nanodroplets suspended in a low index-of-refraction fluorocarbon medium. The index of refraction mismatch between the nanodroplets and fluorocarbon is such that individual hydrosomes can be optically trapped. Using optical tweezers, the hydrosomes are held within a confocal observation volume, and we interrogate the encapsulated molecule by means of fluorescence excitation. Hydrosome encapsulation has an important advantage over liposome encapsulation techniques in that hydrosomes fuse on contact, thereby mixing the encapsulated components. Optical tweezers are used to manipulate the hydrosomes and to induce a fusion event. Custom fabricated microfluidic channels are used to sort the hydrosomes containing different molecule species. We demonstrate the use of hydrosomes as microreactors by fusing two hydrosomes, each containing a complementary single strand of DNA, and observing the subsequent hybridization via FRET (Fluorescence Resonance Energy Transfer).

3042-Pos

Using PNA and LNA as Handles for Tethering Single DNA Molecules

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Single-molecule manipulation (SMM) instruments, such as the optical trap or magnetic tweezer, require a means to immobilize the studied biomolecule on a solid substrate. In the case of DNA, this is accomplished by adding moieties, e.g. biotin, using enzymatic labeling; however, this strategy introduces extra steps in the processing of the sample, and makes direct application of SMM to DNA/protein interactions and chromatin structure difficult. Here, we develop a novel tethering strategy based on the properties of the nucleic acid analogs (NAAs) peptide nucleic acid (PNA) and locked nucleic acid (LNA). PNA and LNA are known to stably bind to double-stranded DNA in a sequence-specific manner, either through triplex formation or strand-invasion. Using a magnetic tweezer, we explore the ability of biotinylated NAAs to immobilize a DNA molecule in a sequence-specific fashion, and to remain bound under applied force. Our results indicate that both LNA and PNA can tether a DNA molecule and withstand mechanical force, but that PNA suffers from non-specific binding, particularly to DNA extremities. We discuss rules for the optimal design of NAA probes for single-molecule experiments.

3043-Pos

Precision Force Spectroscopy of Bacteriorhodopsin

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Single-molecule force spectroscopy studies have produced rich insights into the unfolding of individual proteins and nucleic acid structures. In a typical force spectroscopy experiment, an AFM tip is coupled to a surface-adsorbed protein by pressing the tip into it. Force-extension curves are then generated by retracting the tip at a constant velocity using a piezoelectric (PZT) stage. Force is measured by cantilever deflection. Extension, or more precisely tip-sample separation, is deduced from the PZT stage position used to control the vertical tip position. Thus, this deduced extension is sensitive to the vertical mechanical drift of the AFM assembly (~10 nm/min). We have previously developed an ultrastable AFM in which the tip and the sample positions are independently measured by, and stabilized with respect to, a pair of laser foci in three dimensions. These lasers establish a local reference frame that is insensitive to long-term mechanical drift of the AFM assembly. This new measurement of position is complementary to the cantilever deflection sensing, which measures force. We have now extended the ultrastable AFM capabilities into liquid and can routinely mechanically unfold proteins at slow pulling velocities (2 nm/s), which allows averaging to increase precision. We can also stop pulling altogether and hold the molecule at constant force while independently measuring tip-sample separation ($\sigma = 0.2$ nm, $\Delta f =$ 1-50 Hz). Alternatively, we can stabilize tip-sample separation and measure force ($\sigma = 5$ pN, $\Delta f =$ 1-50 Hz) over 100s of seconds . Using these techniques, we are studying the unfolding and re-folding of bacteriorhodopsin (BR), a model transmembrane protein.

3044-Pos

Fast Millisecond Imaging of Single Fluorescent Protein Molecules Using a Simple "Slimfield" Optical Trick

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Fluorescence microscopy offers a non-invasive probe for biological processes *in vivo*, but there are limitations in sensitivity for commonly used fluorescent proteins typically utilized as genetically encoded fusion constructs as molecular reporters. Here we present a simple optical trick bundled with some relatively straightforward custom-written analysis software which allows quantification of single fluorescent protein molecules over a rapid ~millisecond time scale. This optical trick has been used previously to image bright organic dyes *in vitro* and a similar approach can be used to change confocal volume sizes for use in fluorescence correlation spectroscopy, but never to image single photophysically poor fluorescent proteins in living cells. We have called the illumination mode which results ''slimfield''. Slimfield is cheap and simple and can be implemented on existing commercial microscope systems with relatively little modification. It permits excitation intensities ~100 times greater than those of widefield imaging, facilitating single-molecule detection at high speed. We demonstrate it using many different purified fluorescent proteins in common laboratory use. Controlled *in vitro* experiments indicate single protein molecules over a field of view 30 microns^2 area, large enough to encapsulate single bacterial and yeast cells. Using our custom software we can automate detection and quantification of single molecules using true 2D imaging at ~500 frames per second with a localization precision for these photophysically poor dyes of typically a few tens of nm. We show that you can image the dim enhanced cyan fluoresecent protein (ECFP) and CyPet at a single-molecule level. Simple modifications then allowed us to perform simultaneous dual-color slimfield imaging for use in co-localization and FRET. We then report some preliminary *in vivo* data using bacteria and show ~millisecond time scale functional imaging at a single-molecule level with negligible photodamage.

