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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.

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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.

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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).

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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

water insolubility of HY, one of its major draw-backs. The aggregation and binding of HY in the presence of PVP were studied and digitized fluorescence endoscopic imaging (DFEI) was used to study the effect of the pharmaceutical formulation with the *in vivo* tumor implanted chick chorioallantoic membrane (CAM) model. The combined results from FCS and DFEI studies reveal the coordination of HY-PVP binding, HY disaggregation in the presence of PVP and strengthened HY tumor uptake selectivity. PVP is thus suggested as a potential adjuvant to previously investigated N-methyl pyrrolidone (NMP) in the HY delivery system as well as a replacement for the conventionally used albumin in the HY bladder instillation fluids preparation for clinical use. The findings were then further confirmed by FLIM studies in HY solutions, followed by measurements in MGH and RT112 cells incubated with HY and PVP solutions in dark. Fluorescence lifetime of intracellular HY showed similar trend of prolongation with the presence of PVP as that was observed with FLIM measurements in solutions. The FLIM results, supported by colocalization studies by confocal imaging with fluorescently labeled PVP, strongly suggest that PVP is uptaken in cancer cells despite the large number of contrary reports.

225-Pos Board B104
Brightness Analysis of Nuclear Receptor Interactions in a Cell-Free Expression System
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Brightness analysis with fluorescent fluctuation spectroscopy examines protein interactions in real time. The analysis is based on the principle that the brightness of an oligomer is proportional to the number of fluorophores in the protein complex. We previously applied brightness analysis inside living cells to decipher both homo- and hetero-interactions between proteins. While experiments in cells probe protein interactions in the natural environment, solution studies probe interactions between isolated proteins. In some cases we observed different results in cells than expected from solution studies. In order to identify the origin of such differences, we adapt brightness analysis to solution studies by utilizing a cell-free expression system. Cell-free expression takes advantage of genetic tags for labeling proteins while providing a much more controllable environment than the living cell. We first characterize the behavior of mCherry and EGFP in a cell-free expression system to optimize it for brightness analysis. The samples are measured in microfluidic devices using two-photon excitation. Using this system, we study the oligomerization states and interaction of nuclear receptor proteins RXR and RAR by means of brightness analysis. We determine the binding affinity of these receptor proteins for comparison with measurements made in living cells and on purified protein systems. This work is supported by the National Science Foundation (PHY-0346782) and NIH grant R01GM064589.

226-Pos Board B105
Resolvability of PCH in Two Dimensional Systems
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Although the ability of PCH analysis to resolve the components of mixtures of fluorescent molecules has been carefully studied in three-dimensional systems (Muller, Chen et al. 2000), it has not been investigated in two dimensions. We explored the characteristics of the reduced $\chi 2$ surface of two dimensional binary mixtures, specifically, the principal curvature at the $\chi 2$ minimum as a function of brightness and molecular concentration. Our results are in good agreement with the previously published results.
A potential problem can arise from errors in focusing in two dimensional systems . When data acquisition time and therefore the data record is insufficient to to resolve species in a mixture, independent information about each species, e.g., measurements of brightness, can be used to extract more accurate results. Our experiments on Giant Unilamellar Vesicles (GUVs) labeled with a single lipid analog allow us to estimate brightness variations due to focusing. Combining this information and the PCH from mixtures yield reasonable estimations of the parameters of interest
Muller, J. D., Y. Chen, et al. (2000). "Resolving heterogeneity on the single molecular level with the photon-counting histogram." Biophys J 78(1): 474-86.

227-Pos Board B106
Insights Into The Microscopic Origin Of Anomalous Diffusion From Crowded Solutions
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Subdiffusive motion of tracer molecules has been observed in many crowded environments, ranging from polymer and protein solutions to intracellular fluids. Yet, a clear understanding of the microscopic origins of subdiffusive motion and the variation of the anomaly in crowded media is still missing. To address this point, we have studied the diffusion of tracer molecules in crowded solutions with varying composition using fluorescenc correlation spectroscopy (FCS). Aiming at capturing the essential processes that lead to anomalous diffusion, we observed that none of the artificial mixtures yielded the degree of subdiffusion that has been observed for intracellular fluids, i.e. artificially crowded solutions appear too simple to account for the high degree of anomaly observed in cells. Comparing time and ensemble averages of the tracers' mean square displacement furthermore indicated that ergodicity is unbroken. Hence, the monitored diffusive process cannot be described properly by a (non-stationary) continuous time random walk.

228-Pos Board B107
Bacterial Sec Protein Transport is Rate-limited by Precursor Length: A Single Turnover Study
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An in vitro real-time single-turnover assay for the *Escherichia coli* Sec transport system was developed based on fluorescence dequenching. This assay corrects for the fluorescence quenching that occurs when fluorescent precursor proteins are transported into the lumen of inverted membrane vesicles. We found that: i) the transport kinetics were well fit by single-exponential, even when the ATP concentration was rate-limiting; ii) ATP was utilized during most of the time required for transport; and iii) longer precursor proteins transported more slowly than shorter precursor proteins. Together, these conclusions argue against a model in which precursor movement through the SecYEG translocon is mechanically driven by a series of rate-limiting, discrete translocation steps that result from conformational cycling of the SecA ATPase. Instead, we propose that precursor movement results predominantly from Brownian motion and that SecA regulates pore accessibility through ATP hydrolysis.

229-Pos Board B108
Single Molecule FRET Measurements of Dye-labeled DNA
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Single molecule FRET (sm-FRET) measurements of dye-labeled DNA molecules have become a widely-used tool over the last decade to probe the structure, dynamics and function of nucleic acids. As robust as FRET is, quantitative measurements and absolute distances are seldom extracted, mostly due to difficulties in obtaining the correct value of the transfer efficiency and the orientation factor between the dyes. Moreover, additional complications might arise from undesired interactions between the dyes and the nucleotides, or even between the dyes themselves. We present a systematic study of sm-FRET performed on an extensive set of internally labeled DNA molecules with FRET pairs at various inter-dye distances, spanning the entire dynamic range of transfer efficiencies. An automated two-color scanning confocal microscope allows us to obtain long fluorescence time trajectories at high temporal resolution, from which we extract the corrected FRET efficiencies. By fitting these values to a geometrical model of the double helix we obtain the absolute distances between the dyes as well as the orientation factor. At inter-dye distances smaller than 8 nucleotides apart we observe an increased fraction of abrupt fluorescence fluctuations that we attribute to dye-dye interactions. We note that this phenomenon can distort the apparent FRET efficiency towards lower values, consistent with previous reports. We will show single molecule time traces which have allowed us to characterize and study these interactions in detail.

230-Pos Board B109
Structural Dynamics of SERCA and Phospholamban by Fluorescence and Phosphorescence
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We used fluorescence and phosphorescence to investigate the structure and dynamics of phospholamban (PLB), and its regulation of its inhibited target, sarcoplasmic reticulum calcium ATPase (SERCA). Polarized TIRF of PLB, labeled in the cytoplasmic domain helix with bifunctional rhodamine (BFR), shows that this domain lies parallel to the membrane surface. The structural and functional effects of PLB phosphorylation and mutation are under