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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.

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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.

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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.

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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.

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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 endoplasmic 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.

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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