219-Pos Board B98

Applicability Of An EM-CCD For Spatially Resolved TIR-FCS

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Diffusion constants (DC) of surface near fluorescent particles can be measured by total internal reflection fluorescence correlation microscopy (TIR-FCS). The usage of EM-CCDs instead of photo diodes offers a high degree of parallelization and the possibility of extracting additional information by spatial cross-correlation (TIR image correlation spectroscopy, TIR-ICS).

Since temporal autocorrelation functions of particle fluorescence critically depend on CCD parameters such as pixel size and geometry, binning, sampling rate, and gain, we explored systematically the performance of an EM-CCD as detector in TIR-ICS. We found that variations in the sample geometry can be well described by a structure term (ST). Whereas in axial direction the ST is described by evanescent field depth, the lateral extension of the detection volume was found to be well approximated by a Gaussian fit to the convolution of the CCD pixel geometry with the measured point-spread-function for single pixel read-out. For higher binning we empirically could show a linear relationship between the Gaussian approximation for the lateral ST and the size of the quadratic ROI on the CCD used for detection (binning), with a correction factor (slope) that is independent of the CCD chip used.

To test the performance of CB TIR-ICS we measured diffusion coefficients (DC) and particle numbers (PN) of fluorescent probes of different sizes (Fluorospheres and GFP) at varying viscosities, concentrations, and sampling rates. This allowed calculating the resolution of the method expressed as the minimal relative resolvable difference in PNs or DCs. Distinguishing between different probe concentrations was possible with differences in PN of 30%. In contrast differences as low as 6% in DC could be distinguished at DC-to-sampling-frequency-ratios smaller than 0.5*10ˆ4nmˆ2.

This renders TIR-ICS suitable and ideal for measuring spatially resolved dynamics of proteins in viscous media such as in live cells.

220-Pos Board B99

Imaging Total Internal reflection - Fluorescence Cross-correlation Spectroscopy (ITIR-FCCS)

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Fluorescence correlation spectroscopy has been widely used to understand diffusion, flow processes and dynamics of reactions. Conventional FCS instruments use point detectors thus making multiplexing difficult. EMCCD based detection combined with TIRF illumination allows simultaneous detection of thousands of points. It has been demonstrated that this method, called Imaging Total Internal Reflection-Fluorescence Correlation Spectroscopy (ITIR- FCS), reduces background noise and allows the measurement of correlation functions on each pixel independently. This permits for the first time to observe molecular dynamics on a whole cell membrane simultaneously. This method has been applied to study diffusion phenomena in 2D lipid bilayers and cell membranes. In this work, ITIR-FCS is extended to perform spatial cross-correlations and is referred to as ITIR-FCCS. ITIR-FCCS is used to study flow and diffusion phenomena. Flow properties have been studied by a variety of techniques with suitable spatial resolution. In many of the techniques, there is a trade off between spatial (ICS) and temporal (FCS) resolution. ITIR- FCCS serves as a bridge between these two extremes with a spatial resolution of ~ 300 nm and a temporal resolution of ~ 0.4 ms.

A generalized expression for ITIR-FCCS, allowing the cross-correlation of areas of any size and shape and for diffusion and flow, is derived. Since the expression contains only 5 fit parameters (flow velocities in x and y-direction, diffusion coefficient, concentration, size of the point spread function of the microscope) it is potentially calibration-free. This method was applied to three different systems: Diffusion, Flow, Diffusion and flow. Upon data analysis, flow velocities and diffusion coefficients are determined for all three systems and advantages and limitations are discussed. This technique is a promising tool for the analysis of diffusion and directed motion in cells with good spatial and temporal resolution.

221-Pos Board B100

Analysis Of Diffusion And Binding In Cells Using The Rics Approach

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The movement of macromolecules in cells is assumed to occur either through active transport or by diffusion. However the determination of the diffusion coefficients in cells using fluctuation methods or FRAP frequently give diffusion coefficient that are orders of magnitude smaller than the diffusion coefficients measured for the same macromolecule in solution. It is assumed that the cell internal viscosity is partially responsible for this decrease in the apparent diffusion. When the apparent diffusion is too slow to be due to cytoplasm viscosity, it is assumed that weak binding of the macromolecules to immobile or quasi immobile structures is taking place. In this work we derive equations for fitting of the RICS (Raster-scan Image Correlations Spectroscopy) data in cells to a model that includes transient binding to immobile structures and we show that under some conditions, the spatio-temporal correlation provided by the RICS approach can distinguish the process of diffusion and weak binding. We apply the method to determine the diffusion in the cytoplasm and binding of Focal Adhesion Kinase-EGFP to adhesions in MEF cells. Work supported in part by U54 GM064346 Cell Migration Consortium (MD and EG), NIH-P41 P41-RRO3155 (EG) and P50-GM076516 (EG).

222-Pos Board B101

Effect of Multiple Scattering on the Illumination Profile in Fluorescence Correlation Microscopy

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Knowledge of the size and shape of the illuminated volume is critical when carrying out quantitative studies involving Fluorescent Correlation Microscopy (FCM). Determination of that volume may be difficult when FCM is applied to crowded systems such as cells, tissues, and congested polymer solutions. We thus have carried out Monte-Carlo simulations to address of the effects of multiple scattering on a focused laser beam, using a model of optically non-absorptive spherical scatterers to mimic a concentrated solution of non-fluorescent crowders. We find that as the concentration or size of the nanoparticles increases, the illumining beam spot is broadened in a non-uniform manner. Further, the focal point of the spot shifts towards the incoming beam source. This analysis has been performed for a selection of media conditions, where we vary both bead size and concentration.

223-Pos Board B102

Characterizing Protein Interactions In Different Cellular Compartments By Axial Scan Fluorescence Fluctuation Spectroscopy

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Fluorescence fluctuation spectroscopy (FFS) is a noninvasive tool for measuring protein interactions, concentrations and transport directly in living cells. Brightness analysis of FFS experiments focuses on the photon counts rate of protein complexes and provides a unique approach to quantify homo- and hetero- interactions between proteins. However, FFS theory assumes that the fluorescent particles are uniformly distributed within the optical observation volume. This assumption is violated when measuring thin cytoplasmic sections of cells, because fluorophores only occupy part of the observation volume. Another problem for conventional FFS analysis is the situation where more than one cellular compartment is enclosed by the observation volume. If the concentrations or the interactions of the proteins differ in adjacent compartments, the brightness determined from conventional FFS theory can be strongly biased. We present examples that highlight these biases and introduce a model based on axial scan FFS that avoids these potential pitfalls. The performance of the axial scan FFS and the modified FFS theory is characterized with simple model systems. We apply axial scan FFS to study the protein interactions of the NTF2 protein in the cytoplasm and the nucleus of cells. This work is supported by the National Science Foundation (PHY-0346782) and NIH grant R01GM064589.

224-Pos Board B103

The Study of Interaction of Hypericin And Its Pharmaceutical Preparation by Fluorescence Techniques

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This study reports the combination of fluorescence correlation spectroscopy (FCS) and fluorescence lifetime imaging microscopy (FLIM) in the study of interactions between a photosensitizer, hypericin (HY), and its solvent system prepared with a formulation additive, polyvinylpyrrolidone (PVP), a common pharmaceutical excipient used in HY formulation in order to improve on the