensors. Stefan Marawske, Lei Wang, Markus Richert, Ralf Kühnemuth, Analyzing A board B454 for the United States Department of Energy’s Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy’s Nuclear Security Administration under contract DE-AC04-94AL85000.

3349-Pos Board B454
Analyzing Aβ Aggregates with High Resolution Microscopy
Pia Zifmann, Aileen Susanne Funke, Stephanie Grabowski, Stefan Marawske, Lei Wang, Markus Richert, Ralf Kühnemuth, Eva Birkmann, Claus Seidel, Dieter Willbold.

In a world where more people grow older aging-related neurodegeneration like Alzheimer’s disease (AD) affects more and more people. Today, AD can be diagnosed with certainty only post mortem, detecting insoluble β-amyloid peptide (Aβ) aggregates and neurofibrillary tangles in the patient’s brain tissue. Aggregates consisting of Aβ are a fundamental pathologic feature of AD. Today in many studies, concentrations of monomeric Aβ in body fluids are investigated, especially for diagnostic purposes. Nevertheless, for the detection, quantification and qualification of aggregated pathologic Aβ forms, also in the course of aging, a highly sensitive detection assay system for aggregated Aβ species is necessary. We developed an ultra-sensitive assay for the detection of aggregated protein species out of body fluids. This highly specific and sensitive assay uses confocal fluorescence spectroscopy methods and is sensitive enough to detect single aggregates. For the procedure, pathologic aggregates out of body fluids are immobilized on a glass chip, subsequently fluorescent labeled and detected via confocal spectroscopy. Usually, we are optimizing the assay in concerns of instrumentation (imaging) and microscopy high-resolution and even super-resolution methods. We are developing methods to analyze aggregates via super-resolution microscopy. Setups like PAINT (Point Accumulation for Imaging in Nanoscale Topography) or STORM (Stochastic Optical Reconstruction Microscopy) allow resolutions in nanometer-range. PAINT is based on replacing the point-spread function (PSF) of a fluorophore by a point in the middle of a 2D gaussian fit. First measurements show resolutions of 30 nm. STORM is based on high-accuracy localization of photoswitchable fluorophores. During one imaging cycle, only a small part of the fluorophores is turned on. This allows a high accuracy in determining the fluorophore position by replacing the PSF. The fluorophore positions obtained from a series of imaging cycles can be used to reconstruct the whole image. Actually, we are optimizing the assay in concerns of instrumentation (imaging) and microscopy high-resolution and even super-resolution methods. We are developing methods to analyze aggregates via super-resolution microscopy. Setups like PAINT (Point Accumulation for Imaging in Nanoscale Topography) or STORM (Stochastic Optical Reconstruction Microscopy) allow resolutions in nanometer-range. PAINT is based on replacing the point-spread function (PSF) of a fluorophore by a point in the middle of a 2D gaussian fit. First measurements show resolutions of 30 nm. STORM is based on high-accuracy localization of photoswitchable fluorophores. During one imaging cycle, only a small part of the fluorophores is turned on. This allows a high accuracy in determining the fluorophore position by replacing the PSF. The fluorophore positions obtained from a series of imaging cycles can be used to reconstruct the whole image.

3350-Pos Board B455
Real-Time Hyperspectral Imaging of Multiple Biosensors in Pancreatic Beta Cells
Alicia D. Elliott, Liang Gao, Alessandro Ustione, Tomasz S. Tkaczyk, David W. Piston.

Spectral imaging techniques are important for many biological experiments, particularly live-cell quantitative imaging of multiple fluorescence probes. Existing hyperspectral imaging systems require sequential techniques, limiting the data acquisition rate. A newly-developed snapshot device, the Image Mapping Spectrometer (IMS), acquires full spectral information simultaneously from every pixel in a field with image acquisition rates up to 10 frames per second. The IMS maps adjacent pixels from the object to create space between them in the image, and then uses a grating to spread wavelength content from each pixel into this space. Direct image re-mapping provides the final 3D (x, y, lambda) data cube. Fluorescent protein (FP)-based biosensors are increasingly valuable tools for identifying subcellular dynamic processes in live cells. Many biosensors are based on FP-FRET, and measurements of the resultant small changes in FRET require high quality data. Tracking intracellular free Ca2+ levels is also crucial to elucidating signaling events, but the best Ca2+ indicator dyes overlap in the spectral emission range of the common FP-FRET biosensors. We have used the IMS system to simultaneously image multicolored FPs (i.e., CFP, GFP, YFP) in combination with extrinsic indicator dyes, such as Fluo-4. To demonstrate the speed and resolution of the IMS approach, we monitored both intracellular Ca2+ oscillations and caspase-3 activity during hydrogen peroxide-induced apoptosis. Ca2+ activity was measured with Fluo-4 (emission peak at 517 nm) and caspase-3 activity was measured with SCA7.3.1, a FRET biosensor based on the ECFP (emission peak at 484 nm) and EYFP (emission peak at 527 nm) pair. Using the IMS, the three fluorophores were imaged with sub-second temporal resolution and spectrally unmixed in real-time. This permits direct correlation of Ca2+ activity with other apoptotic signaling events and demonstrates the power of the IMS for measuring dynamic physiological processes.

