Platform AJ: Emerging Single Molecule Techniques II

1889-Plat
First Realization of Single-Molecule Four-Color FRET
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Single-molecule FRET (Fluorescence Resonance Energy Transfer) has provided unprecedented details on fundamental processes in biology. However, information of single inter-fluorophore distances in conventional two-color FRET is insufficient to completely capture the intrinsic complexity of many biological systems. Despite recent developments of single-molecule three-color FRET techniques, there is an ever increasing demand for more advanced FRET techniques, four-color FRET, as single-molecule approaches are being expanded to include biological systems with multiple components.
Here, we report single-molecule four-color FRET technique both in confocal and in total-internal-reflection fluorescence microscopies. Real-time determination of six inter-fluorophore FRET efficiencies allowed us to probe the correlated motion of four arms of the Holliday junction. The technique was also applied to assess the correlation of RecA-mediated strand exchange events at both ends of a synaptic complex. We expect that the technique will have broad applications in measuring the correlated dynamics of complex biological systems.

1890-Plat
Simplex: Single Molecule Approach to Protein Complexes
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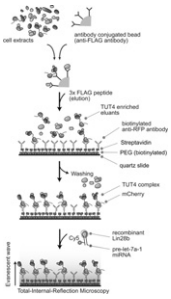
Single molecule approaches were applied to limited systems due to several technical challenges. We report novel technical advance of studying proteins that are directly extracted from human cells. We found that tandem purification (see figure) is sufficient for observing single molecule events without any false positives. We also found that it is essential to have protein expression and extraction procedures optimized in order to have a significant number of single protein complexes observed. It is the first case of utilizing proteins immunopurified, which circumbents the need of purifying proteins from bacterial or other expression systems. Proteins are pulled down from their own cells with natural cofactors bound, which is ideal for studying endogenous functions. We name our technique SIMPlex (SIngle Molecule approach to Protein Complex) anticipating that it will place single molecule approach one step closer to general biology tools. With SIMPlex, we studied the mechanism of TUT4 which cannot be obtained other than with immunoprecipitation. It had been discovered that the maturation pathway of microRNA is suppressed when the polyU polymerase, TUT4, uridylates precursor microRNA. Our direct observation of the elongation process reveals how its cofactor, Lin28, activates TUT4 for microRNA elongation.



1891-Plat
Fast, Simultaneous Multiple Fluorophore Fitting in Single Molecule Super-Resolution Imaging
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Single molecule localization based super-resolution (SML-SR) imaging techniques require repeated localization of many single fluorophores. If, during analysis, successful localization does not require isolated fluorophores, the performance can be improved in one or more of several metrics that result in higher single-frame density of active fluorophores. For example: Data acquisition time can be reduced; A larger number of fluorophores can be localized; There is a higher tolerance on labeling density; and Dyes with higher minimum duty cycle can be used.
We have developed a method that uses the maximum likelihood estimator to localize multiple fluorophores simultaneously within a fitting sub region. We find that for a fitting region of size approximately 6 $\text{sigma}_{\text{PSF}}$ (where $\text{sigma}_{\text{PSF}}$ parameterizes a 2D Gaussian PSF model) localization of up to 5 fluorophores provides a good compromise between fit accuracy and analysis time. For speed, the algorithm is implemented on Graphics Processing Unit (GPU) architecture in a manner similar to our previous single molecule analysis (Smith, Nat. Methods 7, 373-375 2010) and achieves near real-time analysis speed.
We show the performance of multiple fluorophore fitting as a function of (1) maximum allowed number of fitted fluorophores and (2) single-frame active emitter density. We describe the details of the algorithm that allow robust fitting, the details of the GPU implementation, and the other imaging processing steps required for the analysis of (SML-SR) data sets. As a demonstration, we show that our new multi-fluorophore super resolution imaging method reveals actin structure in a HeLa cell under conditions where a high single-frame fluorophore density results in poor reconstructed images using conventional single fluorophore based super resolution imaging techniques.

