in physiological and pathological processes and thus central for the function of biological systems. We describe a fast and reliable ratiometric Fluorescence Lifetime Imaging Microscopy (rmFLIM) approach to analyze the distribution of protein-ligand complexes in the cellular context.[1] Binding of the fluorescently labeled antagonist naloxone to the G-protein coupled μ-opioid receptor is used as an example. To show the broad applicability of the rmFLIM method we extended this approach to investigate the distribution of polymer-based nanocarriers in histological liver sections.

References

(1) Boreham et al. (2011) DOI: 10.1021/ml200092m.

1008-Pos Board B794

Second Harmonic Generation Correlation Spectroscopy for Nanocrystal Characterization

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Many early trials at protein crystallization produce large amounts of sub-diffraction limited crystals. These nanocrystalline showers are challenging to quantitatively characterize by conventional optical methods; however, they can offer important indicators for improving crystallization conditions. Additionally, the advent and availability of ultrafast X-ray free-electron lasers now allows single-pulse diffraction from individual protein nanocrystals for structure determination. However, these and other applications of nanocrystals currently suffer major bottlenecks in sample characterization, limiting their broader utility. Second harmonic generation correlation spectroscopy (SHG-CS) is being developed to address this key characterization need. Under tight focus and high laser intensity, highly-ordered (crystalline) material lacking inversion symmetry; including the vast majority of protein crystals but not simple salt crystals, amorphous protein, solvents, etc.; allow for second harmonic generation, the frequency doubling of light. This provides a way to selectively track crystalline protein particles in solution. The size of the particles can be determined by taking advantage of the fact that the particles diffuse through solution. The amount of time spent in a particular pixel along with the size of the pixel allows diffusion constants to be extracted by autocorrelation. Although in principle, SHG-CS is similar to fluorescence correlation spectroscopy, practical challenges arising from the use of high average power (>100 mW) require constant motion of the beam. The coherence of SHG can also present unique challenges and potential opportunities compared to fluorescence-based correlation methods.

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New Voltage Sensitive Dyes

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To improve the photostability of voltage sensitive dyes (VSDs) we have explored structural modifications such as fluorination, trifluoromethylation, cyanation, and rigidification, on the classic ANEP (aminonaphthylethenylpyridinium) chromophore. Some new dyes show significantly improved photostability in confocal imaging of brain slices and in a photobleaching assay with a suspension of lipid vesicles. Most show high sensitivities to membrane potential changes when tested on a voltage-clamped hemispherical lipid bilayer (HLB) apparatus. The modifications also induce large spectral shifts, up to 50 nm in either blue or red direction, depending on the nature and position of modification. The high sensitivity and photostability of the new VSDs have already produced new applications, such as imaging of electrical signals in single dendritic spines. Because of the range of excitation and emission wavelengths covered by this array of new VSDs, they may be especially useful in multiplexed imaging of membrane potentials together with other fluorescent indicators in live cells and tissues. (Supported by NIH grant EB001963).

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Compressive Mechanics of Hyaluronan Brushes - A Study with a Combined Colloidal Probe AFM/RICM Setup

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Around many biological cells, in the transition zone between the plasma membrane and the extracellular matrix, lies the micrometer thick pericellular coat (PCC): hyaluronan (HA), a long and flexible, charged glycosaminoglycan and HA-binding proteins self-organize into a hydrated gel-like coat which serves crucial mechanical functions of the cell. For a thorough investigation of the physical principles underlying the biological functions of these coats and provided the instrumental limitations in studying these highly hydrated systems in vivo, model systems are useful.

Atomic force microscopy (AFM) is a widely used analytical approach to determine the behavior of molecules or thin films under mechanical force. Colloidal probe reflection interference contrast microscopy (RICM) is an established microinterferometric technique to determine the thickness of soft hydrated films. In this study we combine colloidal probe AFM and RICM to investigate the mechanical properties of a well-defined model system of the pericellular coat: films of hyaluronan that is grafted to supported lipid bilayers (Richter et.al. 2007, JACS, 129:5306-7). The combination provides interaction forces as a function of the absolute distance between the two approaching surfaces, information that cannot easily be obtained with either technique alone. We quantify the thickness of HA films, and their resistance to compression forces as a function of external salt concentration. From the experimental data, and comparison with scaling and mean-field theories, we conclude that grafted HA films are well-described as a polyelectrolyte brush. Addition of cartilage proteoglycan aggrecan induced a drastic increase in thickness and resistance to compression. The novel combined AFM/RICM setup can serve as a powerful tool to quantify the mechanical properties of soft hydrated biopolymer films with precise control of probe sample separation.

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Raman Spectroscopy and Imaging of Biomolecules using Targeted Nanoparticles

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Abstract:
Optical microscopy of biological systems is useful for detecting various structures with varying chemical or structural contrasts. In the past, fluorescent tags have been useful as imaging probes of biomolecules. An alternative is to use optical properties of nanoparticles for contrast and detection. The local electromagnetic fields gained from the excitation of conduction band electronics of metal nanostructures can be used to enhance Raman scattering from molecules in close proximity. This effect, the electromagnetic enhancement responsible for surface-enhanced Raman scattering (SERS), thus provides a sensitive probe of chemical environments. We have coupled tip-enhanced Raman scattering (TERS) with nanoparticle probes to obtain chemical, structural, and spatial information simultaneously. In protein-ligand interactions, our results show signal enhancements from both the ligand, bound to a nanoparticle probe, and the target protein, thus demonstrating this environmental sensitivity. We are exploring these effects to distinguish the differences between the wild type and mutant proteins, as well as investigating intact cell membranes.

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Fast and Accurate FRET Quantification through Computation of the Minimal Fraction of Donor (mf_D) from TCSPC Data

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The study of the spatial and temporal dynamics of molecular interactions using FRET-FLIM is often compromised by the large number of photons required to fit the multiple-lifetime decay of the donor population in each pixel of an image. Long acquisitions prevent interacting dynamics to be detected in an image, while the use of high excitation intensities causes bleaching or unexpected cell responses. The computation of the minimal fraction of donor molecules (mf_D) undergoing FRET is a non-fitting approach that allows quantification of molecular interactions where the complexity associated to fitting a fluorescent decay with fretting and non-fretting components hampers quantification.

We have established the experimental conditions on which quantitative FRET analysis based on mf_D computation is preferable to fitting the fluorescent decay to a model with a weighted non-linear least-squares algorithm. The accuracy of the quantifying parameters as a function of the fraction of donor molecules involved in the interaction and the number of photons was studied for both fitting and non-fitting strategies using simulated TCSPC data that had been validated against actual experiments with FRETting constructs of fluorescent proteins (mTFP1-YFP) expressed in living cells.

In summary, the validity of the non-fitting minimal fraction of donor computation (mf_D) strategy for quantification of TCSPC FRET experiments is demonstrated not only for cases when fitting strategies fail due to the complexity of the decay, but also for simpler models when the number of detected photons is small. The conditions on which quantitative mf_D analysis of FRET experiments allows faster acquisitions than fitting strategies have been established. This approach is well suited for imaging protein interactions in living cells as faster acquisitions result in better resolved spatio-temporal dynamics.