3351-Pos Board B456
In Vivo Fluorescence Imaging of Blood Flow in Mouse Pancreatic Islets
Kurt W. Short, W. Steve Head, Michael McCaughhey, David W. Piston.

Diabetes is a disease resulting from changes in pancreatic islets, which are insulin secreting micro-organs within the pancreas. With increased blood glucose, insulin is secreted from beta cells in the islets in a coordinated pulsatile manner. At the same time alpha cell glucagon secretion is inhibited. Mechanisms controlling these processes at the intercellular and at the inter-islet level remain to be elucidated. We suggest that the three-dimensional organization of islet cells and the dynamics of islet blood flow have a role in regulating insulin and glucagon secretion. This is suggested by observation that the density of blood vessels within islets is much greater than in surrounding pancreatic tissue, and that most individual islet cells are adjacent to a blood vessel. As an initial test of our hypothesis, we have developed a high-speed in vivo fluorescence imaging method to track pancreatic blood flow in a living mouse. We are also developing methods necessary to analyze the large amounts of data generated. Using high speed line scan confocal microscopy the method has full frame sub-micron spatial and less than 10 ms temporal resolution. Islets are located within the pancreas by using mice with GFP-labeled beta cells. Blood plasma is labeled with a fluorescent dextran, allowing mapping of vascular dimensions and pathway. Individual blood cells are fluorescently labeled by osmotic shock loading with an Alexa dye, which allows tracking of the blood flow. We present current results for blood flow under different levels of blood glucose in clamping experiments. Our previous qualitative results have suggested that there are differences in blood flow parameters at different glucose levels. Here, a more quantitative analysis of blood flow velocity, any observed changes in vessel dimensions, and changes in blood flow coverage inside and outside islets is presented.

3352-Pos Board B457
Superresolution Optical Microscopy of Isolated Cardiac Mitochondrial Proteins
Harpreet Singh, Pedro Felipe, Gardeázábal Rodriguez, Rong Lu, Jean Chriostome Bopassa, Yong Wu, Ligia Toro, Enrico Stefanelli.

To study the structural organization of mitochondrial proteins, we applied Stimulated Emission Depletion (STED) microscopy in isolated mitochondria. In STED microscopy, two laser beams are used: one for excitation of fluorophores and the other, with doughnut shape, to deplete them in order to allow fluorescence emission only from the excited volume located at the doughnut’s center. With STED a lateral resolution of ~45 nm was achieved in images of isolated mitochondria. We have demonstrated that protein clusters in the mitochondria can be resolved with a separation power of ~45 nm, and that it is possible to retrieve quantitative information about the number of clusters and density of proteins in mitochondria. This approach can be extended to eis in mitochondria and subcellular organelles. Supported by NIH.

3353-Pos Board B458
Zhuangxiong Huang, Serge Donkers, Nyhke H. Dekker.

Single-molecule fluorescence studies of enzymes that incorporate fluorescently labeled substrate nucleotides typically operate at substrate concentrations well below their Km values. While this is inevitable in conventional fluorescence microscopy, the biological relevance of the insights gained into enzyme mechanism may be compromised. Zero-mode waveguides (ZMWs) provide an excellent solution to this problem by greatly reducing the observation volume. We report the nanofabrication of ZMWs, the surface treatment for controlled immobilization of biomolecules and the reduction of background noise. We also present the development of an assay to monitor in real time the incorporation of fluorescently-labeled nucleotides, which paves the way for the studies of nucleic acid polymerizing enzymes, e.g. DNA/RNA polymerase, reverse transcriptase, telomerase, etc.