a simple construction and fabrication, and robust operation. Together, these features have the potential to make it useful for a broad variety of single-molecule fluorescence experiments.

3238-Plat
Single Plane Illumination Microscopy Allows Fluorescence Correlation Spectroscopy (SPIM-FCS) to be used for Concentration and Diffusion Coefficient Imaging in 3d
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Fluorescence Correlation Spectroscopy (FCS) is a widely used tool for the investigation of molecular dynamics in vitro and in vivo. However, it is mostly used in a confocal configuration, which renders multiplexing difficult. Recently, camera based approaches have been used to measure a large number of correlation functions in parallel. Here, we introduce single plane illumination microscopy (SPIM) as an illumination mechanism for FCS. An illumination objective creates a light sheet in the focal plane of a detection objective, which excites a volume that is comparable to the focal volume of the detection system. The image is collected with a fast EMCCD camera. The signal from every pixel is analysed by calculating a correlation function thus extracting concentrations and diffusion coefficients. Apart from the fact that SPIM illuminates only that fraction of a sample, which is actually measured and thus provides true optical sectioning, it reduces photobleaching compared to confocal setups limiting optical damage to the sample and allowing longer measurement times. We use a laser power in front of the objective of 60 μW and record at least a 32×32 pixel area of an EMCCD. This corresponds to a reduction in laser power delivered to the specimen by at least 3 orders of magnitude compared to confocal FCS. In this work, we demonstrate the use of different combinations of low numerical aperture (NA) illumination objectives for the creation of light sheets with high NA detection objectives for SPIM-FCS. By using microsphere solutions we were able to distinguish the diffusion coefficient of different sized particles and demonstrated that FCS images with 1024 pixels up to 4096 pixels show a clear contrast in concentration and diffusion coefficients in non-homogeneous samples.

3239-Plat
High Throughput Single-Molecule Spectroscopy with Highly Parallel Excitation and Detection
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Single-molecule spectroscopy is a set of powerful techniques for detailed analysis of molecular interactions and motion, but due to the requirement of low concentrations, long acquisition times are required to achieve sufficient statistics. Newly developed detectors permit acquiring data in parallel, resulting in faster acquisition and giving access to shorter time scales for the observation of dynamic phenomena. We present several new single-photon counting detectors and acquisition systems capable of high throughput single-molecule spectroscopy including multi-pixel CMOS detectors and a multi-pixel hybrid photodetector. To take advantage of these detectors, we developed a novel approach for multi-spot excitation utilizing a liquid crystal on silicon spatial light modulator (LCOS), which allows dynamic excitation spot generation corresponding to the detector geometry. We also developed a high throughput field programmable gate array (FPGA)-based parallel acquisition system. We present examples of these approaches for single-molecule applications such as Fluorescence Correlation Spectroscopy (FCS), and demonstrate the feasibility for high throughput single-molecule Förster resonance energy transfer (smFRET) measurements. We also discuss the ways in which our approaches permit measurements of faster dynamic processes. These high throughput developments will significantly expand the power of single-molecule spectroscopy for biophysical and other applications.

3240-Plat
Measuring Multiple Distances within a Single Molecule using Switchable FRET
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Single molecule Förster Resonance Energy Transfer (smFRET) is a powerful method that serves as a nanometer-scale ruler to probe structure and conformational dynamics of biomolecules. In most cases, smFRET measurements utilize a single donor-acceptor pair reporting on a single distance. The ability to monitor FRET between multiple donor-acceptor pairs within a single molecule (multi-pair smFRET) will provide new fundamental insight into the three-dimensional structure, dynamics and interactions of biomolecules and benefit many fields such as structural biology and biosensing. Although multi-pair smFRET approaches have been reported, they are complicated, non-general, and difficult to extend.
Here, we present a novel, flexible, and general multi-pair smFRET method that uses photoswitchable fluorophores as FRET-acceptors. It allows the measurement of multiple distances on an individual molecule such as a protein, protein-DNA complex, or multi-protein complex. Our proof-of-principle experiments were performed on double-stranded DNA fragments labeled with a non-switchable donor and multiple photoswitchable acceptors. Using alternating-laser excitation schemes and stochastic photoswitching of acceptors, we demonstrate the measurement of multiple distances within an individual DNA molecule (as opposed to within a population of single molecules). Our results are complemented and supported by simulations of the photoswitching process and the associated single-molecule fluorescence observables. Moreover, by employing a step-finding algorithm, we achieve high-resolution FRET measurements within an individual molecule. Finally, we show that different mechanisms for photoswitching can be used to the same end, supporting the generality of the concept.

3241-Plat
Colloidal Lenses Enable High Temperature Single Molecule Imaging and Improve Fluorophore Photostability
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Although single molecule fluorescence spectroscopy was first demonstrated at near-absolute zero temperatures (1.8 K), the field has since advanced to include room temperature observations largely due to the use of high numerical aperture objective lenses, brighter fluorophores, and more sensitive detectors. This has opened the door for many chemical and biological systems to be studied at native temperatures at the single molecule level both in vitro and in vivo. However, systems and phenomena that operate at temperatures above 37 °C remain difficult to study at the single molecule level due to the need for index matching fluids with high numerical aperture (NA) objective lenses. These fluids act as a thermal conductor between the sample and the objective and sustained exposure to high temperature can cause the objective to fail. This has prevented the single molecule study of thermophilic organisms, the interactions of their protein repertoire, and the temperature-dependent unfolding kinetics of nucleic acids and proteins. Here we report that high index of refraction micron-sized colloidal lenses are capable of achieving single molecule imaging at 70 °C by incorporating a focusing element in immediate proximity to an emitting molecule; the optical system is completed by a low numerical aperture optic which can have a long working distance and an air interface. TiO2 colloidal lenses were used for parallel imaging of surface-immobilized single fluorophores and to measure real-time single molecule mesophilic and thermophilic DNA polymerase strand displacement replication through an immobilized template at 23 °C and 70 °C, respectively. Fluorophores in close proximity to TiO2 also exhibited a ~40% increase in photostability due to a reduction of the excited-state lifetime.

3242-Plat
Synthesis of Extended Single-Molecule Optical Encoders
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We have designed single-molecule FRET encoders which convert relative motion between two individual biomolecules to a periodic signal. Fundamental signal frequencies obtained with individual DnaB helicase molecules imply unwinding velocities ranging from 200 to 1100 base pairs per second, while low-frequency modulation of peak heights may suggest azimuthal rotation of the helicase. Signal durations show that a single helicase is capable of unwinding many hundreds of base pairs before dissociating. The initial scheme chosen for FRET encoder synthesis was expensive and limited to 5 periods, restricting the duration of the collected signal. We have utilized polymerization-driven self-assembly and rolling circle replication to synthesize inexpensive, extended FRET encoders as long as 150 periods.