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.

Supported by NIH.

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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Board B639

Advances in Fluorescence Resonance Energy Transfer (FRET) microscopy imaging enable the visualization of the spatial distributions of protein interactions inside live cells. Such experiments can give rise to large datasets comprising of multiple images, creating the need for new computational approaches for data analysis and abstraction. In particular, the evaluation of in-vivo protein dissociation constant (Kd) values from the measurements of the spatial distribution of protein concentrations will greatly aid in the quantitative evaluation of protein interactions and in reconstruction of cellular signaling networks. In the current study we present the results of a new algorithm which is developed to estimate Kd from FRET imaging data.

Our algorithm takes FRET images after deconvolution and spectral correction and uses the intensity/concentration values of individual 3D image volume elements (voxels) to compute a probability distribution for Kd preserving spatial information. We first show the performance of our algorithm for a synthetic dataset of bimolecular interactions of labeled proteins both in the absence and presence of endogenous (unlabeled) proteins. We investigate various instrument-specific, cell-specific, and algorithm parameters on the accuracy of the Kd inference. For example, microscopic imaging results in the convolution of the true 3D distribution of fluorescently-tagged molecules with the instrument's point spread function as well as the addition of detection noise, and the influence of these on the calculations are investigated. We study the performance of the algorithm in identification of multiple binding states of proteins. Finally, we demonstrate the application of our algorithm on a real cell data. We also discuss the possible extension of the algorithm that will allow us to infer kinetic information from 4D (x, y, z, t) FRET image data. Our results should facilitate inference into the mechanisms of protein signaling networks in live cells.

795-Pos Tractable Coiled-Coil Tag-Probe System for Fluorescence Labeling of Membrane Receptors in Living Cells

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Board B640

Specific labeling of a protein in living cells using a genetically encodable tag and a small synthetic probe targeted to the tag has been craved alternatively to widely used larger fluorescent proteins. Recently, several tag-probe labeling methods using well-known enzymatic reactions or the formation of peptide-metal complexes have been reported. Despite an expanding repertoire of labeling techniques, each method has some limitations, such as long labeling time, low labeling specificity, few color variation, high toxicity and cell impermeability. Here, we examined the feasibility of heterodimeric coiled-coil peptide pairs (K3-E3, K4-E3, and K3-E4 [1]) as a labeling method for cell surface receptors. The prostaglandin EP3β receptor (EP3βR) was used as a target protein. A tag sequence (K3 or E3) was attached to the N-terminus, and EYFP was fused to the C-terminus to monitor expression and intracellular localization of the receptor, (K3-EP3βR-EYFP and E3-EP3βR-EYFP). Peptide probes (K3, K4, E3, and E4) were labeled by the fluorophore tetramethylrhodamine (TMR). TMR-E3 or TMR-E4 probes (20 nM) added to Chinese Hamster Ovary (CHO) cells transiently expressing K3-EP3βR-EYFP showed no significant labeling, whereas TMR-K3 or TMR-K4 probes (20 nM) added to cells expressing E3-EP3βR-EYFP specifically labeled the receptors on cell membranes (Kd = 64 ± 31 nM and 6 ± 2 nM for TMR-K3 and TMR-K4, respectively). The labeling was quick (< 1 min), nontoxic and available even in culture medium without affecting receptor function. This tractable labeling system will be utilized to diverse applications, such as detection of receptor internalization and oligomerization.

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796-Pos Ca Release and Ca Oscillation in a Single HeLa Cell Triggered by Heat Pulse

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Board B641

As one of the essential parameters determining cellular functions, the temperature affects the rates of chemical reactions, the rate of diffusion process and so on. Here, we found that heat pulse applied by a microheater (Zeeb et al., *J. Neurosci. Meth.* ***139***, 69–77, 2004) induced intracellular free Ca^{2+} concentration ($[Ca^{2+}]_{in}$) increase followed by $[Ca^{2+}]_{in}$ oscillation at the onset of cooling phase. Although cells responded to the repetitive heat pulse, the amplitude of $[Ca^{2+}]_{in}$ increase decreased each time until no response occurred when EGTA was substituted for extracellular Ca. We confirmed that thapsigargin, an inhibitor of Ca-ATPases of endoplasmic reticulum (ER), completely suppressed the Ca response. These results suggest that the Ca release from the ER is the main source of the observed $[Ca^{2+}]_{in}$ increase, and the mechanism is attributable to the temperature dependence of the activities of Ca channel and Ca-ATPase.

797-Pos Quantitative Analysis of the Dynamics of Cell and the Extracellular Matrix with Digital Holographic Microscopy

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Board B642

Principles of digital holography are applied to study the dynamics of cell and its extracellular matrix. Digital holographic microscopy (DHM) has appeared as a unique tool to study the shape and deformation at the nano-scale by resolving differences in refractive index. A digital holographic microscope in transmission mode is designed and built to record two-dimensional holograms on a CCD camera. The digitally recorded holograms are numerically reconstructed based on the angular spectrum method (ASM) providing a better signal to noise ratio in comparison with the traditional Fresnel method. The ASM method also outputs the phase image that is used for quantitative phase-contrast analysis. The phase images are unwrapped using the Flynn's discontinuity algorithm to account for the two-pi ambiguity. The importance of conducting quantitative phase analysis rises up when one needs to reveal the optical thickness profile of a transparent specimen with sub-wavelength accuracy. Digital holography not only offers a simplified and easy to use technique but also has the advantage of yielding quantitative analysis of the phase distribution introduced by the biological samples. The goal of this study is to measure cell-migration induced deformation of collagen matrix. Quantitative phase information concerning cell morphology and volume along with those of the extracellular matrix could be obtained with digital holographic microscopy images. One of the major advantages of DHM is that this method is completely non-invasive and there is no need to dissect the sample or to stain it.