1892-Plat
Photon-By-Photon Analysis of Single Molecule Fluorescence Trajectories Determines an Upper Bound for the Transition Path Time in Protein Folding
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The transition path (TP) is the tiny fraction of an equilibrium molecular trajectory that connects two states of a system, and contains all mechanistic details of the barrier-crossing process. It is a uniquely single-molecule property, and has not yet been observed for any system. In the case of protein folding, the theoretically-predicted duration of the TP is so short (~1 microsecond) that measurement of just the average TP time is challenging for single molecule experiments. We previously estimated an upper bound for the TP time of ~200 microsecond for folding of the two-state, protein G using single molecule Förster-resonance-energy-transfer (FRET) spectroscopy. This time is 10,000 times shorter than the average waiting time in the unfolded state of ~2 seconds (the reciprocal of the rate coefficient). However, there is still a large gap between this experimental value and theoretical predictions. The biggest obstacle to measuring the TP time is that there is an insufficient number of photons emitted during a single TP. To address this problem, we have used a fully-automated data acquisition system to measure a very large number (46,932) of photon trajectories for protein G at high illumination intensities, and carried out a collective photon-by-photon analysis on 151 transitions between folded and unfolded states (the average trajectory lifetime prior to bleaching is ~10 ms). The newly developed method rigorously compares the likelihoods of models with instantaneous and finite TP times to yield an upper bound of ~10 microseconds, significantly narrowing the gap with the theoretically predicted value. The result strongly suggests that protein G folds slowly because of a high ***free-energy*** barrier, and not due to slow diffusion over the barrier caused by a ''rough'' underlying ***energy*** landscape.

1893-Plat
New Applications of Pulsed Interleaved Excitation
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Several years ago, the group of Simon Weiss introduced the concept of alternating laser excitation, which we combined with subnanosecond pulsed lasers in the method of pulsed interleaved excitation (PIE). In PIE, it is possible to determine which excitation pulse is responsible for generating the detected photon. With this additional piece of information, one can, for example, separate donor-only and acceptor-only species from double-labeled species in single-pair Förster Resonance Energy Transfer (spFRET) experiments, remove spectral cross-talk in fluorescence cross-correlation spectroscopy (FCCS) or use it to determine FRET efficiencies from an FCCS experiment.
We have now combined PIE with scanning fluorescence correlation spectroscopy (FCS) and with raster image correlation spectroscopy (RICS). Both scanning FCS and RICS are preferable for fluctuation measurements in living cells where diffusion is slowed and photobleaching is an issue. By scanning, several volumes can be measured in parallel, improving statistics and minimizing photobleaching. However, spectral crosstalk can still be a limiting factor for these experiments, especially when using autofluorescent fusion proteins. Here, we demonstrate the ability of PIE to remove spectral crosstalk for these scanning methods, making scanning FCCS and cross-correlation RICS more sensitive to interactions.
It is also possible to combine PIE with multiparameter fluorescence detection (MFD) to perform highly accurate spFRET experiments. When performing spFRET experiments, there are many factors that can limit the accuracy of the experiment. These include correction for spectral crosstalk, direct excitation of the acceptor, photobleaching during a burst, and uncertainties in the orientation factor. To correct for all these issues, often multiple experiments are performed. We demonstrate that, using the capability of PIE and MFD to

separate different species, all parameters necessary for performing an accurate spFRET experiment can be obtained in a single measurement.

1894-Plat
Single-Molecule Counting with Palm
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Photoactivated Localization Microscopy (PALM) is a single-molecule based imaging technique. By repeated activation and sampling of sparse subsets, densely expressed fluorescent proteins can be resolved in time. Here, we describe how to exploit this property for single-molecule counting in cells. A strategy is developed for differentially activating and optimized imaging of PA-GFP and PA-Cherry. Coupled PA-FPs are used as calibration probes for standardization of single-molecule counting and determination of photoactivation efficiency. With these tools, we demonstrate how single-molecule counting enables to assess stoichiometries and to relate such counts to absolute molecule numbers in a biological specimen.

1895-Plat
Microsecond Single Molecule Tracking: Probing Protein Diffusion at High Spatial and Temporal Resolution
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In recent years advancement in light microscopy have made possible to break the resolution limit and to achieve super- resolution positional accuracy. However, these techniques are limited in their temporal resolution to tens of millisecond, and thus cannot access the faster dynamics relevant for most biological processes. Here we present a versatile technique which allows to track a molecule with nanometer spatial and microsecond temporal accuracy. The method is based on partially overlapping excitation of two spectrally separated fluorophores. A wedge is introduced in the setup so the fluorescent signals from the two emitting species are recorded independently onto different area of the CCD. In this way we record the position of a single molecule with nanometer accuracy at two different points in time. A cross-correlation algorithm is subsequently applied and information on the mean square displacement of the molecule is retrieved. The temporal accuracy is ultimately limited by the diffusion coefficient of the molecule under study, and the accuracy by which the position can be determined. This method was proved using short DNA oligonucleotides and here applied to live cells. We followed the diffusion of GPI anchor protein dually labeled with a refolded split GPF and an Alexa 647 dye attached to the complementary peptide. This approach guarantees the 1:1 stochiometry needed and we were able to track GPI down to microseconds time-resolution, gathering information on the diffusion properties of the anchor in the membrane.
Our results did not exhibit hop-diffusion behavior at short time-scale as previously predicted.

