domains, important for the interaction between CYP3A4 and the membrane. Although the membrane affects specificity of CYP3A4 ligand binding, the structural details of the interaction have not been revealed so far because x-ray crystallography studies are available only for the soluble domain of CYP3A4. Here we report sample preparation and initial magic-angle spinning (MAS) solid-state NMR (SSNMR) of CYP3A4 (Δ3–12) embedded in a nanoscale membrane bilayer, or Nanodisc. The growth protocol yields ~2.5 mg of the enzymatically active, uniformly 13C, 15N-enriched CYP3A4 from a liter of growth medium. Polyethylene glycol 3350-precipitated CYP3A4 in Nanodiscs yields spectra of high resolution and sensitivity, consistent with a folded, homogeneous protein. CYP3A4 in Nanodiscs remains enzymatically active throughout the precipitation protocol as monitored by bromocriptine binding. The 13C line widths measured from 13C-13C 2D chemical shift correlation spectra are ~0.5 ppm. The secondary structure distribution within several amino acid types determined from 13C chemical shifts is consistent with the ligand-free x-ray structures. These results demonstrate that MAS SSNMR can be performed on Nanodisc-embedded membrane proteins in a folded, active state. The combination of SSNMR and Nanodisc methodologies opens up new possibilities for obtaining structural information on CYP3A4 and other integral membrane proteins with full retention of functionality.

Imaging and Optical Microscopy - I

763-Pos Optical nanometer scale gap sensing based on surface Plasmon Resonance (SPR)

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Board B608

Surface plasmon resonance (SPR) can be used to measure the gap distance of thin film, due to its highly sensitive optical properties. SPR is an effective method of detecting the gap distance between the sample and the substrate surface. In this study, we introduce a technique for detecting a nanometer scale gap distance based on SPR. Depending on the gap distance between the sample and the substrate surface, reflectivity is investigated as a function of the incident angle of optical radiation around the SPR angle, which is related to the resolution limit and the propagation length of the plasmons. This method can facilitate the study of searching the mechanism for the contact configuration between a living cell and a substrate surface in vitro and in real time.

764-Pos Precision of Localization Methods for Individual Fluorescent Probes

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Board B609

A fluorescent probe observed through a microscope appears as a diffraction limited spot. It is well known that the Rayleigh criterion is too conservative for determining microscope resolution. The center of a spot can be estimated with a precision that increases infinitely with the number of photons producing the spot; see [Ober et al., 2004 Biophys. J., *86*: 1185–1200; Michalet X., Weiss S., 2006 Proc. Natl. Acad. Sci. USA, *103*: 4797–4798] and references therein. In practice the finite supply of photons limits this precision. So does the choice of statistical estimator. We discuss the relative virtues of estimators and demonstrate the differences experimentally. A popular estimator employs a least-squares fit of a 2D Gaussian to the photon distribution in the image of a spot [Thompson et al., 2002 Biophys. J., *82*: 2775–2783]. We demonstrate that the experimentally observed point-spread function neither is a 2D Gaussian nor the classical Airy point-spread function. We give the theory for the correct function. Then we show that the maximum likelihood estimator based on the experimentally correct point-spread function has only half the variance of the 2D Gaussian estimator, i.e., it is twice as efficient in its use of available photons.

765-Pos Study on the Quantitative Classification for Exfoliate Cells of Lung Cancer in Sputum Smears Stained by Pap Test

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Board B610

Aim: to quantitatively discriminate the lung cancer cells from normal cells in sputum smear stained by Pap test.

Methods: 160 cases sputum samples were collected, include 60 normal cases consisted of 360 cells of columnar and squamous epithelium cells and histiocytes, 100 lung cancer cases consisted of 592 cells from 30 cases of squamous cell carcinoma (SqC), 30 adenocarcinoma (AC), 30 small cell carcinoma (SCC), and 10 large cell carcinoma (LCC). The sputum were smeared and stained by Pap test. Technique of compute image analysis was applied to test the chromatics parameters of R, G, and B and their coefficient, to test morphometry parameters of the cells and the nuclears. Discriminating analysis was done to establish the discriminants.

Results: The test results to the parameter of chromatics and morphometry were given. And the discriminated functions to discriminate the lung cancer cells from normal cells, to discriminate the different subtypes of lung cancer cells, to discriminate the different normal cells were set up based on the parameter of

chromatics and morphometry. It is showed that the accuracy to discriminate the normal and lung cancer cells are separately 87.1% and 98.5, and to the subtypes of lung cancer cells of SqC, AC, SCC, and LCC are respectively 69.7%, 75.2%, 85.5%, and 100%, and to the different normal cells are all 100%.

Conclusion: Carcinoma cells from lung cancer in sputum smear stained by Pap test can be diagnosed based on the discriminated function set up based on the parameter of chromatics and morphometry. A strategy was established to discriminate the exfoliate cells in sputum smear.

National Natural Science Foundation of China; Science and technology planning project of Guangdong Province and Guangzhou city.

