tasks. Biological systems often consist of multiple components due to the a presence of Donor, Donor-Acceptor complex, and fluorescent background. Tools allowing a third component to be removed from lifetime imaging data would represent a significant advance. We will describe a simplified treatment for resolving binary mixtures and a novel approach to ternary and higher mixtures using frequency domain procedures. For binary and ternary mixtures there is no requirement for single exponential decay, meaning that each component can represent a multi-fluorophore mix. For many applications in biology, resolution of the fractional fluorescence contributions from the donor and donor-acceptor components is desirable as this allows activation and related parameters to be observed. From frequency domain data, this can be done in a straightforward fashion without computing lifetimes by using the linear mixing characteristics of the AB-coordinate system. We present the theory and demonstrate the approach using solutions and apply it to a simple biological system. Ternary mixtures work well using the technique, however, the advantages of additional frequencies is limited. We have applied the formalism to A431 cells labelled with quantum dots (QDs) which have three components: QDs, cellular autofluorescence and plate background. The method allowed us to strip the autofluoresence and plate background image leaving only the QDs.

2050-Pos

Using Phasors in Interpreting One- and Two-Photon Fluorescence Lifetime Images of Fruit and Polymer Interfaces

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Phasors prove to be an elegant way of characterizing time-resolved fluorescence images, (Digman et al., Biophys. J., 94, 1483-96, 2008). Fast Flim micro- and macro imaging (Biophys. J., 82, 502a) was applied to: 1. the pre-symptom and early detection of biotic and abiotic stress as well as surface defects and physiological disorders in fruit tissue using photosystem II Chlorophyll a fluorescence and 2. the characterization of conjugated polymer film produced under various conditions for biosensor development. Both Olympus and Zeiss imaging systems were used in conjunction with one photon 488 nm and 80 MHz, typically 15 mW two-photon illumination. For comparison overview color or transmission images were also collected. Several spots spread over the surfaces were used. Images have been analysed using phasors with Globals for Images, aka. SimFCS (LFD, UCI, CA, USA). The potential of the phasor approach as analysis tool for detection of both ageing and physiological stress progression (biological surfaces) and the influence of bleaching and preparation methods (polymer interfaces) is discussed.

2051-Pos

Investigation of the Lipid Metabolism during *Drosophila* Larva Development by Coherent Anti-Stokes Raman Scattering (CARS) Microscopy

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Drosophila melanogaster is one of the most valuable model organisms in studying genetics and developmental biology. The *Drosophila* fat body stores lipids that act as an energy source for the developing animal during its larval stages. Studies on lipid metabolism of the fat body allow us to better understand human energy metabolism and related illnesses.

Coherent anti-Stokes Raman Scattering (CARS) microscopy is a nonlinear optical (NLO) technique which gives three-dimensional imaging based on chemically-selective vibrational scattering signals without any labeling agent. It has been widely used in the imaging of lipids in biological samples due to the strong CARS signal from carbon-hydrogen (C-H) bonds. Here we used CARS microscopy to image the distribution of the fat body in *Drosophila* larva *in vivo* with minimal invasion. Combined with two-photon excitation (TPE) and second harmonic generation (SHG), we could also obtain images of internal organs from autofluorescence and collagen/muscular tissues from SHG simultaneously in the same NLO platform. This study allowed us to visualize the three-dimensional structures of the *Drosophila* larva under the most natural living condition which cannot be achieved by conventional biochemical staining and labeling system. We further investigated the development of the fat body during different larval stages and under various conditions through long-term *in vivo* observations.

To our knowledge, this is the first demonstration on *in vivo* imaging of unstained/label-free *Drosophila* fat body to get new insights into the lipid metabolism during *Drosophila* larva development by using multimodal NLO microscopy.

2052-Pos

Diffusion Measurements of Lipophilic Fluorescent Probes in Fixed Tissue and Living Cells

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By diffusing laterally along cell membranes, lipophilic fluorescent dyes delineate the neural pathways of both wild-type and mutant models. Multicolor imaging studies using a spectrally distinct set of diffusion-matched dyes are needed to further develop our understanding of complex neuronal connections. Previously, a set of dyes with fluorescence emission ranging from the UV to NIR was characterized and used to demonstrate six-color neuronal tracing. Using FRAP and relative distance measurements, transcellular diffusion in fixed tissue was shown to depend on the fluorescent head group. Now to compensate for this head-group-dependent diffusion, the influence of the hydrocarbon chain length has been characterized. Time-scaling exponents and diffusion coefficients within peripheral nerve tissue were compared to measurements in living cell culture. Surprisingly, it was found that the diffusion rates along the nerve increased with increasing hydrocarbon chain length. To elucidate the mechanism of lipid diffusion between cells, additional relative diffusion measurements in cultured living cells were performed by labeling a single cell within an interconnected network and measuring the spread of the fluorescent probe into surrounding cells. Taken together, these studies provide a systematic approach for the design of spectrally-discrete and diffusion-matched fluorescence probes for neurotracing.

