Posters

Emerging Single Molecule Techniques I

122-Pos Board B1

Single Molecule Immuno Pull Down Assay (SiMPull) for Studying Protein-Protein Interactions

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Protein-protein interactions form the cornerstone for most biological pathways. Governed by numerous factors, same protein can associate with a host of different proteins and exhibit diverse functionality. This heterogeneity in complex composition is difficult to probe using bulk assays like immunoblot. Using TIRF microscopy, we have developed a *si*ngle *m*olecule immuno *pull* down assay (SiMPull) enabling direct visualization of protein-protein associations. Using fluorophore labeled antibodies we are able to visualize individual tethered molecules of protein of interest with high specificity. Surface bound antibodies are employed to specifically immobilize a target protein. We are able to pull down protein of interest (bait) from crude cell lysate, eliminating the need for protein purification. The bait protein co-precipitates its interacting partners. The identity of proteins bound to the bait is verified either by using fluorescent protein fusion constructs or through antibodies against anticipated targets. For a multimeric protein complex, fluorophore labeled antibodies against its subunits colocalize in the same diffraction limited spot. Using different dye labels for antibodies against different subunits and multicolor fluorescence colocalization, we are able to ascertain the molecular composition of these complexes. Individual photobleaching events provide us insights about the stoichiometry. SiMPull can be extended to single cell lysate analysis and provides a rapid, sensitive and robust platform for analyzing protein assemblies *in situ*.

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A Single-Molecule System for Detection and Quantification of Proteins with Robust Capture Units and Potential for High Multiplexing

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We have developed a novel sensor technology with powerful high multiplexing detection potential and high sensitivity built around a specialized recombinant DNA molecule, Digital DNA. This digital DNA is constructed with unique identification patterns (barcodes), and serves as a scaffold for specific analyte-recognizing receptors, which can be antibodies, Fab fragments, or camelid nanobodies. The presence of the bound target is detected by binding of a uorescently-labeled secondary antibody. At the current parameters of our DNA-reading technology, the theoretical limit of multiplexing exceeds 10^5. In addition, our microfluidic DNA reader requires a very small sample volume; many assays can be run on sub-microliter-sized samples. Assays have been developed for a half dozen proteins (glutathione-S-transferase, ovalbumin, botulinum toxoid, Venezuelan Equine Encephalitis virus coat, Follicle-stimulating hormone and pokeweed antiviral protein). Studies with these reagents have demonstrated low picomolar sensitivity and the capacity to detect multiple targets simultaneously, and illustrate the potential of this technology to provide solutions for applications in public health, clinical diagnostics, and biomedical research.

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Binding Specificity of Multi-Labeled PNA Probes Studied by Single Molecule Mapping

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We evaluated a set of bisPNA probes carrying one or two fluorophores for their binding specificity to dsDNA by single molecule mapping using Direct Linear Analysis (DLA). In DLA, 50 to 250 kb-long DNA molecules are elongated in a continuous flow to their contour length and individually interrogated by laser light excitation of fluorescent tags. When averaged, optical traces of individual molecules present a physical map of PNA probes binding with a spatial resolution of 4 kb (x um). Average traces are directly related to the occupancies of binding sites, both exactly matched to PNA sequence and sites carrying mismatches. This analysis requires around 10^2 molecules to be detected.

Here we report on specificity of multi-labeled PNA binding, defined as occupancy of the match site relative to the occupancy of a site with a single end mismatch, using a 185.1 kb-long bacterial artificial chromosome 12M9 and E. coli genomic DNA digest. We find that the type and position of the fluorophore on bisPNA determine its affinity and sequence specificity. Moreover, relative placement of fluorophores within the probe also affects probe brightness and consequently confidence of its detection in mapping studies. Fluorophore type and position has to be taken into account when developing probes for the whole genome mapping analysis.

125-Pos Board B4

High Resolution Imaging Via SHREC And SHRImP For Ultra-High DNA/RNA Resolution

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Single-molecule high resolution co-localization (SHREC) (Churchman et al., PNAS, 2005) and Single-molecule high-resolution imaging with photobleaching (SHRImP) (Gordon et al., PNAS, 2004) methods have been developed to measure distances between two fluorophores that are closer than Rayleigh limit ($\approx$250 nm for visible excitation). Combining the two techniques adds another dimension to the power of localization methodology and tens of distances could potentially be resolved by using several fluorophores of different colors each having multiple members.To apply this to DNA, we first stretched double-stranded DNA on a Polyacrylic acid and Polyallylamine coated surface, making the DNA relatively straight. To test SHRIMP, we made a DNA construct with a biotin followed by three Cy-3's at positions 475 bp, 172 bp, and 94 bp, corresponding to distances between Cy3 of 32nm, 58nm, and 90nm. We measured distances of 27 nm, 61nm, and 95 nm, in excellent agreement with the expected distances. To test simultaneously SHRIMP and SHREC, we placed Cy5 at position zero, and two Cy3's at position 94 bp and position 172 bp, and measured their positions using a dual-view imaging system. We determined the distances between Cy3-Cy5 pairs to be 37 ± 5 nm (32 nm expected) and 91 ± 5 nm (87 nm expected), and the distance between Cy3-Cy3 pair to be 56 ± 3 nm (58 nm expected). The agreement is excellent. The next step in this project is to study alternative pre-messenger RNA splicing and to quantify individual splicing variants.

126-Pos Board B5

Dynamic Single-molecule Colocalization Imaging - A New Method For Examining Membrane Protein Association In Living Cells

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Knowledge of the association state of cell membrane proteins is vital for understanding key processes such as signalling pathways and immunological response. However, at present there is a paucity of techniques that are able to accurately measure association on a molecule-by-molecule basis in live cells. One method to follow association on live cells is to label and track individual molecules of interest *in situ*. To this end, we have developed Dynamic Single-molecule Colocalization (DySCo), a technique which identifies the correlated movement of labelled proteins in two-colour channels. Proteins of interest are expressed at low levels with either a yellow or red fluorescent protein, and simultaneously imaged under a TIRF configuration. We then track molecules using a recently developed Bayesian approach, which is able to accurately recover tracks at low signal-to-noise ratios. Finally, we examine the inter-track distances between the two colour channels to determine the level of association. After validating the technique using control samples, we then applied it to the association state of T-cell receptors on the surface of living T cells.

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Characterizing The Equilibrium Blinking Behavior Of Fluorogens With Fluorogen Activating Proteins (FAPs)

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We have recently developed FAPs (fluorogen activating proteins) that can specifically activate the fluorogenic dyes thiazole orange (TO) and malachite green (MG) with nanomolar affinities. Upon binding to FAPs, the otherwise dark fluorogens display thousands of fold increase in fluorescence intensity. The reversible interaction between the fluorogen and the FAP allows the same FAP to bind and activate fresh fluorogens in solution after one fluorogen dissociates from the FAP. Therefore, the binding and unbinding reactions lead to on-and-off (blinking) fluorescent signals at the single molecule level. As a result, this

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.

128-Pos Board B7

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.

130-Pos Board B9

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.

132-Pos Board B11

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.