766-Pos Fluorescence Imaging Of Heat Transfer In Single Living Cells With Thermosensitive Polymer

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Board B611

It is essential to measure the intracellular temperature for the study of the thermogenesis in living cells. Here, we applied the thermosensitive polymer, poly(DBD-AE-co-NNPAM-co-NIPAM), into living cells. It is known that the fluorescence intensity of this polymer in an aqueous solution sharply increases 13-fold during a 6–7 °C increase around the transition temperature. First, we confirmed that this polymer in intracellular solution is insensitive to pH and Ca^{2+}. The transition temperature of this polymer injected into HeLa cells was a few degrees lower than that of outside the cell. We also found that this polymer in living cells was sensitive to an increase of the extracellular Ca^{2+}, K^{+} and DMSO concentration. Next, we measured the speed of temperature wave inside and outside the HeLa cells. The speed of the response to the rapid heating and cooling applied by a microheater (Zeeb, et al., *J. Neurosci. Meth.* ***139***, 69–77, 2004) was the order of tens of milliseconds. Our results suggest that

1. the thermal diffusion coefficient, which is equal to the thermal conductivity divided by the heat capacity, of a living cell is lower than that of an extracellular solution and
2. the thermal properties of the cytoplasm and nuclei were almost equal.

767-Pos Using Single Cell Measurements of Protein Diffusion to Study Recovery from Osmotic Stress in live Escherichia coli

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Board B612

A powerful advantage of single molecule techniques is the capability to measure distinct, individual events that otherwise might be masked by the average measurement of a bulk solution. Similarly, multiple measurements on a single cell before and after changing conditions provide valuable information that would be unavailable in a "batch" measurement. We utilize this latter methodology by plating cells in a perfusion chamber that enables us to quickly vary growth conditions and make measurements on both short and long time scales. Previously, we measured the diffusion of green fluorescent protein (GFP) in the cytoplasm of live *Escherichia coli* by fluorescence recovery after photobleaching (FRAP) under two different conditions. When increasing the growth medium osmolality while giving the cells time to adapt to their new condition, we found only a small decrease in mean diffusion coefficient $<D>$ (from 14.8 ± 3.4 to 6.1 ± 2.4 μm^2-s^{-1}). When we rapidly upshifted the osmolality and prevented the cell from recovering (plasmolysis), the decrease in $<D>$ was 30 times larger (to 0.20 ± 0.16 μm^2-s^{-1}). These batch measurements do not trace the recovery of diffusion, only average values for the entire population. With our single cell measurements, we determine both the volume of the cytoplasm and GFP diffusion up to 10 times for each cell to follow the recovery in real time. Following the addition of K^{+} and glucose, the increase in GFP diffusion appears to track the increase in cytoplasmic volume within a cell. There is heterogeneity across cells. We have also begun looking at the effects of osmoprotectants on the time course of protein diffusion and volume recovery.

768-Pos Surface Plasmon Resonance Imaging for Studying Cells and Extracellular Proteins

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Board B613

We present an application of surface plasmon resonance imaging (SPRI) as a technique to quantitatively measure cell and protein interactions at a surface. SPRI essentially is an evanescent wave imaging technique sensitive to changes of refractive index at the sensor surface. SPRI allows for quantitative label-free imaging

measurements of protein mass changes and cell-substrate interactions. This technique is designed to help understand the process of extracellular matrix remodeling by measuring and quantifying the interactions of cells with their extracellular protein environment. We investigated extracellular protein deposition by vascular smooth muscle cells growing on a fibronectin-patterned surface. Phase contrast and fluorescence microscopy aid interpretation of SPR images. Using SPRI, cells, cell-matrix interactions, and surface proteins can be imaged. As little as 30 ng/cm^2 of deposited cellular protein can be detected, which is equivalent to ~3 pg of protein over the area of a cell. This technique allows for label-free study of live cells and their matrix remodeling activity.

769-Pos Protein Diffusion in the Cytoplasm and Periplasm of Live *E. coli* Grown at Various Osmolalities

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Board B614

Bacteria are expert at adapting to their surroundings. *Escherichia coli* can grow in fresh and brackish water, an osmotic difference of about 5 Osmolal. The water content and biopolymer volume fraction of both the periplasm and cytoplasm change considerably over this range. The mean diffusion coefficient $<D>$ is a quantitative measure of the time scale for interactions and a probe of the physical nature of these regions. Using fluorescence recovery after photobleaching (FRAP), we have measured the translational diffusion coefficient of three different probes: cytoplasmic green fluorescent protein (GFP), GFP targeted to the periplasm as a *torA::gfp* fusion, and GFP-tagged RNA polymerase (RNAP). These studies as well as cytoplasmic volume measurements have been done at varying osmolalities. The recovery of the fluorescence profile for RNAP in cells grown in Lennox L Broth (LB) at 0.26 Osm was on the order of 20 seconds, implying a $<D>$ of 0.03 $\mu m^2 s^{-1}$. The recovery is incomplete, consistent with the idea that RNAP that is actually transcribing DNA is immobile. For the same growth conditions, $<D>_{GFP,\ cyto}$ was 6.1±2.4 $\mu m^2 s^{-1}$ and $<D>_{GFP,\ peri}$ was 5.4±1.8 $\mu m^2 s^{-1}$. When growth occurred at 1.03 Osm due to additional NaCl, $<D>_{GFP,cyto}$ and $<D>_{GFP,peri}$ were 10.0±3.0 $\mu m^2 s^{-1}$ and 3.3±1.3 $\mu m^2 s^{-1}$ respectively. This contrasts with growth in MBM at 0.28 Osm and 1.0 Osm where $<D>_{GFP,cyto}$ changes from 14.8 to 9.9$\mu m^2 s^{-1}$ respectively. This effect could be due to osmoprotectants in the LB as well as a growth rate dependent response. This prompted the use of Neidhardt's defined rich media (EZRDM) for the three probe comparison of $<D>$ with growth osmolality. We present the results of $<D>_{GFP,\ cyto}$, $<D>_{GFP,\ peri}$, and $<D>_{RNAP}$ grown in EZRDM at different osmolalities.