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798-Pos Visualizing Molecular Orientation in Ternary Model "Raft" Membranes Using Combined Atomic Force and Total Internal Reflection Fluorescence-Linear Dichroism

(TIRF-LD) Microscopy

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Board B643

It is currently thought that cellular membranes may contain specialized domains, ranging in size from ~ 40 nm - 2 μm, characterized by different spatial-temporal arrangements of the constitutive lipids, cholesterols, and proteins. These so-called "lipid rafts" are hypothesized to play important roles in regulating signal transduction, cellular transport, and lipid sorting. A complete understanding of the physical mechanisms that control the size, conformation, and lifetime of membrane rafts is however lacking. We report here the application of total internal reflection fluorescence-detected linear dichroism (TIRF-LD) microscopy in combination with atomic force microscopy (AFM), to spatially resolve and probe localized lipid domain order in substrate-supported DOPC/DSPC/cholesterol model membranes containing varying amounts of cholesterol. The use of polarized light in TIRF-LD and the dichroic nature of fluorescent membrane probes permits the recovery of a diffraction-limited and time-averaged measure of the fluorophore's absorption dipole orientation in a membrane phase via an order parameter (), while AFM provides an unambiguous determination of membrane phase topography. Our results show that, at low cholesterol concentrations (5–30 mol%), a 1–5 degree orientational difference between liquid-ordered and liquid-disordered membrane phases can be detected at room temperature with ~1 degree precision, depending on the amount of cholesterol present as well as the partitioning behaviour of the fluorescent membrane probe. At high cholesterol concentrations (50 mol%), phase separation was not observed by fluorescence or topography, and the order parameter increases or decreases, depending on the location of the probe's dipole within the membrane (interface or hydrophobic core). We also examined the effect of an anti-microbial peptide (indolicidin) on domain order and topography using this combined imaging technique.

799-Pos Fluorescence Spectral Imaging Of Exocytosis Following Fertilization Of Sea Urchin Eggs

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Board B644

The wave of exocytosis that follows fertilization of sea urchin eggs can be followed by an increase in the membrane fluorescence and a decrease in the second harmonic generation (SHG) of a well known voltage sensitive dye, di-4-ANEPPS (Millard, A.C., M. Terasaki, and L.M. Loew. 2005. Second harmonic imaging of exocytosis at fertilisation. *Biophysical Journal*. *88*:L46–8.). The mechanism for this sensitivity is investigated using fluorescence spectral imaging in a confocal fluorescence microscope for di-4-ANEPPS as well as a series of new dyes synthesized in this lab that are even better indicators of exocytosis. We were particularly interested in determining if the fluorescence change is related to the sensitivity of the emission spectra of these dyes to different lipid compositions, as evidenced by strong spectral shifts in lipid vesicles of varying composition. We also investigated the dependence of the fluorescence change on dye concentration. Our study revealed that there was a relatively small change in the position of the spectrum, but a large change in the fluorescence intensity that was dependent on the concentration of the various dyes. The fluorescence from the sea urchin eggs before fertilization is self-quenching, i.e. the fluorescence intensity decreases with increasing dye concentration. Upon the fertilization of the sea urchin eggs, the fluorescence intensity increases significantly if the dye concentration is over 1 uM. These results indicate that the insertion of new membrane into the plasma membrane during exocytosis relieves the dye self-quenching. The self-association of dye molecules prior to fertilization could also

underlie the high SHG signal from the membrane and the reduction of this self-association resulting from a reduction of the dye surface density might explain the high sensitivity of SHG to exocytosis.

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800-Pos A Novel High Throughput Screening Platform for Selecting Zinc Sensors by Fluorescence Resonance Energy Transfer

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Board B645

Fluorescent proteins are widely used in biotechnology with applications as fluorescent labels for protein tagging and recently in protein-based sensors for specific ligands, such as divalent cations. These sensors have been used to define binding interactions; as well as to measure local fluctuations in ligand concentration, most notably in calcium signaling research. However to further advance the utility of these sensors, sensors and fluorescent proteins with improved biophysical properties are needed. Moreover, improved strategies are needed to screen sensors in response to added ligand. To address this need, our lab is developing a novel high throughput screening platform using microfluidic technology to screen and select individual mammalian cells expressing fluorescent sensors. We have developed fluorescent sensors for Zn^{2+} where Zn^{2+} binding causes a conformational change and hence change in fluorescence resonance energy transfer (FRET). Our microfluidic screening platform is geared towards detecting the change in FRET upon Zn^{2+} binding in individual cells expressing members of a directed sensor library. The sorting platform has been engineered for gravity flow which allows precise control of the hydrodynamic focusing stream location and width. This is essential for maintaining sorting accuracy. Cells are interrogated before and after addition of ligand, and those cells which exhibit the desired properties are deflected into the collection stream by a diode bar laser. We envision this versatile platform can be expanded to sort sensors based on pH stability or kinetic lability.

