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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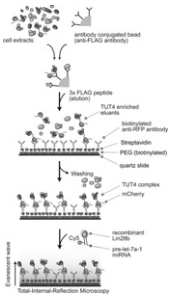
Single molecule approaches were applied to limited systems due to several technical challenges. We report novel technical advance of studying proteins that are directly extracted from human cells. We found that tandem purification (see figure) is sufficient for observing single molecule events without 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 sigma$_{PSF}$ (where sigma$_{PSF}$ 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