770-Pos Single-molecule Observation of DNA/helicase Interaction by Novel Microscopy

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Board B615

Escherichia coli UvrD is a member of the helicase SF1 superfamily which plays a crucial role in nucleotide excision repair and methyl-directed mismatch repair. There is a general consensus that the enzyme unwinds a duplex DNA from a 3′ end single-stranded DNA (ssDNA) tail, a gap, or a nick and translocates along ssDNA in a 3′–5′ direction. However, conflicting models for the detailed unwinding mechanism have been proposed.

To understand the unwinding mechanism of UvrD more in detail, we have been developing novel microscopy that simultaneously allows the manipulation (stretching or twisting) of single DNA molecules and the visualization of the enzyme that are interacting with it.

Recently, we have juxtaposed single molecule imaging system with magnetic tweezers by carefully developing the optical system. With this microscopy, we have imaged single UvrD molecules interacting with single DNA tethered by magnetic tweezers. We will present the progress on the microscopy and some results obtained by the observation of the DNA/UvrD interaction.

771-Pos Epsin is a Cargo-Specific Adaptor for the Clathrin-Mediated Endocytosis of Influenza Virus

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Board B616

During clathrin-mediated endocytosis, adaptor proteins recognize specific internalization signals on cargo receptors, recruiting cargos into clathrin-coated pits (CCPs) or initiating clathrin coat assembly around the cargo molecules. Here we identify epsin, a clathrin-, ubiquitin-, and phospholipid-interacting protein, as a cargo-specific adaptor for the clathrin-mediated endocytosis of influenza virus. Using live-cell imaging to monitor the entry of individual virus particles, we observed specific recruitment of epsin to the binding sites of influenza viruses in synchrony with the assembly of clathrin-coated pits. Epsin knockdown by siRNA or over-expression of dominant-negative epsin mutants either lacking the ubiquitin-interaction motifs (UIMs) or consisting of only the UIMs inhibited the

clathrin-mediated endocytosis of influenza virus. These treatments had, however, no effect on the entry of several classical ligands for clathrin-mediated endocytosis, including transferrin (Tfn), low-density lipoprotein (LDL), and epidermal growth factor (EGF). These results suggest that epsin binds to the ubiquitinated receptors of influenza virus and functions as a cargo-specific adaptor for influenza viral entry.

772-Pos Anomalous Diffusion In The Extracellular Space Of Rat Cerebellum *In Vitro* Suggests The Presence Of Dead Spaces

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Board B617

Extracellular space (ECS) of the brain forms a channel for transport of signaling molecules, cellular metabolites and therapeutic agents. Diffusion of these substances is slower in comparison with a free medium; this hindrance is often described by the tortuosity factor ($\lambda = (D/D^*)^{1/2}$ where D is the free diffusion coefficient and D^* is the effective diffusion coefficient in tissue). In most brain regions, the tortuosity measured with small extracellular probes is about 1.6 (λ_{tissue}) while the theoretical upper limit for a 3D medium composed from uniformly spaced convex elements is only 1.23 (λ_{theory}). One explanation for $\lambda_{tissue} > \lambda_{theory}$ is based on the dead-space hypothesis proposing that the concave dead spaces in the ECS are responsible for diffusion delays that raise λ above its theoretical limit. So far this hypothesis has been supported by experiments in the ischemic brain slice where swelling cells create dead-end pores. In normal brain tissue, dead spaces may be formed by concave glia processes mapping neuronal elements. Here we employed the integrative optical method (IOI) to measured the diffusion of fluorophore-labeled dextran (*MW* 3 kDa) in the GL of rat cerebellum (400 μm-thick slices), where large concave glia processes are abundant. Unlike in any other brain regions, D^* decreased with time which can be described as anomalous diffusion. Because the theoretical studies indicate that anomalous diffusion may result from the traps distributed in the medium and the dead spaces function as the traps, anomalous diffusion observed in our experiments likely reflects the presence of dead spaces in the GL. In conclusion, our experiments in the GL of cerebellum suggest the existence of dead spaces in brain under normal conditions.

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773-Pos Imaging Neurofilaments and their Networks in vitro

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Board B618

Neurofilaments (NFs) are cytoskeletal proteins expressed in neuronal cells, and are believed to play a role in the determination and maintenance of the neuronal cell shape and mechanical integrity. NFs self-assemble to a flexible cylinder from 3 protein subunits: NF-Low, NF-Medium, and NF-High. At high concentrations, the filaments interact between themselves through their unstructured tails that branch out of the filament core and form a viscous gel. NF networks and single NFs self-assembled under various conditions were imaged using transmission electron microscopy, atomic force microscopy, and fluorescence tagged optical microscopy. These images have allowed us to study the properties of the self-assembled filaments, the filament flexibility as well as visualize the NF networks. We find that the length distribution of NFs during self-assembly is temperature, pH, and ionic strength dependent. Combinations of these techniques allow for the determination of key contributing factors towards the interactions governing the NF assembly in the neuronal cytoskeleton.