801-Pos Quantitative Imaging of Zinc Secretion From Pancreatic Islets

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Board B646

Insulin is stored in the secretory granules of β-cells in pancreatic islets, where it is co-crystallized with zinc ions in a 3:1 ratio (insulin: Zn^{2+}). Upon secretion from normal islets, insulin and zinc ions are released into the extracellular space in this 3:1 ratio. We hypothesize that the insulin-to-zinc ratio is perturbed by mutations of the *SLC30A8* gene, which encodes for the zinc transporter ZnT-8 that is present exclusively in secretory vesicles of pancreatic β-cells. Deletion of this gene leads to impaired glucose-tolerance in knock-out mouse models. In order to image insulin secretion from islets, droplet microfluidics is utilized to collect secretions with minimal dilution, and lock-in spatial filtering of droplets allows highly sensitive measurements via confocal fluorescence microscopy. Using the fluorescent zinc indicator, FluoZin-3, this approach results in sensitive and quantitative imaging of zinc as it is secreted from live pancreatic islets during glucose-stimulated insulin secretion (GSIS). Furthermore, droplet-confined secretions can be stored in tubes, which can later be used to quantify insulin using radio-immunoassays or enzyme-linked immunosorbent assays (ELISA). We are utilizing these methods to investigate insulin storage defects that occur in diabetic mice, particularly those with mutations of the *SLC30A8* gene.

802-Pos Fluorescence Imaging Of Intracellular ATP Using A FRET-based Probe

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Board B647

Adenosine 5′-triphosphate (ATP) is an energy source of all living organisms, and plays important roles in muscle contraction, cell motility, intracellular transport and various biological reactions. However, spatio-temporal dynamics and distribution of ATP within single living cells are not well understood, because it has been difficult to quantitatively monitor intracellular ATP. We will report here the visualization of intracellular ATP using a newly developed fluorescent resonance energy transfer (FRET) probe. The probe consists of cyan (CFP) and yellow (YFP) fluorescent proteins connected by a regulatory ε subunit of F_oF_1-ATP synthase, which selectively binds but does not hydrolyze ATP. Probes with ε subunit from different bacteria showed different affinity to ATP, from the order of μM to mM. Among them, the probe with *Bacillus subtilis* ε subunit had suitable affinity for detecting intracellular ATP (usually the order of mM). By introducing some modifications including circular permutation of YFP, the probe finally showed about 150% increase in YFP/CFP ratio at saturated ATP *in vitro.* Next, we recorded fluorescence from living HeLa cells expressing the probe under microscope. Although ratio of YFP/CFP emission from living cells is virtually constant without stimulations, it decreased significantly after addition of 2-deoxyglucose and oligomycin, inhibitors of ATP synthesis, indicating that it can detect changes in intracellular ATP. The probes will enable us to analyze ATP within single living cells during various cellular processes. Furthermore, these probes may be used for investigating many ATP-consuming or producing processes *in vitro*.

803-Pos Dynamic Recording Of Intracellular Glucose Imaged In Single Cells Using A Fret-based Glucose Nanosensor

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To study intracellular glucose homeostasis, a nanosensor, termed FLIP, with a Kd of 600μM and which undergoes changes in Fluorescence Resonance Energy Transfer (FRET) upon interaction with glucose has been expressed in 4 mammalian cell lines: COS-7, CHO, HEK293 and C2C12. Upon addition of extracellular glucose, the intracellular FRET ratio decreased rapidly indicating increase in intracellular glucose. The kinetics were fastest (τ=5 to 15s) in COS and C2C12 cells and slower (τ=20 to 40 s) in HEK and CHO cells. Upon removal of extracellular glucose, the FRET ratio returned to its initial value at similar rates (τ =15 to 40s) in the four cell types. Glucose uptake was blocked by the glucose transporter (GLUTx) inhibitor cytochalasin B, and was unaffected by the Na/Glucose transporter inhibitor phlorizin. Glucose clearance was inhibited by the glycolytic inhibitor iodoacetate (IAA). Using β-escin to permeabilize the cell, we found that the glucose gradient across the plasma membrane was strongly dependent on the rates of glucose uptake versus glucose clearance. With 10mM extracellular glucose and a high rate of glucose clearance, intracellular glucose level fell below 100μM when glucose uptake rate was low, while it exceeded 0.5mM when glucose uptake was high. Cells cultured in high glucose maintained lower basal intracellular glucose levels than cells cultured in physiological glucose, attributed to "reciprocal regulation" of glycolysis and gluconeogenesis. Experiments performed with C2C12 cells demonstrated that differentiation caused a shift from fast glucose uptake to slow glucose uptake in the absence of insulin.

804-Pos Imaging signal transduction within the Islet of Langerhans

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Board B649

Islets of Langerhans are coordinated multicellular micro-organs within the pancreas that are centrally responsible for the regulation of blood glucose levels. In the islets, β-cells secrete insulin in response to elevated blood glucose, which triggers glucose uptake, while α-cells secrete glucagon in response to low glucose, which induces glucose release. Insight into the mechanism of insulin/glucagon secretion is required to understand diabetic phenotypes, and for development of appropriate therapeutics. Besides responding to blood glucose levels, insulin and glucagon secretion are also regulated by autocrine and paracrine signalling between α- and β-cells. Biochemical approaches to study regulation of hormone secretion within the islet are hampered due to the inability to study α- and β-cells individually, and the use of dispersed cells has been shown to poorly reflect the true behavior within the islet.

