S phase accumulation. Redox changes with tocopherol or ferricyanide or increases of the ATP energetic pool with pyruvate will suppress autophagy allowing for DNA and cell repairs and completion of cell cycles for cell proliferation. This process of unlocking the cell cycle with EMF to optimizing the effect of the topical treatment has previously been shown increase drug chemotoxicity (taxol, DiHydroArtemisinin, …) in tumor cell lines.

3653-Pos Board B514
Microwave Sensor for Measuring the Growth of Biomaterials
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The propagation of electromagnetic waves in a transmission line is affected by the dielectric permittivity and thickness of the material surrounding the line. We present a technique that can determine the growth of biomaterials from measurements of wave propagation characteristics. It utilizes a coplanar transmission line as sensor and an Agilent Automatic Network Analyzer for measurements in the time domain. The test material is placed on one side of the sensor. Numerical analysis indicates that the phase velocity, effective dielectric constant, and characteristic impedance show dependence on the thickness of the test dielectric. Experimental verification was obtained using water as the test dielectric. This technique can be used for continuous monitoring of the growth or thickness of biomaterials.

Imaging & Optical Microscopy: Superresolution Imaging & Single Molecules

3654-Pos Board B515
Simultaneous Observation of the Three-Dimensional Orientation and Position of a Single Fluorescent Probe
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We have developed a new microscopy system for simultaneously measuring the three-dimensional orientation and position of a fluorescent probe based on the principal of polarization analysis. This method requires only that one collect fluorescence counts from a single fluorescent probe at four different polarizations such that only relatively simple optics and mathematical equations are required to yield the orientation. Orientations can be determined with less than 10° accuracy at 33ms time resolution except for a situation where a probe is nearly parallel to optical axis. We used the newly developed microscopy and quantum rods as our fluorescent probes to simultaneously measure myosin V movement along an actin filament and rotation around its own axis. Myosin V was seen to rotate 90° around its own axis for each step. From this result, we suggest that in the two-headed bound state the necks are perpendicular to one another, while in the one-headed bound state the detached trailing head is biased forward in part by rotating its lever arm about its own axis. This new microscopy system should have particular applicability to biophysical studies that investigate single molecule orientation dynamics.

3655-Pos Board B516
Accurate Quantification Methods for Single Molecule Localization
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Super-resolution and single molecule imaging methods often rely on photon counts for quantification of precision and image processing to improve particle detection and localization. However, there is still no consensus on an accurate photoelectron calibration method for EMCCDs. In order to directly measure the light response of an EMCCD, we illuminate each sensor individually with highly attenuated laser light and compare pixel values to readout from a calibrated photomultiplier tube in photon counting mode. The data is then compared to manufacturer specification sheets as well as various published calibration methods and results from EMCCD theory. Meanwhile, smoothing and background subtraction are often used to improve the quality of single molecule data, but the effects of such routines on localization and spot-finding have not been rigorously studied. Furthermore, precision is usually calculated based on a simple model of nonlinear least-squares Gaussian fitting of the point spread function in which the effects of denoising are not at all considered. We apply several methods for image filtering and background subtraction to synthetic images representative of PALM/STORM data and measure their effects on localization precision, bias and spot-finding (quantified using Type I and Type II error rates).

3656-Pos Board B517
Fluorescence Excitation and Imaging of Single Molecules Near Coated Surfaces: A Theoretical Study
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Microscopic fluorescent samples of interest to cell and molecular biology are almost always in aqueous medium near a solid surface. Frequently, that surface is coated with a thin film such as: a lipid monolayer, bilayer, or multilayer; a collagen or agarose layer deposited before cell plating or deposited by the cells themselves; acrylamide gel to immobilize beads or single molecules (such as in commercial preparations for nucleic acid sequencing); or a cell wall interposed between the substrate and cellular organelle. Both excitation and emission of fluorescent single molecules near film-coated surfaces are strongly affected by the proximity of the coated surface, the film thickness, its refractive index, and the fluorophore's orientation. For TIR excitation, multiple reflections in the film lead to unique resonance peaks in the evanescent intensity vs. incidence angle curve. For emission, multiple reflections coupled to the fluorophore's near field emission create a distinct intensity pattern in the back focal plane (BFP) of a high aperture objective. In principle, these features should allow retrieval of information about local film thickness and refractive index, and about fluorophore axial position and 3D molecular orientation. This entirely theoretical analysis with computer-generated images explores these possibilities. Supported by NIH grants 2R56NS038129-11 and 1R21NS073686-01 to DA and Ronald V. Holz.