1896-Plat
Nano-Scale Spatial Organization of Plasma Membrane Revealed by Pair-Correlation Analysis
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TIRF-based photoactivated localization microscopy (PALM) offers a powerful tool to interrogate the spatial distribution of proteins in the plasma membrane (PM) at scales inaccessible by conventional light microscopy. We employed PALM to map out the nanoscopic organization of PM and construct a mechanistic perspective of PM organization. Cluster analysis of PALM data is complicated by the multiple appearances of a single protein with associated stochastic uncertainty in position, and reversible blinking of individual fluorophores. We developed a statistical algorithm to rigorously analyze PALM-data by performing spatial pair auto- and cross-correlation on the entire ensemble of detected molecular peaks, and subsequently separating out the contribution resulting from multiple appearances of the same molecule from that arising due to the actual lateral organization of the protein molecules. Using this approach, we could describe protein organization in an accurate and quantitative way, and extract reliable physical parameters like cluster-radius and number of molecules in a cluster. We examined the distribution of a diverse set of PM-proteins carefully chosen based on their different membrane-anchors and differential partitioning in phase-separated PM vesicles. Correlation analysis of PALM data revealed distinct steady state organization of these proteins: Transferrin Receptor-PAGFP and VSVG-PAGFP were organized into discrete clusters of ~150nm and 70nm radius, respectively. PAGFP-GL-GPI, Lyn-PAGFP and Lat-PAGFP, in contrast, exhibited random distribution. Interestingly, cross-correlation of 2-color PALM images showed dramatic reorganization of actin into ring-like structures around antibody-crosslinked PAGFP-GPI clusters (~100-200nm); similar actin-structures can modulate protein-platforms during cell-signaling. The combination of PALM and correlation analysis provides a robust and quantitative approach to study PM reorganization during various physiological processes.

Platform AK: Membrane Active Peptides

1897-Plat
Probing Structural Features of Alzheimer's β-Amyloid Ion Channels in Membranes Using Aβ Mutants
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A current hypothesis for the pathology of Alzheimer's disease (AD) proposes that amyloid-beta (Aβ) peptides induce uncontrolled, neurotoxic ion flux across cellular membranes. The resulting inability of neurons to regulate their intracellular concentration of ions, in particular calcium ions, has been associated with cell death and may thus contribute to cognitive impairment typical for AD. The mechanism of the ion flux is not fully understood since no experimentally based Aβ channel structures at atomic resolution are currently available, and few polymorphisms have been predicted by computational models. Structural models and experimental evidence suggest that Aβ channel is an assembly of loosely-associated mobile β-sheet subunits. Histidines were proven to be on or near the mouth of the Aβ pore, but no other amino acids have been tested. Using planar lipid bilayers, we present a study showing that amino acidic substitutions can be used to infer which residues line the pore and are water accessible. For example, the substitution of F19P is capable of undermining the amyloid structure such that bilayer membranes exposed to it do not support ion channel formations for prolonged periods of time. This and other structural information on or in membrane are needed to aid drug design aiming to control unregulated Aβ ion fluxes.
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1898-Plat
Evolution of Membrane Leakage by Pre-Amyloidogenic Oligomers of Islet Amyloid Polypeptide
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Islet amyloid polypeptide (IAPP) is the primary amyloid component found in patients suffering from type II diabetes. IAPP has been implicated in the death of pancreatic beta-cells, resulting in a greater strain on the remaining insulin producing cells and contributing to further progression of the disease. While fibers are the most obvious characteristic of any amyloid disease, it has been found that smaller soluble oligomers are the most likely source of cytotoxicity. Previous research has shown that IAPP oligomers are capable of permeabilizing lipid bilayers under physiological conditions. Permeabilization of cellular membranes resulting in ionic imbalance and subsequent apoptosis is a leading theory for the cause of beta-cell death in type II diabetes. In order to better understand the mechanism by which IAPP causes cell death we have examined the evolution of membrane-bound leakage states over time. Using a combination of bulk and single molecule techniques we have uncovered a multi-phasic leakage mechanism in which we observe two clear time regimes of leakage behavior induced by soluble pre-amyloidogenic oligomers. Our results give us further insight into the mechanism by which IAPP is capable of inducing membrane leakage.

1899-Plat
***In Situ* Mapping of Membranolytic Peptide-Membrane Interactions by Coupled Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy-Atomic Force Microscopy (ATR-FTIR-AFM)**
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The mechanisms by which membranolytic peptides may exert their action are highly complex and specific. They may involve peptide self-association, membrane-induced refolding, or targeting of specific lipid domains or components. Insights into these behaviours are critical for the *de novo* design of peptide-based antimicrobial agents. To better understand these mechanisms, we have applied coupled attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR)-atomic force microscopy (AFM) to directly