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774-Pos Protein Diffusion in the E. Coli Cytoplasm and Periplasm under Osmotic Stress

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Board B619

In order to grow, bacterial cells must maintain facile diffusion of globular proteins within a cytoplasm that is dense with biopolymers. Increases in external osmolality can increase the mean biopolymer volume fraction $<\phi>$ from ~0.16 for growth at low osmolality to ~0.35 or even higher. We have used fluorescence recovery after photobleaching (FRAP) to compare the effective axial diffusion coefficient D of green fluorescent protein (GFP) within the cytoplasm of *E. coli* cells osmotically stressed to reach large values of $<\phi>$ in two different ways. For *plasmolyzed* cells harvested from growth at 0.28 Osm and resuspended at higher osmolality in medium lacking K^+ and nutrients, $<D>$ decreases by a factor of 75 as $<\phi>$ increases from 0.16 to 0.33. Remarkably, for cells *adapted* to growth at osmolality ranging from 0.28–1.45 Osm, $<D>$ decreases by only a factor of 2.4 as $<\phi>$ increases from 0.16 to 0.36. To explain these strikingly different effects of large $<\phi>$, we propose that the measured D is a *composite* of slow GFP diffusion within a central domain containing the nucleoid(s) and much faster diffusion within a peripheral, ribosome-rich domain. The data suggest that cells adapted to growth at high osmolality maintain this segregation, enabling relatively facile GFP diffusion. In contrast, plasmolysis rapidly removes as much as half the cytoplasmic water, causing a larger fraction of the cytoplasm to be occupied by nucleoid(s) and thus dramatically slowing GFP diffusion. Recovery of ribosome-nucleoid segregation may thus be an important step in the adaptive response of cells to sudden osmotic stress. We will also present recent results probing the rotational relaxation of cytoplasmic GFP by time-resolved fluorescence anisotropy, diffusion of GFP within the periplasm, and diffusion of GFP-labeled RNA polymerase.

775-Pos Imaging the Cell Membrane using Fringe-Field Electric Impedance Tomography

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Board B620

Fringe-Field Electric Impedance Tomography (Fringe-Field EIT) is a technique which can be used to reconstruct a spatially selective image of a single cell membrane; this image is indicative of integral protein distribution, protein configuration, lipid distribution and lipid composition around a single cell. In order to accomplish these aims, a device with eight planar electrodes was constructed using a combination of rapid prototyping, thick film and microfabrication technologies. Cells were positioned within the recording channel and radiofrequencies (1kHz-10MHz) were passed between groups or pairs of electrodes to interrogate the cell membrane. A simple algorithm was used to transform data into an effective dielectric image of the membrane. Measurements around phantom disks showed that Fringe-Field EIT is capable of producing a spatially resolved electrical shape of an object in the recording channel. Measurements were also performed on the *Xenopus Oocytes. Oocytes* are polarized cells, in which the animal half of the cell has a variable concentration of protein expression from the vegetal half of the cell. Evidence suggests that calcium gated chloride channels, the granule pigment, and microvilli exist in greater quantity in the animal hemisphere of the oocyte (Dascal, 1987). Measurements around *Xenopus Oocytes* using Fringe-Field EIT indicate that there is in fact a significant difference in effective dielectric properties between the animal and vegetal hemispheres at high frequencies of interrogation (e.g. 1MHz). These data might reflect charge movements associated with membrane protein reconfigurations in response to a high frequency stimulus. Thus, in addition to providing a 2-dimensional electrical image of a cell membrane, data indicates that Fringe-Field EIT may provide insight into the difficult task of characterizing membrane bound proteins in living cells.

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776-Pos Imaging The Anisotropy Spectrum Of Mixtures Of Anti-human IgG-FITC, R6G, and Fluorescent Beads

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Board B621

We report the development of a system for combining the capabilities of fluorescence imaging spectroscopy and fluorescence anisotropy which enables the measurement of the anisotropy of fluorophores in 4-dimensions: (x, y, λ, r). We adapted existing anisotropy technology by introducing a spectrograph and linear scanning translation stage. The parallel and perpendicular polarized components of the fluorescence signal are recorded, leading to estimations the wavelength dependent steady-state anisotropies. The system was tested with Rhodamine 6G solutions containing 10% and 61% glycerol giving results that compare favorably with literature values. The advanced capabilities of the method were used to test mixtures of Anti-Human IgG-FITC (γ-chain specific) and Rhodamine 6G. Finally, the anisotropy spectra were measured in all 4 dimensions on mixtures of 0.5 μm polystyrene fluorescent beads and Rhodamine 6G. This capability should allow better measurements of receptor aggregation in studies using homo-transfer among proteins tagged with CFP and YFP as well as an improved ability to measure multiply labeled species in studies of cell biophysics.

777-Pos Clearing the Fluorescent "Fog" in Images using a Time-reversed Quasi-diffusion filter

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Board B622

A common task in fluorescence microscopy is to count the number of objects (cells, nuclei, clusters of ryanodine receptors) in an image. While these objects may be spatially separate, a fluorescent "fog" between them makes it difficult to separate one object from another with computer-based techniques. A typical way of reducing or eliminating this fog is to use a threshold filter that eliminates all pixels below a certain value. This method suffers the problem of eliminating the desired objects in some proportion to the fog. Now imagine the foggy image to be the initial data to a diffusion problem (with zero-flux boundary conditions).