2053-Pos

Real Time Monitoring of Endogenous Messenger RNA Using Linear Antisense Probe

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In eukaryotic cells mRNA plays a key role in gene regulation. However, the function of mRNA is not fully understood because direct analysis of endogenous mRNAs in living cells has been difficult. We developed a method for the observation of endogenous mRNA in living cells using two fluorescently labeled linear antisense 2′*O*-methyl RNA oligonucleotides. When those two antisense probes, each is labeled with different fluorescent dyes, are hybridized to an adjacent sequence of the target mRNA, the distance between two fluorophores becomes close and FRET occurs.

Here we applied linear antisense probes to the real time monitoring of endogenous mRNA, which will be useful in understanding the function of mRNA as well as the intracellular localization. First, two kinds of linear antisense probes were microinjected into the cytoplasm of living COS7 cells and the FRET signal from cells was recorded over time to examine the kinetics of the hybridization reaction with c-*fos* mRNA. The hybridization reaction of linear antisense probes proceeded quickly and time constants of linear antisense probe was estimated to be less than one minute. When using Molecular Beacon, the conventional probe for endogenous mRNAs, it took more than one hour to complete the hybridization. Next, the induction of c-*fos* mRNA in the cytoplasm of COS7 cells was investigated in real time using linear antisense probes. As a result, the elevation of c-*fos* mRNA expressed in the cytoplasm was observed within one hour after the stimulation with PMA (phorbol 12-myristate 13-acetate). In conclusion, we showed the linear antisense probes are advantageous in monitoring of mRNAs due to their prominent kinetics in hybridizing with target mRNAs in living cells.

2054-Pos

3D-Frap of PAGFP Reveals Inhomogeneity in Cytoplasmic Structures between the Major Rod Photoreceptor Compartments

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Introduction: Diffusion of signaling proteins is thought to be essential for photoreceptor physiology, allowing, for example, regulation of the photoresponse through dynamic sequestration of key transduction proteins in either of the two major photoreceptor compartments, the inner and outer segments (IS and OS, respectively). The influence of the physical nature of the cytoplasm in these compartments on protein mobility is not known, but is essential for understanding photoreceptor function. We have thus developed a novel approach to quantifying protein mobility in 3D in live photoreceptors.

Methods: Transgenic *Xenopus laevis* expressing the photoactivatable variant of GFP, PAGFP, exclusively in rod photoreceptors were generated using established methods. Live retinal slices were imaged with a custom-built

multiphoton/confocal microscope. A source of photoactivated PAGFP was generated within selected compartments using multiphoton excitation at 820nm and its dissipation was monitored with 488nm confocal scanning. Images were compared with the output of a 3D diffusion model to estimate effective radial and axial diffusion coefficients.

Results: PAGFP diffusion in the IS was isotropic and faster than in the OS, $D_{IS} = 5.2\ \mu m^2\ s^{-1}$. In the OS PAGFP diffusion was anisotropic, with faster radial diffusion, $D_{OS\text{-}radial} = 3.5\ \mu m^2\ s^{-1}$, and slower axial diffusion, $D_{OS\text{-}axial} = 0.19\ \mu m^2\ s^{-1}$.

Conclusion: PAGFP diffusion in both compartments was substantially retarded relative to aqueous solution, $D_{aq,PAGFP} \sim 90\ \mu m^2\ s^{-1}$, and cultured Chinese hamster ovary (CHO) cell cytoplasm, $D_{CHO,PAGFP} \sim 20\ \mu m^2\ s^{-1}$. Moreover, axial diffusion of PAGFP in the OS was hindered to a larger extent than expected from the geometry of disc membranes that span the compartment orthogonal to the cylinder axis. These results suggest that the photoreceptor cytoplasm possess higher density of cytoskeleton and/or macromolecules.

2055-Pos

Lipopolysaccharide Regulation of Dendritic Cells Activation and Life Cycle: in vitro and in vivo Studies Towards Antitumor Immunoactivity

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Dendritic cells (DCs) are key regulators of innate and adaptive immune responses that can be exploited in the immunological treatment of many type of cancers. Recently, we have demonstrated that lipopolysaccharide (LPS) is able to regulate DC life cycle through the activation of a CD14-dependet pathway [1]. Once activated with LPS, DCs become also able to prime Natural Killer (NK) cells to exert their anti-tumoral activity as demonstrated both *in-vitro* and *in-vivo* using mouse models in which melanoma tumors were sub-cute implanted. Using two-photon microscopy, we are currently extending these experiments to the direct investigation of the interaction of LPS-activated DCs with NK cells in *in vivo* condition at the level of peripheral lymph nodes.