3657-Pos Board B518
Super-Resolution Imaging of Membrane Topology using Small Molecule Lipophilic Dye
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Plasma membrane of any cell supports a vast array of biomolecules on its surface. These are crucial in response and adaptation to the external environmental cues of the cell. The process of determining these cues is done by a plethora of plasma membrane associated proteins including receptors, channels and pores. The spatial organization including compartmentalization of these proteins is critical for cellular function. In order to generate a high spatial resolution topological map of a plasma membrane we use wide-field fluorescence super-resolution imaging technique known as Stochastic optical reconstruction microscopy (STORM). STORM works on the principle of stochastic switching of single-molecule fluorophores and precise localization of the centers of these spots. Here we labeled the plasma membrane using a small molecule lipophilic carbocyanine dye, DiD, which can photoswitch under similar conditions as previously used for Cy5 photoswitching. We stained Drosophila Schneider 2 cells with DiD and imaged them under both fixed and live conditions using 3D STORM imaging and/or single-particle tracking modes. We observed plasma membrane features including clathrin coated pit-like structures. Further experiments using multicolor STORM to image DiD together with membrane proteins will shed light on how membrane protein distribution is correlated with membrane topology.

3658-Pos Board B519
Three Dimensional Single Molecule Localization using Phase Retrieved Pupil Functions
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Single particle tracking and single-molecule localization based super-resolution techniques rely on the precise and accurate localization of single fluorescent molecules. For two dimensional imaging, relatively simple models of a microscope point spread function (PSF), such as the two dimensional Gaussian, are adequate for most common imaging approaches. However, three dimensional localization is hampered by the fact that the image of a molecule near the focal plane contains little information about its axial position. In recent years, several three dimensional (3D) imaging techniques have been demonstrated that improve the localization of single fluorescent molecules along the axial direction, such as astigmatic imaging[1], a double helix point spread function (DH-PSF)[2], and dual focal plane methods[3]. Although these methods implement very different optical setups, they use either a simple Gaussian models, theoretical PSFs that do not account imaging system aberrations, or large, unwieldy, experimentally acquired 3D PSFs that can be prone to artifacts. Here we introduce a localization algorithm based on phase retrieved pupil functions. Pupil functions can contain information about specific aberrations present in the imaging system and can be used to calculate realistic 3D PSFs from a small set of Zernike polynomial coefficients that describe the pupil magnitude and phase. This compact representation of the 3D PSF allows the PSF to be efficiently calculated as needed in an iterative update method implemented

on GPU hardware. We demonstrate the use of phase retrieved pupil functions for 3D localization in both the astigmatic and dual focal plane setups.

Reference

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3659-Pos Board B520

Imaging of Molecular Complexes on the Surfaces of Living Cells by Uniform Total Internal Reflection-Based Fret Microscopy

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Abstract

Fluorescence Resonance Energy Transfer (FRET) microscopy measures the interaction between donor and acceptor labeled proteins in living cells. Currently, these measurements are limited by out-of-focus contributions, which degrade the image. Total internal reflection (TIR) microscopy can selectively excite molecules within about 150 nanometers of the glass-water interface thereby eliminating out-of-focus fluorescence. However, the interference fringing of the coherent laser illumination used in TIR creates artifacts that prohibit quantitative imaging methods that require multiple laser illuminations such as FRET. Axelrod and collogues demonstrated that for through-the-lens TIR that rapid azimuthal spinning of a collimated beam in the back focal plane eliminates interference fringes. Here we describe multicolor, depth-matched, 360-degree spinning TIR illumination to enable quantitative, high-resolution FRET imaging of molecular complexes near the plasma membranes of living cells. We have devised new methods for normalizing spatial variations in illumination and calibrating this field for quantitative FRET analysis of protein interactions on the plasma membrane. Initial applications to Rac1 activation demonstrate a novel spatial organization of these signaling activities during adhesion of macrophages to the coverglass.

3660-Pos Board B521

Super Resolution Microscopy of Membrane Receptor Aggregation and Actin Restructuring during Staphylococcus Typhimurium Infection

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Infection of a host cell by Staphylococcus Typhimurium begins with initial contact and adhesion between host and pathogen, followed by injection of bacterial effector proteins into the host cell cytoplasm via the Type III Secretion System (TTSS) needle complex and subsequent endocytosis of the entire bacterium into the host cell cytoplasm. Conventional fluorescence microscopy has identified key host receptors, CD44 and $\alpha5\beta1$ integrin, that aggregate near the site of infection. However, due to the small size of the bacteria (~ 2 μm) and the diffraction limit of conventional optical microscopy, these aggregates appear as unresolved fluorescent ''blobs,'' making it impossible to determine the exact number of receptors present or whether there is any particular spatial arrangement of the receptors that facilitates bacterial adhesion or entry into the cell. In addition to membrane receptor aggregation, injected bacterial effectors immediately hijack local control over actin polymerization, causing membrane ruffling to occur via renegade actin filament restructuring at the site of injection. Previous studies using conventional fluorescence microscopy have revealed the entangled nature of restructured actin filaments during initial invasion and subsequent endocytosis of the invading bacterium. However, higher resolution imaging may reveal spatial details between restructured actin and the invading bacterium unobtainable by conventional microscopy. In this work, we have used single molecule localization super resolution microscopy to image CD44 and $\alpha5\beta1$ integrin aggregation as well as actin restructuring in HeLa cells fixed at various time points after initial infection. The super resolution images reveal spatial details unobtainable by conventional optical imaging methods, and may lead to further insights into the role of aggregated membrane receptors as well as the spatial relationship between restructured actin filaments and the invading bacterium.