3045-Pos

Zero Mode Waveguides: a Powerful Tool for Single Molecule Optical Studies

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Single-molecule fluorescence studies of enzymes that incorporate fluorescently labeled substrate nucleotides typically operate at substrate concentrations well below their K_m values. While this is inevitable in conventional fluorescence microscopy, the biological relevance of the insights gained into enzyme mechanism may be compromised. Zero-mode waveguides (ZMWs) provide an excellent solution to this problem by greatly reducing the observation volume. We report the nanofabrication of ZMWs, the surface treatment for controlled immobilization of biomolecules and the reduction of background noise. We also present the development of an assay to monitor in real time the incorporation of fluorescently-labeled nucleotides, which paves the way for the studies of nucleic acid polymerizing enzymes, e.g. DNA/RNA polymerase, reverse transcriptase, telomerase, etc.

3046-Pos

Comparison of Three-Dimensional Imaging Configurations for High Resolution Microscopy Measurements

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In recent years, a class of fluorescence microscopy imaging techniques has emerged which enables the imaging of single fluorophores at high resolution by reducing the problem of resolution to one of localization. The photoactivated localization microscopy (PALM) technique, for example, constructs finely resolved images by way of accurately localizing closely spaced fluorophores that are detected separately in time by successively photoactivating small and stochastically different subsets of fluorophores.

Due to the optical microscope's poor depth discrimination capability, the resolution of three-dimensional (3D) versions of techniques like PALM is limited by the z-localization accuracy of a single fluorophore, which can be especially poor when a fluorophore is near-focus. An imaging technique that overcomes the near-focus problem is multifocal plane microscopy (MUM) (Prabhat, P. et. al., IEEE Trans. Nanobiosci., 2004), which allows the simultaneous imaging of a fluorophore from distinct focal planes. Images from multiple focal planes enable MUM to accurately localize a near-focus fluorophore (Ram, S. et. al., Proc. SPIE, 64430D1, 2007) and to support high accuracy 3D localization over a wide depth range.

Here we compare 3D fluorescence imaging configurations which employ different combinations of conventional excitation, PALM excitation, conventional emission, and MUM emission. Using a Cramer-Rao lower bound-based 3D resolution measure (Chao, J. et. al., Opt. Commun., 2009), comparisons are made in terms of the accuracy with which the distance separating two closely spaced fluorophores can be estimated. Such distance information can be important as it can help to characterize the interaction between two biomolecules. Our results show that configurations incorporating PALM excitation provide superior distance estimation accuracies for fluorophore pairs characterized by small distances of separation and orientations near parallel to the optical axis. Meanwhile, configurations incorporating MUM emission provide the best accuracies for near-focus fluorophore pairs.

3047-Pos

Investigating Structural Heterogeneity in Dimers of Amyloid-β Peptide

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Recent evidence suggests that soluble amyloid-β (Aβ) oligomers as small as dimers may be linked to the progression of Alzheimer's disease. We have used single-pair FRET measurements to investigate heterogeneity in surface-tethered dimers of Aβ40, probing for preferred structures. Dimers are prepared by combining monomers singly labeled with donor and acceptor dyes; dimers prepared in solution (prior to surface-tethering) and on the functionalized surface have been examined. Donor and acceptor fluorescence are separated onto two detectors, such that co-localized spots in two-color images are indicative of at least two associated peptides. Dimers are verified based on the observation of single-step photobleaching in each detection channel; larger oligomers are excluded from analysis. By measuring donor and acceptor fluorescence as a function of time, we have determined time-dependent FRET efficiencies for dozens of individual dimers, permitting insight into inter-dye distances and dimer structures. These results are further complemented by comparison to published structures of simulated Aβ40 dimers. Together, experiment and simulation may reveal a subset of preferred structures for Aβ40 dimers.

3048-Pos

High Density Single Particle Tracking Using Bayesian Multi-Fluorophore Fitting through Vertices

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Single particle tracking (SPT) has become a common technique for studying protein and lipid behavior in cell membranes. Using photo-stable fluorescent probes such as quantum dots, SPT is now being applied using multi-color strategies in order to look at nano-scale protein-protein interactions. However, the sparse labeling of each spectral species needed to limit the occurrence of fluorophores overlapping within the diffraction limit reduces the probability of overlap from opposing spectral species and the frequency of observed interactions.

We present a single particle tracking method that allows a higher density of labels by performing a Bayesian multi-fluorophore fit to particles that are spaced closer than the diffraction limit. We call a single event of overlapping trajectories a 'vertex.' We use knowledge of the particles' intensities, diffusion constants positions before and after the vertex, and possibly blinking rates to improve the multi-fluorophore fits through the vertex. We examine the accuracy of the trajectories and fits as a function of label density. Furthermore, we show that under a range of frame and blinking rates, fluorescence intermittency from blinking probes can improve the accuracy of multi-fluorophore fits. We show that single particle and Bayesian multi-particle fits can be performed at more than 10^5 fits per second using an iterative fitting method implemented on GPU architecture.

