2044-Pos
Fab Fragments Versus Full IgGs in Stimulated Emission Depletion (STED) Nanoscopy
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STED microscopy is an optical far-field microscopy technique which breaks the diffraction limit by quenching excited fluorophores into the dark state and thus reducing the effective (excitation) point spread function. The state of the art STED microscopes achieve resolution below 20 nm.
Standard labelling techniques use full IgG antibodies to tag specific proteins, an interaction similar to a lock and key, by two antigen binding sites. On a single protein scale IgGs therefore can potentially induce protein clustering, falsifying the results with respect to the cell or particle's natural environment. This effect is not observable in the standard confocal microscopy. However, in STED and other nanoscopic techniques protein clustering will lead to artifacts, which must be considered in an image interpretation.
In contrast Fab fragments have only one antigen binding site and clustering doesn't have to be taken into account.
We show the difference between full IgGs and Fab fragments on the nanoscopic scale by analyzing Env molecules on the surface of HIV particles and CD4 on the surface of JC53 cells. We demonstrate that Fab fragments result in images closer to the true structure of biological samples.

2045-Pos
Long-Term Super-Resolution Imaging of Actin Cytoeskeleton in Dendritic Spines Using a Low-Affinity Photoactivatable Probe
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The transmission of signals across synapses requires the precise interaction of a large number of different synaptic proteins such as neurotransmitter receptors, adhesion, scaffold, signaling and cytoskeletal proteins. In small central excitatory synapses, this molecular machinery is contained in specialized cellular compartments called dendritic spines. Plastic changes in the strength of synaptic neurotransmission include alterations of the spine morphology. The shape of dendritic spines is determined by the actin cytoskeleton and is highly dynamic. Rearrangements of the actin network occur in response to synaptic activity. Thus, actin plays an important role in morphological aspects of synaptic plasticity.
The visualization of the precise spine morphology has been hampered by the limited spatial resolution of conventional wide field optical microscopy (typically in the range of 300 nm). The recent development of nanoscopic imaging methods makes it now possible to achieve a spatial resolution below the diffraction limit of light. Here, we have implemented photoactivated localization microscopy (PALM) to study the organization of the actin cytoskeleton within dendritic spines at 25 nm resolution.
To this aim we have generated a low affinity actin probe that consists of an actin-binding peptide (ABP) fused to a tandem Eos photoconvertible fluorescent protein (tdEos). ABP-tdEos was expressed in hippocampal neurons, where it binds reversibly to actin, thus allowing for long-term live imaging of the spine cytoskeleton at a spatial resolution beyond the diffraction limit of light. By reconstructing super-resolution images we have quantified morphological parameters of dendritic spines. Furthermore, we have studied dynamic changes of dendritic spine morphology over 30 minutes at a temporal resolution of 50 s. Using this approach we determined changes in the actin distribution within spines in response to pharmacologically induced synaptic activity.

2046-Pos
Photounbinding of Fluorescent Proteins
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Fluorescent probes are commonly used in biological experiments. Despite their great success story over the last century, fluorescent conjugates can not only visualize but also influence the properties of the molecules under study.
Recent studies have shown that fluorescently labeled antibodies can be dissociated from their antigen by illumination with laser light; the same has been observed for protein-peptide binding, including toxins. The mechanism responsible for the photounbinding effect however remains elusive. Here, we give insights into the mechanism of photounbinding and discuss bio (medical) applications of photounbinding.
We present studies of the photounbinding of labeled calmodulin (CaM) from a set of CaM-binding peptides with different affinities to CaM. Our results suggest that photounbinding is linked to photobleaching and a 'radiative' process requiring a fluorescent label. Interestingly, the photounbinding effect becomes stronger with increasing binding affinity, however, does not induce breakage of covalent bonds. We show that by writing a simple rate law for the dissociation process that takes into account the effective concentration of the fluorescent molecule, the affinity of binding and the laser intensity, it becomes possible to describe the intensity dependence of the photounbinding of our data. The proposed model assumes that an intermediate (transitional) complex is formed before the unbinding occurs and is consistent with the labeled-protein undergoing a conformational change resulting in a distinct dissociation constant which is in turn responsible for the unbinding.
We believe that detailed knowledge about the molecular processes involved in photounbinding will not only allow a systematic improvement of quantitative fluorescent studies, but also open the door to inducing or inhibiting molecular interactions by light and thus the development of novel tools, such as drug activation or delivery.

2047-Pos
3D Nanoscopic Optical Imaging of Subcellular Protein Organization and Neuronal Dendritic Morphology
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Single molecule localization microscopy enables biological samples to be optically observed with sub-diffraction resolution. Here we report new progress in 3D nanoscopic imaging of cellular and subcellular structures by our newly developed virtual volume photoactivated localization microscopy (VVPALM), in which a tilted mirror is used to generate side view of biological samples in addition to the front view, therefore providing precise single molecule localization in three dimensions. VVPALM brings advantages including high efficiency to use detected photons, minimum vulnerability to optical aberration, and simplicity of implementation. VVPALM is ideally compatible with weak chromophores, such as fluorescent proteins. We have used VVPALM to probe the localization and organization of various bacterial proteins, which are fused with photoactivatable proteins, inside or on the membrane of single *Escherichia coli* cells with sub-100 nm resolution in 3D. We have also combined VVPALM with label-free PAINT (points accumulation for imaging in nanoscale topography) technique to measure the nanoscale morphology of rat neuronal dendrites. Advances in multi-color nanoscopic imaging for protein 3D colocalization will also be presented.

2048-Pos
Photoactivation Localization Microscopy (PALM) on Orai1 Channels
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Store Operated Calcium Entry (SOCE) is a crucial mechanism for many cellular signalling processes. The two major proteins involved in SOCE are Orai1 (located in the plasma membrane) and STIM1 (located in the membrane of the endoplasmatic reticulum (ER)). Upon depletion of the calcium stores in the ER, the STIM1 co-clusters with the Orai1 in the plasma membrane which results in a calcium influx into the cell.
In order to investigate the distribution of the Orai1 in the plasma membrane we used Photoactivation Localization Microscopy (PALM). PALM is a technique that allows overcoming the diffraction limit by photo-activating only a small subset of fluorophores with a laser pulse. At shallow illumination conditions, the active fluorophores are spatially well separated and can be localized with a precision of a few ten nm. Sequential activation, readout and photobleaching allows for recording a complete image of the sample. The Orai1 subunits were fused to photoactivatable GFP (paGFP) and expressed in Chinese hamster ovary cells. PALM revealed submicrometer clusters of Orai1, which show a high degree of colocalization with STIM1-mCherry, as confirmed by two-color microscopy.

2049-Pos
Using Ab-Space to Remove Background Components from Images in Systems of Multiple Fluorophores
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The linearization of the mixing of fluorescence intensity afforded by the AB-space formalism, simplifies a number of Frequency domain lifetime imaging

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