Running time backwards would cause the fog in the "valley" (region between objects) to run against the gradient up to the "mountain" (the bright object), thereby clearing the image. A straight-forward implementation of time-reversed diffusion is unstable. Here we develop a simple, stable time-reversed quasi-diffusion image filter. We call this a quasi-diffusion filter because the pixel flux need not be in the direction of the maximum gradient. While this distorts the image (irrelevant for counting) it speeds up convergence and adds stability to the filtering process.

778-Pos A Bayesian Approach to Fluorescence Intermittency Based Localization Microscopy

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Board B623

In fluorescence "localization microscopy", a high resolution (10–20 nm) image is constructed by finding the position of each fluorophore

in a sample. Typically photo-activation, photo-switching, or inherent fluorescence intermittency is used to prepare a sparse field of fluorescent state emitters, allowing localization of each emitter to 10–20 nm. The field is reset and imaging continues in this manner until all fluorophores are localized.

Here we present an approach to localization microscopy that does not require isolated fluorescent state emitters, but can accurately localize fluorophores when the bright state fluorophore density is 10–100 emitters/micron^2. This approach has the potential of speeding acquisition times by allowing a higher duty cycle of probes, relaxing experimental or probe requirements needed for generating a sparse field, and through extension of the technique, allowing imaging of dynamic behavior. Fluorophore positions are found by a Bayesian parameter estimation that uses prior information about the fluorophore bright state intensity and switching rates. This computationally intensive task is largely performed on a graphics processing unit (GPU) using the recently released Nvidia 'CUDA' platform. We experimentally demonstrate this technique using labeled IgE bound to FcεRI on RBL-2H3 cells, where the label is either quantum dots or Cy3-Cy5 dye pairs.

779-Pos Single-Particle and Ensemble Studies of the pH Dependence of Quantum Dot Fluorescence Emission

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Board B624

Semiconductor nanoparticles, also known as quantum dots (QDs), offer unique advantages for cell studies that employ fluorescence microscopy. However, they also suffer from the potential drawbacks of fluorescence blinking and the presence of a dark fraction that do not fluoresce. To date, most studies have focused on transport of QD-labeled extracellular membrane surface receptors, but in some studies intracellular components have also been labeled. As the pH of intracellular compartments generally differs from that of the extracellular environment, it is important to understand how the fluorescence properties of the QDs are influenced by changes in this parameter. Here we present comparative single-particle and ensemble studies of QD fluorescence properties over a pH range covering most physiological conditions. We studied the blinking, spectral properties, fluorescence lifetime and photostability as a function of pH and salt concentration. We found that with decreasing pH, the fluorescence intensity decreases, which was related to an increase in the dark fraction of QDs as well as a decrease in the single-particle brightness. Furthermore, we found that the blinking statistics were also affected by pH and were dependent on the surface functionalization of the QD. In contrast, no pH-dependent shift in the QD fluorescence spectrum was observed, suggesting that the emitting core size was unaffected. We also detected a time-dependent reduction in the intensity of an ensemble of QDs, which became more pronounced at lower pH, and which was accompanied by a change in the fluorescence lifetime of the QDs. We have used the results of these experiments to propose a physico-chemical model that explains the pH effects on the QD fluorescence properties. This work will be of importance for researchers who use QDs for quantitative fluorescence microscopy studies within the pH-variable intracellular environment.

780-Pos Fluorescence Anisotropy Imaging

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Board B625

We designed and constructed optical system for fluorescence anisotropy imaging of cells, particles and molecules. The system includes the inverted microscope (Olympus-71), two calcite prisms (CPs) inserted in excitation and emission optical paths, and CCD camera. The excitation CP creates two illumination spots, where one spot is illuminated with vertically and the other with horizontally polarized light beams, respectively. The emission CP splits fluorescence image of each spot into two images formed by horizontally and vertically polarized emission lights. CCD camera records simultaneously four images so the fluctuation in excitation light did not affect anisotropy values and, the most important, that two polarized components corresponded exactly to the same state of a sample. The later is particularly important when anisotropy of single quantum dots (QDs) is recorded since QD "blinks" and it is impossible to measure anisotropy by recording sequentially one and then the second components. The system allows to do time-resolved (in millisecond range) fluorescence anisotropy imaging of variety of fluorescent objects. The interactive software has been developed to reconstruct fluorescence anisotropy images of two samples (or spots) from their orthogonally polarized fluorescence images recorded by CCD camera. The program calculates the intensity and anisotropy values for each pixel, generate anisotropy images, calculates average anisotropy and intensity, and plot a histogram for anisotropy or intensity values. Using this technique we analyzed fluorescent properties of thousands single QDs attached to the glass surface or to actin filaments. We studied the binding of fluorescent molecules to the macromolecular structures in cells. It makes possible to localize the bound molecules and estimate fractions of free and bound fluorescent molecules.