We are developing fluorescent protein biosensor tools to study signaling within intact islets by fluorescence microscopy. Using two-photon excitation microscopy and these genetically encoded biosensors we can image the signal tranduction pathways in α- and β-cells that result from autocrine and paracrine signaling. The biosensors consist of a fluorescent protein fused to a protein-domain with affinity for important 2nd messengers. In particular, we have used lipid-binding domains specific for $PI(3,4,5)P_3$, $PI(4,5)P_2$, or DAG, which can be used to assay the activation of PKB/Akt, PLC and PKC; respectively. These proteins are known to be important for insulin and glucagon secretion arising from autocrine or paracrine stimulation. To introduce these biosensors into intact islets, we are constructing lentiviral vectors, which use α- or β-cell specific promoters to direct expression and synthesis of the biosensor to the respective cells. The use of different colored fluorescent proteins will further allow simultaneous imaging of α- and β-cells to study directly the autocrine and paracrine signaling.

805-Pos FRET Phasor-FLIM Analysis of Homotypic and Heterotypic Non-covalent Interactions of Membrane Receptors in Living Cells

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Board B650

It is commonly believed that the best approach to measure FRET in cells is through the measurement of the fluorescence lifetime quenching of the donor as done in FLIM. However, FLIM is still restricted to few laboratories because of the cost of the instrumentation and the difficulty of data analysis. Several assumptions are introduced in the analysis such as the use of average lifetimes, as many fluorescent proteins and the autofluorescence show multi-exponential decays in cells. The phasor approach is introduced to simplify the way data are analyzed in FLIM. Changing the data representation from the classical time delay histogram to the phasor representation provides a global view of the fluorescence decay at each pixel of an image. In the phasor representation we easily recognize the presence of different molecular species in a pixel or the occurrence of FRET also when the cellular background is relevant. We show the application of phasor-FLIM to determine the homodimerization of the urokinase plasminogen GPI-anchored receptor, uPAR, in stable clones of HEK293 cells. We also followed

the effects of stimulatory and inhibitory agents on the assembly of the endothelial adhesive platforms members, VCAM and ICAM receptors with their tetraspanins partners in human primary endothelial cells, having variable and transient co-expression of donor and acceptor.

806-Pos *In-vivo* Imaging of Pancreatic Islet Electrical Activity

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Board B651

The pancreatic islets of Langerherns' are integral to maintaining glucose homeostasis thorough secretion of hormones such as insulin and glucagon. Glucose-stimulated insulin secretion can be characterized by a chain of metabolic and electrical activity that lead to elevated intra-cellular calcium activity ($[Ca^{2+}]_i$) and subsequent insulin granule release. Over a physiological range of glucose, islet electrical activity and $[Ca^{2+}]_i$ oscillations lead to coordinated pulsatile insulin release, which is seen at the cellular, islet, and even whole animal level. The mechanisms and importance of this synchronous oscillatory insulin release is of great debate, however in many diabetic models, the pulsatility is disrupted or lost entirely. Until now, we have focused on measuring $[Ca^{2+}]_i$ and insulin secretion in isolated islets. We are furthering these studies by imaging in the intact pancreas as a more physiological model of islet function. To this end, we present work towards imaging intra-islet electrical activity *in vivo.* We image $[Ca^{2+}]_i$ activity in a perfused pancreas which has previously been loaded with the calcium indicator dye Fluo-4. To locate islets within the pancreas, we utilize transgenic mice in which a small subset of cells in the islet express YFP controlled by the TTR promoter, or in which the majority of cells express DsRed controlled by the mouse insulin promoter. At normal glucose levels we can visualize electrical activity occurring in random cells with transient increases in $[Ca^{2+}]_i$, consistent with alpha cell activity, whereas at high glucose levels we observe synchronized and oscillatory electrical activity consistent with beta cell activity. By simultaneously measuring insulin secretion from the entire perfused pancreas, we can correlate function with the oscillating electrical activity, and thus assess putative mechanisms underlying inter-islet synchronization of insulin release.

807-Pos A Novel Fluorescence Lifetime Imaging System that Optimizes Photon Efficiency

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Board B652

Fluorescence lifetime imaging (FLIM) is a powerful microscopy technique for providing contrast of biological and other systems by differences in molecular species or their environments. However, the cost of equipment and the complexity of data analysis have limited the application of FLIM. We present a mathematical model and physical implementation for a low cost Digital Frequency Domain FLIM (DFD-FLIM) system which can provide lifetime resolution with quality comparable to time-correlated single photon counting methods. Our implementation provides data natively in the form of phasors. Based on the mathematical model, we present an error analysis which shows the precise parameters for maximizing the quality of lifetime acquisition, as well as data to support this conclusion. The hardware and software of the proposed DFD-FLIM method simplifies the process of data acquisition for FLIM, presents a new interface for data display and interpretation, and optimizes the accuracy of lifetime determination.