3661-Pos Board B522

Colocalization Analysis of Mutant and Wildtype Desmin using Dual Color Super-Resolution Microscopy

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Light microscopy is arguably the most important technique to study living systems since it is minimally invasive and, therefore, allows imaging of cells and tissues under physiological conditions. However, the resolution of conventional far-field light microscopy is limited by diffraction such that only structures larger than ~200 nm can be resolved, which is insufficient for many applications including colocalization analysis. Stimulated emission depletion (STED) microscopy and photoactivation localization microscopy (PALM, FPALM) are recently developed techniques capable of providing an optical resolution down to a few tens of nanometers. We have applied these techniques to study the effect of co-expression of mutant and wild-type desmin on filament assembly.

Functioning as an important structural component, the desmin protein forms intermediate filaments found in cardiac, skeletal and smooth muscle cells. Mutations in the gene encoding for desmin were found in arrhythmogenic right ventricular cardiomyopathy (ARVC) patients. As yet it is unknown how desmin mutations contribute to the arrhythomgenic phenotype of ARVC. Some of these mutations cause desmin aggresomes. Therefore, we have investigated how coexpression of mutant and wild-type desmin disturbs filament assembly using dual color super-resolution microscopy. For *in vivo* dual color labeling, we employed mEosFP*thermo*, a monomeric variant of the green-to-red photoconvertible protein EosFP, and mIrisGFP, a green-only variant of the photoswitcher/-converter mIrisFP. These studies allowed assessing the impact of different ARVC-related desmin mutations on filament assembly.

3662-Pos Board B523

Fast Maximum Likelihood Algorithm for Localization of Fluorescent Molecules

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In super-resolution fluorescence microscopy, one key objective is to localize fluorescent molecules as quickly and precisely as possible. The most common approaches involve fitting an image of a molecule to a point spread function, often a Gaussian for simplicity. The separable property of the 2D Gaussian function allows us to separate the tasks of estimating the x and y coordinates. We did this by summing the columns of an image, then we used a maximum likelihood algorithm to estimate the position of the molecule along the x axis. Because we were able to separate the Gaussian, our computational time went from $O(L^2)$ to $O(L)$ where L is the width (in pixels) of the pixel array. This algorithm gives us precision close to the Cramer-Rao Lower Bound, and is robust against variations of pixel size, window size, or displacements of the molecule relative to the center of the computational window.

3663-Pos Board B524

Development of Methods to Observe a Living Cell and Macromolecules in Aqueous Solution with Scanning Electron Microscope at Nanometer Resolution

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In electron microscopy, transparency of specimens against a beam of electrons in TEM or intensity of secondary electrons and so on induced by an incident electron beam in SEM is translated into contrast. Any material surrounding a specimen, which prevents electron beam passing or detection of secondary electrons, obstructs to create an image. Hence, electron microscopy intrinsically requires high voltage electron beam irradiation of specimens and high vacuum under 10-4 Pa in the cell for specimens. Water in samples must be replaced with some resins or completely dried up. These conditions make it difficult to observe wet or living samples like enzymes retaining catalytic activities or living cells in aqueous solution. To image wet and living samples using electron microscopy at nanometer resolution, we are developing a new wet cell for SEM whereby living cells and enzymes can be maintained in aqueous solution. A carbon thin layer with thickness of 20 nm was made by vacuum evaporation. We applied it as a diaphragm withstanding a pressure gap for separating a specimen in solution at atmospheric pressure from high vacuum environment. Cells and enzymes were placed on its surface of the atmospheric side. They were imaged using SEM. The EM photographs show detailed structures of the cell membrane and the enzymes.

3664-Pos Board B525

Benchmarking Quickpalm and Other Molecule Localization Software for Super-Resolution Microscopy

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Image processing software is used to estimate the positions of fluorescent molecules in super-resolution microscopy images. The rejection methods (for distinguishing single-molecule and multi-molecule images) and fitting algorithms (for estimating molecular positions) used determine the quality and reliability of the reconstructed images. We benchmarked and analyzed the ImageJ plug-in called QuickPALM. To do this, we used simulations of