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781-Pos Nanoscale Dynamics of Hemagglutinin Clusters in Live Cell Membranes Quantified by Fluorescence Photoactivation Localization Microscopy

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Board B626

While mediating cellular function, the dynamic organization of cell membranes spans many orders of magnitude in length scale. For example, the clustered distribution of the influenza protein hemagglutinin (HA) is responsible for membrane fusion and entry of the influenza virus into host cells. Using fluorescence photoactivation localization microscopy (FPALM), we have visualized the lateral distribution of HA in living and fixed fibroblast cell membranes with a localization-based resolution of ~30–40 nm to show that the sizes of these clusters range from ~40 nm to ~2.5 μm. This novel technique also yields time-resolved nanoscale dynamics and single molecule information for large ($> 10^4$) numbers of molecules. With molecular positions measured every ~100–150 ms, quantification of the motion of molecules that are visible for successive frames reveals motion of HA on ~100 nm length scales. These results demonstrate FPALM as a nanoscopy technique that is applicable to living biological systems. We also present a comparison of background subtraction methods for localization of single molecules in the presence of a background signal with spatial and temporal variation.

782-Pos Dynamic Measurement of Nano-displacement and Tension Force in Myofilamets of Individual Sarcomeres by Means of Second Harmonic Generation Microscopy

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Board B627

A new method of measuring nano-displacement in the central region of myosin filaments in myocyte has been developed. The nano-displacement is proportional to the tension force applied to the myofilaments during myocyte contraction. The dynamic tension force measurement in individual sarcomeres can be performed within contracting myocytes *in vivo*. The method is based on the second harmonic generation (SHG) microscopic imaging of myocytes. The second harmonic is efficiently generated from the anisotropic (A-) bands of myocytes where myosin filaments reside. However, the central region of the A-band usually has lower intensity of SHG signal due to the antiparallel arrangement of the myosin molecules. The second harmonic radiation from oppositely oriented myosin molecules interferes destructively leading to the decrease in the SHG signal intensity in the far field. During stretching, the increase in separation between oppositely oriented myosin molecules leads to the constructive interference of the SHG signal and the evolution of double peaked profiles into a single peak with higher intensity. Therefore, the SHG intensity variation can be associated with the myosin displacement and tension force applied to the myofilament. The SHG microscopic imaging experiments were performed on *Drosophila melanogaster* larva myocytes undergoing rhythmic contraction. The SHG intensity oscillations closely followed the periodic changes in sarcomere length and tension force on the myofilaments during regular contractions.

783-Pos Influence Of 3d Bleach Distribution In Frap Experiments In Conventional And 2P Excitation Schemes

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Board B628

Fluorescence recovery after photobleaching is a classical tool for quantitative evaluation of 2D diffusion processes [1]. The quantitative analysis of such experiments requires the development of analytical models capable to describe the experimental conditions. When diffusion in 3D environments is considered, the description of the initial condition produced by the perturbation is crucial, as the usual approximations can lead to deviation in the measurement of the kinetic parameters of the labeled molecules. Furthermore the experimental distribution of fluorescent molecules depends on the intensity that produces the perturbation as fluorescence saturation would play a role [2]. In this work we measured the experimental 3D bleaching distributions in conventional and two-photon excitation and analyzed the deviations from the idealized cases. The measurement of these pattern in immobile samples revealed that the approximation of the confocal bleaching intensity distribution as Gaussian can lead to relevant errors. On the opposite side the two-photon bleach volume seems well described by such approximation, even when fluorescence saturation effects arise [3]. These data has been used for finite elements simulations mimicking FRAP experiments and compared with model FRAP curves. The results show that two photon excitation provides a better fit to the idealized bleaching patterns even in saturation regime, resulting in correct estimations of diffusion coefficients within the 20%. This result has led both to the extension to the two-photon case of models formerly developed for confocal excitation, both to the creation of procedures oriented to two-photon FRAP.

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784-Pos Spatially-resolved Fluorescence Correlation Spectroscopy Using A Spinning Disk Confocal Microscope

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Board B629

We describe an extension of fluorescence correlation spectroscopy (FCS) using a spinning disk confocal microscope. This approach can spatially map diffusion coefficients or flow velocities at up to ~10^5 independent locations simultaneously. Complex media–e.g., a tumor, cell nucleus, or extracellular matrix–are spatially-heterogeneous, making this spatially-resolved technique an ideal tool to understand hindered diffusion. There have been a number of recent extensions to FCS based on laser scanning microscopy. Spinning disk confocal microscopy, however, can be much faster at high resolution–potentially up to 1000 Hz at full resolution for the fastest available cameras–and without temporal delays between pixels. We show how to correct for a pixel size effect not encountered with standard or scanning FCS, and we introduce a method to correct for photobleaching. Finally, we apply this technique to microspheres diffusing in Type I collagen, which show nontrivial spatially varying diffusion caused by hydrodynamic and steric interactions with the collagen matrix.

785-Pos Second-Harmonic-Generation Microscopy Monitors Activity of Cystic Fibrosis Transmembrane Conductance Regulator Channel

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Board B630

Cystic fibrosis is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, which encodes a chloride channel essential for proper maintenance of epithelial cells membrane potential. The rate of membrane potential changes in response to an activation of the channel is a direct read-out of its activity.

We use second harmonic generation (SHG) microscopy in combination with voltage sensitive dyes to probe the membrane potential. This approach is faster than patch-clamp measurements and allows measuring the activity of the channel in response to different cell treatments. SHG is only detectable from non-centrosymmetric ordered structures. Thus, only dye molecules incorporated into one leaflet of the cell membrane are visible. Molecules in solution or the cytosol do not exhibit an SHG signal, reducing the background dramatically compared to standard fluorescence measurements. Because SHG does not involve electronic states of the dye, it is a much faster process than fluorescence. We use these differences in the time domain to gate the signal, thus improving the signal-to-noise ratio even further.