NIH, PHS 5 P41 RR03155

808-Pos Design, Synthesis and Applications of New Functionalized Optical Switch Probes

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Board B653

New functionalized optical switch probes based on the nitrospirobenzopyran (NitroBIPS) photochrome are described. NitroBIPS harboring homo- and hetero-bifunctional reactive groups were synthesized, and their optical switching properties studied in vitro and in vivo. All probes undergo rapid and reversible, 1- and/or 2-photon-driven transitions between a colorless spiro (SP) state and a colored merocyanine (MC) state - the excited MC-state can also decay back to the ground state with emission of a red photon and serves as a sensitive readout of the state of the switch in cells. NitroBIPS-phallodiin was used to label F-actin in vitro and in vivo and serves as a novel probe for Optical Lock-in Detection (OLID) imaging microscopy. Bismaleimido- optical probes were used to crosslink cysteine residues with protein dimers and should prove useful for controlling protein conformation and dynamics and protein-ligand interaction.

809-Pos 3D Particle Tracking in 2-Photon Microscopy Using Orbital Scanning Expanded

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Board B654

The 3D spatial position of a particle can be determined by scanning the excitation volume of a 2-photon microscope in a three-dimensional orbit around the particle and by subsequently analyzing the fluorescence intensity profile along the orbit. [1] If the analysis is computed very rapidly in comparison to the orbit time, it is possible

to use it in a feedback loop to constantly monitor the particle's position and re-center the orbit at the particle, in the case the particle moves. The individual feedback steps reconstitute the particle's 3D spatial trajectory. Trajectories of organelles in vivo, for example, have been obtained in this way with a spatial and temporal resolution in the order of 10 nm and 32 ms respectively. [2]

In this presentation we turn to some important capabilities inherent to the described orbital scan technique, which provide additional information, obtained from the tracked particle simultaneously with the tracking. Firstly, it is possible to obtain information about the particle's shape (e.g. elongation) through the evaluation of the recorded intensity transients in every orbit. Secondly the same intensity transient also contains information about the emission lifetime of the particle, if the information is recorded in synchronization with the excitation pulse train. Thirdly, because the excitation beams and detectors are always "locked-in" with the particle, it is possible to analyze the emitted light (e.g. spectrum) - or even to interfere with the particle as it moves, for example by modifying the excitation light.

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810-Pos Differential Evanescence Nanometry (DiNa): Live Cell Fluorescence Measurements with 10 nm Axial Resolution on the Plasma Membrane

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Board B655

To resolve components within a diffraction-limited object, the average axial positions of two different sets of fluorescent molecules within it are simultaneously tracked. The axial positions are then subtracted from each other to resolve the separation of the two sets of fluorophores. This method tracks the dynamic changes in the separation of the two sets of fluorophores using sequential acquisitions with total internal reflection and wide field illumination and can be used to measure the formation of small structures on living cells. We first verified that we can achieve a resolution of 10 nm and then used the method to follow the location of clathrin and its adaptor AP-2 as they are recruited to a diffraction-limited coated pit during its assembly on the plasma membrane. We find a gradual increase in the axial separation corresponding to the average distribution of clathrin and AP-2 up to a final value of 30 nm just prior to coated pit pinching and formation of the coated vesicle.

811-Pos Towards High Resolution Optical Mapping Of Proteins Bound To DNA

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Board B656

The ability to determine the precise loci and distribution of nucleic acid binding proteins is instrumental to our detailed understanding of cellular processes such as transcription, replication and chromatin reorganization.

Traditional molecular biology approaches and above all Chromatin immunoprecipitation (ChIP) based methods have provided a wealth of information regarding protein-DNA interactions. Nevertheless, existing techniques can only provide average properties as they are based on the accumulation of data from numerous protein-DNA complexes.

We propose a single molecule approach for direct visualization of proteins bound to their template DNA. Fluorescent Quantum-dots are used to tag proteins bound to DNA, and the complex is deposited on a glass substrate by stretching the DNA to a linear form. A known locus on the DNA template is labeled with a second quantum-dot and the distance between the two points is measured optically to determine the absolute location of the protein binding site. Finally, an atomic force microscope is used to validate the optical data.

812-Pos A Novel 3d Confocal Tracking Microscope: Combining The Advantages Of Orbital Tracking And Wide-field Imaging

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Board B657

Complex transport mechanisms in live cells are not restricted to a single plane. Therefore, it is of great interest to explore dynamics in 3D. To this purpose, we have developed a tracking microscope based on laser scanning confocal microscopy to follow fluorescent particles in 3D. For x-y positioning, we used the previously developed orbital tracking approach of E.Gratton[1], where the laser is scanned or orbited about the particle. By analyzing the modulation of the fluorescence intensity, the x-y position of the particle is determined and, via a real-time feedback routine, the orbit of the laser is re-centered on the new position of the particle. We have enhanced and extended the capabilities of orbital tracking by incorporating a new approach for determining the z position. Two detection channels are used, where the confocal pinholes have been shifted to detection regions slightly above and below the image plane. The z-position of the particle is determined from the difference of the two signals. As the detection of the z position is done simultaneously, the objective

only needs to be moved when the particle travels in z. This has the additional advantage that the focal plane follows the tracked particle. Exploiting this advantage, we have installed an additional 2 color wide-field setup to visualize the local environment of the particle. Interactions of the tracked particle with other particles or cellular structures such as microtubules can be recorded simultaneously. We present first experiments where we utilize this method to investigate the entry of artificial viruses in HuH7 cells.

