FAP-fluorogen system is ideal for generating a renewable, photostable signal and providing high photon flux. Since the localization accuracy is in principle limited by the number of photons collected from fluorescent probes, our module holds great promise for achieving super-resolution imaging (See abstract by Lidke, K.A. et al.). In this study, we characterize the blinking behavior of FAP-fluorogen pairs at equilibrium. We show that the on-time is highly dependent on the excitation power density and the off-time is controlled by the free dye concentration and therefore, is limited by diffusion. By adjusting the dye concentration in solution and the intensity of incident power, the on and off rates of the binding reaction are controllable and could be optimized to resolve the objects in time and space. Clones of the FAP with point mutations show different blinking behaviors, which suggests that these amino-acid residues could play an important role in FAP-fluorogen binding and indicates that structural differences of the FAP at the binding interface can change the photochemical and photophysical properties of fluorogen.

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Single Molecule Anisotropy Imaging with Fluorescence Photoactivation Localization Microscopy

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Recently, localization-based microscopy techniques have proven their versatility and opened a window into nanoscale structures in biological systems. Furthermore, knowing the orientations of individual molecules that make up the structure will provide more information on molecular interactions and hence the underlying mechanism of the process of interest. Here we present the principles and techniques incorporated into Fluorescence Photoactivation Localization Microscopy (FPALM) to simultaneously measure single molecule positions and anisotropies. Single molecule anisotropy distributions were experimentally measured and theoretically calculated using Monte Carlo simulations. Results are presented for two model systems consisting of either immobilized or freely rotating photoactivatable molecules. Next, the technique was applied to image fixed mouse fibroblast cells expressing Dendra2-actin with an effective lateral resolution of 17nm, based on the number of detected photons and the local density of molecules. The images showed trends in anisotropy distributions of filamentous actin. Cells treated with cytochalasin-D before fixation and imaging showed changes in cell morphology such as fewer distinct filamentous structures, and significant differences in the measured single-molecule anisotropy distributions.

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Fluorescence Correlation Spectroscopy In Live Bacillus Subtilis Cells: An In Vivo Study Of Transcriptional Regulation

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Fluorescence correlation spectroscopy (FCS) is a useful technique for characterizing the mobility and concentration of fluorescent molecules both in vitro and in vivo (1). We utilize two photon FCS to characterize the concentration and mobility of fluorescent molecules within living cells of Bacillus subtilis. Autocorrelation functions were measured in bacteria expressing green fluorescent protein(GFP) under the lac promoter in both nutrient rich and nutrient poor culture medium. Although considerable heterogeneity was evident from cell to cell, on average, both intracellular concentration and mobility were found to be dependent upon culture medium and Isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration. We also investigated bacteria expressing GFP under control of native promoters for transcription factors (TF) involved in the regulation of the carbon metabolic cycle in Bacillus subtilis. The GFP concentration, which should be related to TF concentration, was investigated for single cells and cell populations under different metabolic conditions. Some photobleaching was observed during the course of the measurements as a depletion in the average fluorescence intensity. This is due to the small size of the bacteria (~10 fL) and low basal expression levels of GFP (~100 nM) in the absence of IPTG. Methods to take this into account during data analysis are discussed.

1. Schwille, P., U. Haupts, S. Maiti, and W.W. Webb. 1999. Molecular dynamics in living cells observed by fluorescence correlation spectroscopy with one- and two-photon excitation. Biophys J 77:2251-2265.

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Full Fluorescence Correlation Analysis - From Picoseconds To Seconds-For The Study Of Biomolecular Interactions And Dynamics

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Fluorescence correlation spectroscopy (FCS) is a widely used technique for providing quantitative information on many important cellular processes, such as translocation, molecular association, and diffusion. When carried out at the single molecule level using recent advances in time correlated single photon counting, photon coincidence analysis can be used to correlate intensity fluctuations on time scales from seconds and hours down to picoseconds. This method not only reveals the diffusional and molecular association properties of molecular complexes in the microsecond to millisecond regime, but also enables the characterization of dynamics and photophysics on several different time scales. Photon bunching in microsecond regime can be used for the study of fast conformational changes as well as internal photophysics like singlet-triplet transitions. On the nanosecond time scale, fluorescence lifetime and rotational diffusion dynamics are accessible. Furthermore, we show that photon coincidence analysis down to the picosecond regime can be used to quantify a small number of molecules; hence, providing quantitative information on the stoichiometry of molecular complexes. We present here a generalized approach for the full correlation analysis from time scales of hours down to picoseconds, and demonstrate its utility in biological applications. We provide results of this technique applied to the study of apolipoprotein interactions with rHDL and the dimerization properties of a novel red fluorescent protein probe, phytofluor red 1 (PR1).

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Fluorescence Correlation Spectroscopy with Sub-Diffraction-Limited Resolution Using Near-field Optical Probes

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Fluorescence Correlation Spectroscopy (FCS) is a powerful technique for studying single molecule dynamics. Because of the requirement for a small number of fluorescent molecules in the excitation volume, typical FCS set-ups based on confocal optics are limited to relatively low fluorophore concentrations (nanomolar and below). This limitation has prompted a range of approaches for reducing the excitation volume, particularly for biological samples where higher concentrations are often encountered. We have recently demonstrated that application of near field optical probes enables FCS measurements with sub-diffraction-limited resolution [1]. As a proof-of-principle experiment, we have measured the diffusion of Oregon Green-labeled DHPE in a DOPC bilayer supported on glass. Using a near field probe with an aperture diameter of 140 nm we have achieved a reduction in the diffusion time and excitation area of approximately an order of magnitude, as compared to confocal FCS. Further, we have shown that a simple analytical expression based on a step function excitation profile is appropriate for fitting the correlation data obtained with near field probes. Additional experiments aimed at extending the initial measurements of lipid diffusion to more complex membranes and further reducing the observation area using an optimized probe design will be presented. The use of near field probes has considerable potential for observations on cellular membranes which possess submicron features such as lipid domains and macromolecular assemblies, because the small axial extent of the near-field will minimize excitation of auto-fluorescence from the cytoplasm.

[1] Dusan Vobornik, Daniel S. Banks, Zhengfang Lu, Cécile Fradin, Rod Taylor, Linda J. Johnston, *Appl. Phys. Lett.*, in press, 2008.

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Single Molecule Lifetime Probability Distribution Analysis (τ-PDA)

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Time correlated single photon counting signals are compiled into arrival time histograms from which lifetime distributions are generated using a variation of the probability distribution analysis (PDA) technique which has previously been used to analyze two color fluorescence resonance energy transfer signals and anisotropy signals. The PDA techniques produces lifetime distributions which are well described using statistical methods, making the assignment of error bars and the detection of heterogeneities and dynamics easier. In this work, simulations of fluorescence signals from freely diffusing single molecules are used to compare PDA analysis of lifetimes with maximum likelihood estimator (MLE) derived distributions of the same data. The simulations incorporate Raman scattering, dark counts, afterpulsing, and for the case of two color fluorescence resonance energy transfer signals, crosstalk. Results are presented demonstrating the effectiveness of lifetime PDA analysis in homogeneous, heterogeneous, and dynamic systems.