SHG microscopy uses a near-infrared laser as light source, thus increasing the viability of the host cells.

786-Pos Fluorescence Correlation Spectroscopy Complementing High Content Screening

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Board B631

We use fluorescence fluctuation measurement techniques, such as Fluorescence Correlation Spectroscopy (FCS) and Photon Counting Histogram (PCH) to determine concentration, diffusion properties, oligomeric state and interactions of fluorescently labeled proteins in living cells. We complement this information with high-resolution image data. Fluorescence fluctuation measurements require long acquisition times to reach acceptable signal-to-noise ratios, and can result in photophysical artifacts (e.g. bleaching) if taken at an individual cell. Therefore, we want to shorten the measurement time per cell and increase the number of different cells investigated. This approach makes it possible to screen subsets of yeast libraries containing proteins fused with Green Fluorescence Protein (GFP).

We developed an automated system to collect large data sets and combine the fluctuation information with high content screening data. The system takes an overview image to extract the position of cells of interest using threshold and pattern recognition algorithms. At these positions, the automated microscope acquires high-resolution confocal images and fluctuation data on multiple points within the cell.

The system is based on a Carl Zeiss LSM 510 - ConfoCor3 and the programming language IDL. We developed our own software to analyze the data and combine it with commercial packages.

787-Pos Image Photon Counting Histogram (IPCH) - a New Approach to Image Analysis

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Board B632

Spatial fluctuations of fluorescence intensity within a confocal image encode valuable information about the system in question. Image Correlation Spectroscopy (ICS) employs autocorrelation analysis to evaluate the magnitude, width and frequency of these fluctuations and resolve the system in terms of the occupation number and character of the spatial distribution. As a technique complimentary to ICS, we developed IPCH analysis that yields the number of particles per unit area and allows the distinction between species based on the difference in their molecular brightness. Theoretical background is presented. The theory was tested on computer generated PCHs that mimic the histograms expected to be obtained from real life images. Capabilities of the new method are discussed.

788-Pos Number and Brightness Analysis for EMCCD Cameras

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Board B633

Recently, techniques for the determination of particle number and brightness (N&B analysis) have been developed for confocal mi-

croscopy. We extend this technique to brightfield and TIRF microscopies with high sensitivity EMCCD cameras. The high signal to noise ratio and speed of these systems provides unique opportunities to follow changes in molecular brightness and concentration within a dynamic system over time as well as the spatial localization of these events. Nevertheless, these systems are also affected by saturation and nonlinearity effects which must be avoided in order to accurately measure changes in brightness. As with other analog systems, stability is a key factor and must be accounted for in the measurement of particle fluctuations. Despite these drawbacks, we show that the method can accurately measure particle brightnesses over several orders of magnitude independently of bleaching and other concentration gradients. In addition, this method provides a sensitive measure of immobile and mobile populations due to the lack of fluctuations in the former.

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789-Pos Physical And Chemical Variables Which Influence Quantum Dot Blinking: A Comparison Study Of Histogram And Image Correlation Spectroscopy Methods

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Board B634

Fluorescence blinking of quantum dots (QDs) can be a limitation for using these nanoparticles in quantitative biophysical applications. The mechanism of blinking is still not fully understood in spite of many physical studies that have employed a number of analytical methods. The complex blinking statistics have usually been studied by measuring intensity time traces of single QDs followed by determining the distribution of "on" and "off" time durations from histogram analysis after applying an intensity threshold. However, new approaches involving measuring correlation functions of the fluorescence fluctuations, such as fluorescence correlation spectroscopy (FCS) and its imaging analogue, image correlation spectroscopy (ICS) have been recently applied. The correlation techniques provide a new set of statistical parameters, but it is important to determine the relationship between the parameters derived from the various analytical approaches and ultimately link them to an underlying physical mechanism that can account for the blinking. We present an experimental study of QD blinking where we systematically varied the excitation conditions (source power and wavelength) and QD composition (chemical modification of the QD shell and ligand). We used both the histogram and ICS approaches to monitor the effects on the QD blinking and, specifically, on the parameters extracted by the two analytical methods. We found that the duration of "on" times reduces and the duration of "off" times increases upon exciting uncapped CdSe (no ZnS shell) QDs with higher energy photons. A similar effect was observed upon conjugation of dopamine ligands to the QD surface. The correlation functions found by the ICS technique were also affected by these variables. The extracted parameters from both analysis methods were compared to each other and to proposed physical models for fluorescence intermittency.

790-Pos Using Quantum dots as a Dual Mode Microscopy Probe for Molecular Studies In Situ

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Board B635

Probing the spatiotemporal response of individual intracellular proteins and multi-peptide complexes is essential in understanding the integrated response of cells. Although dynamic information can be captured using optical microscopy, most conventional spatial resolutions are limited to around 200 nm, which is significantly greater than the size of molecules. One mode of microscopy that overcomes this resolution limitation is the electron microscope, which enables *in situ* protein labeling and allows for single or sub-nanometer resolution to be obtained. Transmission electron microscopy though is limited by the inability to capture dynamic molecular responses. Here, we have demonstrated the ability to use quantum dots for both modes of microscopy through a single labeling technology, which allows both dynamic and high resolution visualization with optical and electron microscopy. We visualized core-shell CdSe/ZnS quantum dots within *Dictyostelium discoideum* using both microscopy modes through a bacterial nutrient protocol, which enables the quantum dots to enter living cells without the need of an artificial transporter system for assisted internalization. Optical imaging was first used to visualize the spatiotemporal behavior of actin filaments using phalloidin conjugated quantum dots. The same cells were then imaged using a transmission electron microscope to examine the detailed intracellular distribution down to a single nanometer size scale. These results have potential applications in a variety of areas including biophysics, cell motility, cancer metastasis, and cell structure.