References

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813-Pos New Near-IR fluorescent Probes for Membrane Physiology

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Board B658

AminoNaphthylEthenylPyridinium (ANEP) dyes are among the most sensitive fast-response probes for membrane potentials (~10% per 100 mV). To extend the working spectra to longer wavelength two series of Near-IR fluorescent probes have been developed: one features bridges with longer conjugation length; the other features more electron-rich amino(oligo)thiophene donors. Besides advantage of longer wavelength, these dyes show fluorescence voltage sensitivities up to 18% per 100 mV when tested in a voltage-clamped hemispherical lipid bilayer. Second harmonic generation from membranes stained with several of the dyes also show strong and fast responses to steps in membrane potential. Microinjection of water soluble variants of these dyes makes possible the intracellular staining of axons and thin dendrites in single neurons in brain slices. Electrical activity can be recorded optically from these thin processes with unprecedented sensitivity. Additionally some dyes show dramatic emission spectral shifts with varying lipid compositions, displaying especially strong sensitivity to cholesterol concentration. They have been applied to probe lipid variations along the membranes of differentiated neuroblastoma cells. The spectral images obtained with di-4-ANEPPDHQ suggest that the neurite and growth cone of these cells are enriched in cholesterol and/or lipid ordered phase membrane patches.

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814-Pos Dynamic In Vivo Imaging Of Blood Flow In Pancreatic Islets

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Board B659

Pancreatic islets are highly vascularized and uniquely organized at the cellular level. The arrangement of the different cell types as well as the tortuous nature of its vascular patterns suggests a coupling between morphology and function within the islet. Here we present methods for real time imaging of intra islet blood flow *in vivo* using fluorescence microscopy. By using confocal microscopy (Zeiss LSM510 META), we were able to identify consistently islets in mice expressing green fluorescent protein (GFP) in beta cells. We used this data to reconstruct images of the 3-D islet architecture within the pancreas of an anesthetized live mouse. To visualize the direction and velocity of blood flow in the islet microvasculature, we took two approaches:

1. intravenous injection of a pulse of fluorescent dextran or
2. perfusion of sulforhodamine-labeled red blood cells.

For real-time imaging, we used a high-speed, line-scanning pseudo-confocal microscope (Zeiss LSM5 Live), which allowed us to acquire images faster than 200 frames/s in a single z-plane or approximately 70 frames/s in a multi-plane z-stack time series. Three-dimensional data was analyzed using a novel temporal profiler that allowed visualization of the fluorescence intensity time-course at regions of interest in the islets. Initial results show that different islets contain different flow patterns (i.e. some have polar flow from one end to the other, while others have a radial flow pattern from the center of the islet outward), and that regions of the islet change their blood flow under differing physiological stresses. The novelty of our methods include not only the speed at which we were able to image islet blood flow *in vivo* but also the micron scale resolution we were able to attain in the living mouse which has not been attainable by other methods.

815-Pos In-Vivo Multiphoton Imaging and Investigation of Hepatic Physiological Processes

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Board B660

In this work, we present the design and application of an intravital hepatic imaging chamber for imaging and investigating hepatic physiological processes in-vivo. The imaging chamber is made of 6V-4Al titanium alloy and can be installed on a mouse. The sinuoids in the liver can be imaged by the addition of rhodamine-dextran while hepatic metabolism can be visualized by the processing and excretion of 6-carboxyfluorescein diacetate (6-CFDA). In normal mice, the 6-CFDA processing and excretion processes are complete within about an hour while such processes in mouse livers with bile duct ligation are severely interrupted. The intravital hepatic chamber may be applied to the investigation of biophysical, physiological, and pathological processes of the liver in-vivo and help to gain unprecedented insights to hepatic functions.

816-Pos Skin Lesions Imaging by Multiphoton Microscopy

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Board B661

We investigated different kinds of human cutaneous *ex-vivo* skin samples by combined two photon intrinsic fluorescence (TPE), second harmonic generation microscopy (SHG), fluorescence lifetime imaging microscopy (FLIM), and multispectral two photon emission detection (MTPE). Morphological and spectroscopic differences were found between healthy and pathological skin samples, including tumors. In particular, we examined tissue samples from normal and pathological scar tissue (keloid), and skin tumors, including basal cell carcinoma (BCC) and malignant melanoma (MM). By using combined TPE-SHG microscopy we investigated morphological features of different skin regions. Further comparative analysis of healthy skin and neoplastic samples was performed using FLIM, and MTPE. Finally, the use of aminolevulinic acid as a contrast agent has been demonstrated to increase the constrast in BCC border detection. The results obtained represent further support for *in-vivo* non-invasive imaging of diseased skin.

817-Pos Non-contact Optical Measurement Of Nanometer Range Deformations In Squid Giant Axon Structure During Activity

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Board B662

We demonstrate non-contact optical measurement of action potential related changes in squid giant axon using spectral-domain optical coherence tomography, which detects intensity and phase of interference along a full depth-profile. The experimental setup also measures cross-polarized transmission- and reflection-mode light intensities, 90-degree scattering, and the electrical action potential, simultaneously. In the experiments, cross-sectional images of the axon in a nerve chamber are displayed in real-time. Then, by stopping the lateral scanner, a single depth profile of interest is probed for functional interrogation. The transient deformations detected by differential phase measurement are correlated with the other optical indications of neural activity. Coincident with the action potential, there is an approximately 0.5 nm increase and decrease in the distance between the upper and lower surfaces of the axon. This study also investigates the transient deformations in presence of different environmental (i.e. temperature) and physiological (i.e. ionic concentrations) conditions. We found that cooling increases the amplitude of these deformations significantly. The results, with further investigation, may be useful for functional neural imaging.