DCs and NK cells,labeled with different fluorescent markers, are tracked continuously in the lymph nodes while the structure of the lymph node is monitored by second harmonic generation microscopy. The analysis of the traces and the comparison of the experimental results to statistical and simulative models of the lymphocytes motion allows to elucidate their dynamic behavior at different times after the activation of the DCs shedding new light on the DCs - NK cells interaction.

[1] Zanoni, I.; Ostuni, R.; Capuano, G.; Collini, M.; Caccia, M.; Ronchi, A.E.;Rocchetti, M.; Mingozzi, F.; Foti, M.; Chirico, G.; Costa, B.;Zaza, A.; Ricciardi-Castagnoli, P.; Granucci, F. (2009) CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation. ***Nature,*** 460:264-269.

2056-Pos

Growing Lung A549 Epithelial Cells on Metallic Surfaces

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Fluorescence can be greatly enhanced near metal surfaces due to many-fold increased brightness and photostability of fluorophores located near metallic nanoparticles or nanostructures. Further more, fluorophores deposited on a plane of a 50 nm thick silver or gold mirrors, show directional fluorescence in a form of hollow cone. These favorable properties of fluorophore-plasmonic interaction can be utilized in high-sensitivity imaging of cellular processes. However, the cell growth strongly depends on the nature of the substrate and is often very difficult on bare metal surfaces. In our study we examined suitability of different metal surface coatings for growing lung A549 epithelial cells. Six different surfaces were tested - glass, silver mirror, silver mirror coated with SiO_2, gold mirror, gold mirror coated with SiO_2 and silver fractals on glass. The glass coverslips with five different metallic surface coatings and one control were placed in a tissue culture plates containing DMEM-High Glucose media. Suspension of $0.5x10^6$ cells/plate was deposited on the slides and the cell cultures were placed in 37°C, 5%CO_2 incubator. Cell growth was monitored every 24 hours. No substantial differences in cell morphology were found whether they were on the regular microscopic glass slide or slide covered with metals, but cells initially grew significantly slower on fractals compared to other ''smooth'' surfaces. The findings demonstrate feasibility of growing A549 cells on metal-coated glass surfaces and opens the opportunity for imaging live A549 cells using metal enhanced fluorescence.

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2057-Pos

Fluorinated Voltage Sensitive Dyes for SHG and Multiphoton Microscopy

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Dyes based on hemicyanine chromophores have high membrane affinities and the ability to report on local membrane environment and act as sensors of membrane voltage. Laser-scanning second harmonic generation (SHG) microscopy utilizing such voltage sensitive dyes has shown considerable promise as an imaging modality, possessing several advantages over fluorescence for the optical mapping of membrane potentials. The addition of electronegative fluorine atoms to the chromophore is intended to lower both the ground and excited state energies, so as to make dyes less susceptible to photobleaching. Improved photostability will allow extension of the duration of optical recording measurements, permit the use of more intense laser excitation, and minimize photodamage to the biological sample. The effect of fluorination on photostability and dye performance has been systematically investigated for a series of newly synthesized dyes and found to depend critically on the location of the substituent within the chromophore. Voltage-clamped neuroblastoma cells stained with these dyes were imaged with 1064nm excitation, allowing sensitivities and response kinetics of SHG and two-photon fluorescence to be determined simultaneously for several fluorinated dyes. Our results suggest that voltage sensitive dyes can be developed which have large SHG signal changes, sufficient photostability, and the requisite speed for use as a practical tool for measuring electrical activity in biological systems.

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2058-Pos

High Throughput Screening of Biosensor Domains: Visualizing Dynamic Activation of Src Kinases in Live Cells

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Biosensors that report activation of native, unmodified proteins can help delineate complex cellular processes with minimal perturbation of normal behavior. However, sensors for endogenous proteins are rare, in part due to the absence of readily available binding reagents that are selective for the active form of the targeted molecule. To address this issue we combined high throughput screening of engineered state-specific binding elements with a fluorescence-based reporting system that turns these elements into biosensors. We have targeted Src family kinases (SFKs) since they are key signaling nodes that control numerous cellular functions, including migration and adhesion. Also, the multiple roles performed by Src kinases and their involvement in several signaling networks suggest spatio-temporally regulated pools of SFK activities. Phage display screening was used to generate fibronectin domain III (FN3) binders that selectively bind SH3 domains from Src family kinases. Pull down experiments demonstrated that an FN3 binds selectively to active Src kinases. Using merocyanine dyes developed in our lab for live cell imaging, we have converted this FN3 into a sensitive fluorescence-based biosensor for activation of Src family kinases. The new sensors reveals patterns of Src activation in migrating cells and in cells stimulated with growth factors. In migrating cells, a distinct band of Src activation was observed at the leading edge. This transient activation coincided with protrusion. We also observed precisely timed Src activation in linear and circular dorsal ruffles. In keeping with our overall aim of developing a generally applicable strategy of sensor design, we have generated multiple binders using HT screening and are developing biosensors specific to individual Src family kinases.