791-Pos Three Dimensional Observations Of Vesicle Transports With Nm Accuracy In Living Cells Using Dual Focus Imaging Optics (DIO)

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Board B636

Nanometeries of vesicle transports are critical issue to understand the molecular mechanisms of motor proteins in a living cell. Development of three dimensional (3D) measurement system has

been necessary to achieve this, because the vesicles in cells move cubically on the cytoskeletal networks. In this study, we developed a new optics, which enables us to obtain simultaneously the two images at distinct focus. The 3D positions could be calculated from the differences of the fluorescent intensities between the two images of the individual fluorophore of quantum dots. The spatial precisions were 2 nm on XY plane and 6 nm on z axis. Temporal resolution was determined by the frame rate of the camera we used.

We observed the movements of the vesicles including HER2 (Human Epidermal factor Receptor 2) in KPL-4 cell line, in which HER2 was overexpressed. Anti HER2 antibody labeled with a quantum dot (antiHER2-QD) was endocytosed inside the cell. The vesicles including antiHER2-QD were transported toward the nucleus on GFP-microtubule. The vesicles were moved with successive 8 nm steps without rotating around the microtubule, and along a protofilament at most time. Dynein, which is minus-end motor, plays a role in the inside transport of the endocytic vesicle on a microtubule. We observed the vesicles transfer among protofilaments when the direction changed. The results in this study suggests that dynein transports the vesicles with 8 nm step along a protofilament and transfers from one protofilament to the other when the vesicle meets obstacle.

The 3D single particle tracking using DIO in this study can be applied to wide field in Cell biology and Biophysics.

792-Pos Quantitative Determination of Protein-protein Spatial Correlation in Fluorescence Microscopy

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Board B637

Determining the spatial colocalization of two fluorescent-labeled proteins is an important methodology to define macromolecular complexes in optical microscopy. Two proteins are considered positively colocalized, if they are associated forming part of the same macromolecular complex and cannot be separated due to the limited spatial resolution. On the other hand, if they tend to be present in different complexes, they are negatively colocalized. One widely used method of colocalization analysis overlaps two images with labeling two proteins with two distinct colors, respectively, and identifies the area with combined colors as colocalized. This simple method, however, has to deal with the contribution of various noises. Initially, user-chosen thresholds were used to remove the random noise, which inevitably introduces human bias. Methods of automatic thresholding were then proposed, but failed to process image with high noise. The fact that the noise is not spatially correlated is utilized by the Image Cross-correlation Spectroscopy (ICCS) method to effectively remove the contribution of random labeling. Another issue in colocalization is that, images of cells often show positive correlation and negative correlation in different areas, which can cancel out each other in a global result, and the overall amount of colocalization alone is not capable of describing the correlations between proteins comprehensively. To solve these problems, we divide the image to smaller, but statistically significant regions and find the correlation coefficient in each of them, using the ICCS. The statistics of these coefficients can give us a more comprehensive description of protein-protein correlation in cells. This method provides a quantitative, user-independent determination of both positive and negative correlations, and minimizes the influence of random noises. This method is tested in treating both simulation and experimental data.

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Imaging and Optical Microscopy - II

793-Pos E0GFP-mCherry: a Novel FRET Pair for Quantitative FRET Imaging

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Board B638

Fluorescence Resonance Energy Transfer (FRET) is a powerful tool to elucidate protein-protein interaction and protein modifications inside living cells. The recent development of spectral variants of the Green Fluorescent Protein (GFP) suitable for FRET has valuably improved the application of this technique inside living systems simplifying the labeling of proteins with fluorescent markers. While most studies have used cyan- and yellow-emitting fluorescent proteins as FRET donors and acceptors respectively, this pair of fluorophores suffers from problems of pH-sensitivity and cross-talk. In this work we demonstrate how to achieve quantitative measurement of FRET efficiency utilizing the green donor E^0GFP with the DsRed-derived acceptor mCherry. The photophysics of this two proteins has been explored revealing good spectral overlap (R_0 = 51Å), an independence of the emission spectra from H^+ and Cl^- ions and low bleeding between channels. To verify the ability of E^0GFP and mCherry to carry out energy transfer, three tandem constructs have been cloned. In these molecules the donor and the acceptor are separated by aminoacidic linkers of different length to obtain different FRET efficiencies. Two methods for the quantification of FRET efficiency, Acceptor Photobleaching and Fluorescence Lifetime Imaging Microscopy (FLIM), have been optimized for this pair and compared with a reference method, the enzymatic cleavage of the linker. In particular FLIM is suited for analysis inside living systems and for this reason this method has been applied for the determination of FRET efficiency of E^0GFP-mCherry constructs transfected in HeLa cells. The consistence of the results with the reference method (both *in vitro* and *in vivo*) confirm that this new pair can be used for more effective quantitative FRET imaging.

794-Pos Quantitative Inference of Protein Binding Affinities from FRET Imaging Data

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