818-Pos Imaging Axonal Transport with Iron Nanoparticles

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Board B663

Neurons are faced with the challenge of transporting proteins, vesicles and organelles, required for normal growth, development and maintenance, over relatively 'long' distances between the cell body and the synapse. Axonal transport overcomes this challenge. Defects in axonal transport have been hypothesized to play a contributing role in the progression of several neurodegenerative diseases, including Alzheimer's disease (AD). However, very few minimally invasive methods exist for the real-time imaging of these transport defects for several reasons: the diameter of an axon is typically <1 micron and the particles used for imaging must be non-toxic and easily internalized by neurons. Imaging with a superconducting quantum interference device (SQUID) using magnetic nanoparticles is one method that offers much better resolution than currently available methods and meets the above expectations.

Iron nanoparticles were synthesized by a previously developed chemical reduction reaction at pH 9.5. Briefly, ferric chloride was reduced by sodium borohydride. Polyacrylic acid (PAA) was used as a dispersing agent to prevent aggregation and palladium ions were used as nucleation sites for iron nanoparticle formation. TEM images of synthesized iron nanoparticles showed monodisperse particles <10 nm in diameter on average.

Nanoparticles were diluted in cell culture media in ratios between 1.1 mg/mL and 0.02 mg/mL and applied to cultured mouse neuroblastoma (N2a) cells. An MTT assay was conducted to obtain a dose response curve for cytotoxicity, which showed optimal dilutions between 0.02–0.2 mg/mL. PDMS microchannels with various heights and widths were fabricated using standard soft lithography procedures. N2a cells were initially cultured in tissue culture dishes before being seeded into PDMS microchannels and grown to confluency. SQUID imaging of nanoparticles demonstrated the feasibility of real-time imaging in both wild-type and AD neurons with higher resolution than standard MRI.

819-Pos The Use Of Voltage Sensitive Dyes In Optical Coherence Tomography For Depth Localization Of Neural Activity

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We report an optical method for depth localization of neural activity using voltage sensitive dyes as contrast agents in optical coherence

tomography. Absorption and scattering changes induced by voltage sensitive dyes during action potential propagation are utilized in spectral domain optical coherence tomography, which records single-scattered light intensity simultaneously from multiple depth points along a depth profile. Squid giant axon were stained with voltage sensitive dyes in near infrared region and placed in a nerve chamber. The optical system first imaged the cross section of the axon in real-time and then monitored a single depth profile of interest over time. Our experiments showed that intensity of the detected light changes significantly in the lower and upper surfaces of the axon due to electrical stimulation. The optical signal coincides with the electrical action potential and is also synchronous with the transmitted light intensity changes that are measured simultaneously. With further investigation our results might open a new avenue in the dye imaging technology to detect neural activity at different depths in situ.

820-Pos Pharmacodynamic Studies of Ligand-induced Clustering of EGF Receptors Using Fluorescence Moment Image Analysis

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Board B665

Epidermal growth factor (EGF) receptors are believed to be upregulated in many types of tumors and the activation steps of signal transduction pathways are known to involve receptor oligomerization. Consequently, this class of receptors is a target of pharmaceutical intervention. A complete understanding of the signaling pathways will require in situ measurement of oligomer sizes and the kinetics of aggregation so it is of fundamental importance to quantify distributions of the receptor oligomerization states in intact cells.

Pharmacodynamic studies of ligand-induced clustering of EGF receptors were carried out and the distributions of aggregates were measured using two-population moment analysis of fluorescence microscopy images of CHO-K1 cells expressing an EGFR-eGFP construct. The accuracy of the moment analysis necessitates both high signal to noise ratio, and sufficient spatial sampling. To meet these criteria we chose to examine the basal membrane of these cells which provides a large surface area for fluorescence imaging required for the moment analysis.

The measurements show that the oligomerization state of the EGF receptor depends largely on the concentration of the growth factor ligand and to some degree on the expression level of the receptors on the surface of the CHO-K1 cells. We observe differences in the time of onset and spatial distribution of the receptor clusters on the basal membrane depending on whether the concentration of the growth factor ligand is below or above the Kd value of the receptor. These results, in combination with fluorescent ligand accessibility studies to the basal membrane, were used to propose a tentative model that distinguishes receptor clustering occurring as a result of direct ligand activation from clustering which occurs due to downstream processes and other artifacts.

