**3064-Pos Board B756**

**Optically Modulated Fluorescent Proteins Enhance Sensitivity in Live Cell Imaging**

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Fluorescence microscopy is a widely used non-invasive tool for investigating intracellular structure and processes. Although the palette of fluorescent proteins has revolutionized detection and dynamics in molecular and cellular biology, the limited brightness, low spectral discrimination, and high cellular autofluorescence continue to limit applications. In contrast to photoswitch-based methods, we have developed a spectroscopic method to recover fluorescence from photoaccessible dark states via long-wavelength secondary laser co-illumination. We selectively recover the higher energy fluorescence by modulating this secondary laser without modulating background autofluorescence. Using hypothesized photoreversible isomerizations of the chromophore, we have identified specific fluorescent protein mutants, ranging from blue to red, capable of optical modulation. Employing these methods, we have demonstrated the ability to recover the modulated signal, with >10-fold improvement, from background in live cells. Such modulation schemes enable new imaging modalities for probing intracellular kinetics and equilibria of low-abundance proteins.

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**Mechanisms of Multiphoton Bleaching of Red Fluorescent Proteins**

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Two-photon laser scanning microscopy (TPLSM) has several advantages over one-photon confocal microscopy, including deeper tissue penetration, higher signal-to-background ratio, and less photodamage in the out-of-focus volume. However, due to very high instantaneous light intensities in the focal volume, the probability of further, stepwise resonant photon(s) absorption increases dramatically, leading to very efficient bleaching of a probe. To deal with this challenge one has to understand the underlying mechanisms. Here we measured the power dependence of multiphoton bleaching rates of several red fluorescent proteins expressed in live *E. coli* cells under two-photon microscope conditions. To clarify the photophysical mechanisms, we also used much lower repetition rate (1 kHz) and different pulse durations in experiments *in vitro* with Ti:Sa amplifier excitation. Our experimental data supported by quantum mechanical calculations of the chromophore in protein environment are consistent with the mechanism of ultrafast (<150 fs) singlet-singlet stepwise absorption of one or two additional photons following initial simultaneous two-photon absorption. In the DsRed2 protein, the third photon absorption most probably results in an ultrafast electron transfer (ET) from the anionic chromophore to an excited Rydberg state of a nearby positive amino acid residue (e.g. K163+). The transient radical state of the chromophore tends to accept an electron from the deprotonated E215- amino acid, thus promoting the first step of the recently established decarboxylation reaction. In mFruits proteins, the third photon promotes an ET from the chromophore to the 4S Rydberg state of nearby K70+ residue with its subsequent photoionization by the fourth photon.

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**Quantitative Imaging of Protein Secretions from Single Cells in Real Time**

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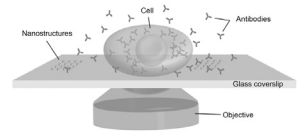
Cell-to-cell signaling often involves the secretion of proteins, which creates spatial and temporal concentration profiles in the extra-cellular environment for the receiving cells to detect and interpret. Real-time measurements of secreted protein concentrations at the single cell level are thus fundamental to understanding these communications pathways but have proven difficult to realize in practice. Here we present a label-free technique based upon nanoplasmonic imaging and high-affinity binding which enabled the measurement of individual cell secretions in real time. When applied to the detection of antibody secretions from individual hybridoma cells, the enhanced time resolution revealed two modes of secretion: one in which the cell secreted continuously and another in which antibodies were released in concentrated bursts that coincided with minute-long morphological contractions of the cell. From the continuous secretion measurements we determined the local concentration of antibodies at the sensing array closest to the cell. The technique is incorporated on to a wide-field microscope which enables real-time transmitted light and fluorescence imaging of the cells as well. We anticipate this technique will be broadly applicable to the real-time characterization of both paracrine and autocrine signaling pathways.




**3067-Pos Board B759**

**A Fluorescence Approach to Discrimination of Aggregated Amyloid Proteins**

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We present the design and synthesis of a new class of fluorophores with sensitivity to both environment viscosity as well as environment polarity, a property known as solvatochromism. We recently reported the ability of these fluorophores to discriminate, based on fluorescence emission, protein amyloid aggregate deposition in *ex vivo* neuronal tissue (Cao *et al*., *J. Am. Chem. Soc.*, **2012**, *134*, 17338-17341). Here, we have further modeled the solvatochromic relationship of these probes with environmental permittivity and have used these results to extrapolate the permittivity values of the probe binding pockets within several amyloid proteins. Additionally, we have begun to examine the utility of these fluorophores as potential diagnostic tools for detection of neurodegenerative pathologies.

**3068-Pos Board B760**

**Live Synaptic Mapping of Vertebrate Whole Brain with Light Sheet Microscopy and Endogenously Labeled Synapsin-2B Protein**

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A synaptic map of vertebrate brain is a crucial step for understanding the molecular basis for neuronal networks development and modification. However, conventional two-photon, point-scanning microscopy presents challenges for in vivo, whole-animal studies of brain synaptic structure and dynamics in higher organism due to reduced axial resolution, and inadequate temporal resolution for developmental or behavioral studies, as well as excessive photodamage to the sample. Application of two-photon, light sheet microscopy overcomes these barriers, approaching isotropic 3D resolution and faster volume scan speed (>10x), while simultaneously reducing phototoxic effects. Using a protein trap zebrafish line, in which Synapsin-2B is expressed as a fluorescent fusion protein from the endogenous locus, combined with two-photon light sheet microscopy enables live, synaptic mapping of the whole vertebrate brain. This work provides the basis for further research in imaging brain dynamics and development, for example fast, high-resolution whole-brain activity imaging.

**3069-Pos Board B761**

**A Multi-Emitter Localization Comparison of 3D Superresolution Imaging Modalities**

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Single-molecule localization-based superresolution imaging is complicated by emission from multiple emitters overlapping at the detector. The potential for overlapping emitters is even greater for 3D imaging than for 2D imaging due to the large effective 'volume' of the 3D point spread function (PSF). Overlapping emission can be accounted for in the estimation model, recovering the ability to localize the emitters, but with the caveat that the localization precision has a dependence on the amount of overlap from other emitters. It is interesting to consider if a particular 3D imaging modality has a significant advantage in facilitating the position estimation of overlapping emitters. We compare variants of two commonly used and easily implemented imaging modalities for 3D single-molecule imaging: astigmatic imaging; dual focal plane imaging; and the combination of the two approaches– dual focal plane with astigmatism. We quantify the ability for a particular 3D modality to facilitate estimation with overlapping emitters using the Cramér-Rao lower bound (CRLB). The CRLB is used to calculate the theoretical best localization precision under a multi-emitter estimation model. We investigate the performance of these 3D modalities under a wide range of conditions including various distributions of collected photons per frame, background counts, pixel sizes, and camera read-out noise values.