3049-Pos

Dynamical Observations of Brownian Motions on Single Protein Molecules Using Diffracted Electron Tracking (DET)

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In order to improve both monitoring super-precisions of conformational changes and stability of the dynamical signal intensity from single molecular units under in vitro physiological conditions, we have proposed new single molecular techniques using shorten wavelength, for example, X-rays, electrons, and neutron. Diffracted X-Ray tracking (DXT) has been developed for obtaining the information about the dynamics of single molecules. This method can observe the rotating motion of an individual nanocrystal, which is linked to specific sites in single protein molecules, using a time-resolved Laue diffraction technique. However, this method needs a very strong X-ray source, so we began to develop a compact instrument for monitoring the motions of the single protein molecules, using the electron beam instead of the X-ray. In this work, we demonstrated three-dimensional tracking of single nanocrystals labeled with individual single molecular units using Scanning Electron Microscope (SEM). We called Diffracted Electron Tracking (DET). Instead of the Laue diffraction using white X-ray, the Electron Back-Scattered Diffraction Pattern (EBSP) in SEM is adopted to monitor the crystal orientation of the nanocrystals linked to the single protein molecules.

We used SEM (JSM-7000F TYPE A, JEOL) to monitor EBSP from the labeled gold nanocrystals (Diameter size= 30-60nm) in the thin aqueous solution (thickness is about 100nm). We observed dynamical EBSP during 3s in each integrated time of 30ms. We determined three Euler angles from EBSP mapping of the observed gold nanocrystals. Thus, we observed three-dimensional Brownian motions of the labeled gold nanocrystal. Additionally, we checked the relationship between the sizes of the labeled nanocrystals and Brownian motions from dynamical EBSP data.

3050-Pos

Single-Image Measurements of Monochromatic Subdiffraction Dimolecular Separations

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One of the current challenges in single-molecule imaging is the dynamic separation measurement between two subdiffraction-separated identical fluorophores. The combined intensity profile of two subdiffraction separated fluorophores can be approximated by a 2D Gaussian function, and we have developed a simple method to measures the dimolecular separation using this combined Gaussian intensity profile. By measuring the standard deviation (SD) of the convolved Gaussian image we show that we can (1) differentiate dimers from monomers and (2) measure the dimolecular separation with a known precision depending on the number of detected photons, all using a single image of milliseconds exposure time. We have constructed diagrams showing (a) the number of photons required to differentiate dimers from monomers, (b) dimer SD vs. separation, and (c) the precision in the measured dimer separation. This study demonstrates a simple method that allows dynamic measurements of monochromatic subdiffraction dimolecular separations. For example, a single image of 10,000 collected photons in 100 ms can determine the dimolecular separation down to 70 nm with a precision of 20 nm.

3051-Pos

Single Molecule Studies of Protein Surface Adsorption Kinetics

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The fact that most protein-based biotechnological and medical procedures involve protein-surface interactions demands that the protein-surface adsorption process be studied in greater detail to prevent undesirable nonspecific adsorption of proteins to surfaces. To solve this problem, the mechanisms responsible for protein adsorption to surfaces must be identified and quantified. Unlike conventional bulk measurements where adsorbed proteins of one kind cannot be differentiated from another, single-molecule imaging studies can identify the adsorption mechanisms using real-time imaging of the adsorption process. Here we report on Total Internal Reflection Fluorescence (TIRF) Microscopy imaging of single Streptavidin-Cy3 molecules interacting with hydrophobic fused-silica surfaces. The results reveal the different mechanisms responsible for protein-surface adsorption, and their kinetics. We have observed reversible and irreversible adsorptions due to the intrinsic interaction of proteins with surfaces at the water-surface interface, and irreversible adsorptions due to the protein deposition process at the air-surface interface. We will discuss the extent of contribution to total surface-protein adsorption for each adsorption mechanism.

3052-Pos

De-Noising Single Molecule FRET Trajectories using Wavelent and Bayesian Techniques

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Extracting quantitative information from low signal-to-noise single molecule FRET trajectories remains a significant challenge. In particular, biological systems that derive their function from shallowly defined or continua of states are not amenable to recent Markovian analysis algorithms. A method to de-noise single-molecule fluorescence resonance energy (smFRET) trajectories using wavelet detail thresholding and Bayesian inference is presented. Bayesian methods are developed to identify fluorophore photoblinks in the time trajectories. Simulated data are used to quantify the improvement in static and dynamic data analysis. Application of the method to experimental smFRET data shows that it distinguishes photoblinks from large shifts in smFRET efficiency while maintaining the important advantage of an unbiased approach. Known sources of experimental noise are examined and quantified as a means to remove their contributions via soft thresholding of wavelet coefficients. A wavelet decomposition algorithm is described, and thresholds are produced through the knowledge of noise parameters in the discrete-time photon signals. Reconstruction of the signals from thresholded coefficients produces signals that contain noise arising only from unquantifiable parameters. The method is applied to simulated and observed smFRET data, and it is found that the denoised data retain their underlying dynamical properties, but with increased resolution.