821-Pos Super-resolution Imaging And Lithography By Interfering Surface Plasmon Waves

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Board B666

Studies have been undertaken to overcome the diffraction limit for imaging and lithography. We have developed the standing-wave surface plasmon resonance fluorescence (SW-SPRF) microscopy by combination of standing-wave total internal reflection fluorescence (SW-TIRF) microscopy and surface plasmon resonance (SPR) or surface plasmon-coupled emission (SPCE). The SW-TIRF technique uses evanescent standing waves in the lower refractive index media with object of interest. The SW-TIRF takes advantage of the excitation wavelength in the factor of two allowing higher lateral resolution. Surface plasmon can be generated by reflecting light on the gold surface through the cover glass at a specific angle inducing collective excitation of electrons in the metal. The SPR contributes a better signal-to-noise ratio by inducing an enhanced evanescent electric field to excite fluorophores. With the SW-TIRF instrument, lateral resolution of approximately 100 nm was obtained. In this study, we aim to develop imaging and lithography methods with less than 50 nm resolution using corrugated gold surface to induce surface plasmon waves with larger wave number compared to uncorrugated one. Wave number matching is required to couple the energy of excitation light to that of corresponding surface plasmon. This matching process requires proper optimization of parameters such as grating constant, perturbation depth, incidence angle of the beam, and excitation wavelength. The fabrication of the corrugated gold surface is done by e-beam etching. For lithography, nano-patterns are produced on azo dye thin films, Congo-Red dye with spin-coating, exposed by an interference of evanescent waves propagating on a substrate. The resultant patterns are measured with AFM. For imaging, sub-diffraction limited fluorescent particle would be used for point spread function measurement to verify imaging at tens of nanometer resolution.

822-Pos Measuring Monomer Dimer Distributions In Tissue From Histogram Analysis Of Fluorescence Images

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Board B667

We present a novel analysis technique that can measure the densities and oligomerization states of fluorescent macromolecules within individual images obtained using conventional single detection channel laser scanning microscopy. The method is based on fitting intensity histograms from images with super-poissonian distributions to obtain density maps of fluorescent molecules and their molecular brightness in cellular samples. The approach represents a transposition of the temporal photon counting histogram (PCH) to the spatial domain and can thus be applied to analysis of chemically fixed tissue as well as live cells. The technique does not rely on spatial correlations, which frees it from biases due to subcellular compartmentalization that can be problematic for spatial image correlation based approaches. Analysis of computer simulated images and immunochemically stained $GABA_B$ receptors in spinal cord samples shows that the approach provides accurate estimates of monomer/dimer distributions over a broad range of densities within limits set by spatial sampling (areas of 6 μm^2) and the yield of the fluorophore. We use this method to show, for the first time, that the G-protein coupled receptor for substance P (NK-1r) forms almost exclusively homodimers on the plasma membrane in native spinal neurons, in contrast to within the cytoplasmic compartment where it is composed primarily of monomers. The density of NK-1r homodimers on the surface of the membrane was estimated to be 38±7 μm^{-2}. Triggering receptor internalization with capsaicin caused a measurable decrease in homodimer density at the membrane to 21±8 μm^{-2}. Independent immunocytochemical analysis using electron microscopy confirmed the differential distribution of NK-1r monomers and homodimers in distinct subcellular compartments. This new method opens the door for quantification of protein oligomer distribtions with tissue samples prepared by standard immunocytochemistry.

Imaging and Optical Microscopy - III

823-Pos Design Sensitive Protease Sensors for Living Cell Imaging

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Board B668

Proteases are essential for regulating a wide range of physiological and pathological processes and for regulating quality control of life cycles. Currently there is a strong need to develop protease sensors that are capable of quantitatively measuring protease activity in real time and monitoring activation and inhibition of enzymatic activity in various subcellular compartments. In this paper, we report a novel strategy to create protease sensors by grafting an enzymatic cleavage site into a chromophore-sensitive location of the enhanced green fluorescent protein (EGFP). Our designed protease sensors exhibit a large ratiometric optical signal change, and a wide dynamic range in both absorbance and fluorescence, as a response to the action of proteases. These engineered protein variants exhibit high enzymatic selectivity and kinetic responses that are comparable or better than current small peptide probes. Additionally, our developed protease sensors can be utilized for real real-time monitoring of cellular activation of zymogens and the effects of inhibitors in living cells. This designed strategy opens a new avenue for developing other specific protease sensors to investigate enzymatic activity in real time, diagnose diseases related to proteases *in vitro* and *in vivo,* and screen protease inhibitors with therapeutic effects.

Keywords

Enhanced green fluorescent protein, protease sensor, ratiometric change, protease activity and protease inhibitor

824-Pos Fluorescence Microscopy Investigations of Ligand Propagation and Accessibility to the Basal Membrane of Adherent Cells

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Board B669

Fluorescence microscopy methods including total internal reflectance (TIRF), and confocal laser scanning microscopy (CLSM) allow researchers to obtain images of fluorescently labeled components of cell membranes. As such these methods are often used to examine interactions occurring between membrane receptors and ligands such as antibodies and growth factors. For quantitative biophysical applications based on these imaging methods, one often assumes that the maker of interest has the ability to access all areas of the membrane equally. Our findings suggest that there is limited accessibility of ligands under the basal membrane of adherent cells plated on bare glass. We present a detailed examination of the extent to which ligands are able to propagate under adherent cells which have been grown on a variety of biologically compatible substrates. Furthermore, we examine the steric properties that limit basal membrane accessibility using a number of typical fluorescent labels, including antibodies, fluorspheres, small organic dyes and quantum dots. Finally, we examine the kinetics of the process by fluorescence photobleaching studies using both fluorescent ligands, and GFP-labeled cells, on the different substrates. Taken together we determine which of the substrates examined provides the ideal balance between cell growth/proliferation and ligand propagation for quantitative fluorescence microscopy studies on the basal membrane.

825-Pos An Automated System For Screening Pharmaceutical Agents As Potential Multiphoton-excited Cancer Contrast Reagents

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