946-Pos

Superresolution Imaging Using Fluorogen Activating Proteins by Sted Nanoscopy and Equilibrium Localization Microscopy

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Our center has recently developed the Fluorescence Activating Protein (FAP) technology for genetically targeted fluorescent labeling of proteins in live cells. FAPs are single chain antibodies that can specifically activate the fluorogenic dyes thiazole orange (TO) and malachite green (MG) with nanomolar affinities. When bound to FAPs, the otherwise dark fluorogens generate thousands of fold increase in fluoresce intensity. The fact that FAPs are small in size (12 to 25 kDa) and genetically encoded makes them an ideal fluorescence tag for live cell imaging. Since no appreciable background from the fluorogen alone is detectable, no washing steps are required to remove the excess fluorogen. Moreover, different FAP-fluorogen combinations can result in spectrally resolvable probes for multi-color imaging. We have demonstrated the utility of these probes for both ensemble and single molecule based superresolution methodologies.

Because malachite green has a similar quantum yield and spectral characteristic to the STED-efficient dye Atto647N, we tested the feasibility of using MG-FAPs in STED imaging. We show that STED imaging could be performed on both live and fixed and permeabilized cells expressing MG-FAP. When an engineered, cytosolic expressible MG FAP H6.2-MG was fused to the N-terminus of actin, actin filaments with FWHM of 110-122nm were observed, an approximately 3-fold resolution improvement compared to a confocal image. The reversible interaction between the fluorogen and the FAP allows the same FAP to bind and activate fresh fluorogen from solution after one fluorogen dissociates from the FAP. Consequently, multiple binding and unbinding cycles result in a characteristic intermittency, leading to a high photon flux and photobleaching-resistant system. Using low concentrations of the fluorogen gives rise to sparse labeling of objects for localization microscopy with a localization accuracy of <10 nm.

947-Pos

The Use of Quantum Dot Blinking to Optimize of 3D Nanoscopy

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Fluorescence microscopy is a widely used tool in many molecular and cellular biologically research, as it enables the observation of specific components or processes in living cells, tissues, and whole organisms. However, the limited resolution of fluorescence microscopy leaves many biological structures too small to be studied in detail. Although subcellular structures often ranges as small as nanometers, most optical microscopes have lateral and axial resolutions of ~ 200 nm direction and ~ 500 nm respectively. Currently, novel imaging methods, stochastic optical reconstruction microscopy (STORM), have broken the diffraction limit resulting in significant improvements in resolution by switching a fluorescent molecule ON (bright) or OFF (dark). Most recently, STORM has spread the observed area from two (XY) to three (XYZ) dimensions by applying cylindrical optics (3D-STORM). However, they are constrained by the specific optics or fluorescent probes.

To simplify the 3D-STORM method, we optimized both of the optics and the probe. The Z position of the fluorophore was represented to be ellipticity of point spread function of it by setting cylindrical lens after imaging lens. This ellipticity dependence was responsible for the focusing length of the cylindrical lens. We optimized the ellipticity dependency by verifying a distance between two cylindrical lenses (concave and convex). The appropriate distance was 10 mm and the 3D resolutions of the position determination of fluorophore are 10 nm (XY) and 40 nm (Z) when the fluorophore emitted 1000 photons. Next, we applied quantum dot (Qdot) to be a 3D-STROM because Qdot has intense and stable fluorescence, and especially blinks stochastically. Since the fluorophore rarely emits fluorescence in STORM method, we improved the Qdot whose ON events were rare. In this meeting, we will discuss our method, which is performed with Qdot , in detail.

Emerging Single Molecule Techniques I

948-Pos

Rotating Magnetic Particles Probe: A New Technique to Measure Interactions Between Protein-Coated Particles and a Substrate

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We demonstrate a new rotating magnetic particles probe technique to measure the interactions between protein-coated magnetic particles and a substrate. The rotational behavior of particles is quantified in a rotating magnetic field and allows the study of association kinetics. By using multiple particles in parallel, good statistics are obtained in a single measurement.

We describe the rotating particles probing technique and its application to a study of binding between streptavidin-coated particles and a glass substrate, as a function of ionic strength, pH and protein blocking. An increase of binding is observed for increasing ionic strength and decreasing pH. The results are in agreement with calculations of the electrostatic interaction between a spherical particle and a wall. For low ionic strength, the particles stay at a finite distance from the substrate due to the electrostatic repulsion between the negatively charged particles and the negatively charged glass substrate. For increasing ionic strength, the electrostatic repulsion is shielded and the particles come in contact with the substrate, generating non-specific binding between streptavidin and glass. When the pH is decreased below the pI of the particles, the particles become positively charged, resulting in high binding due to the negative charge on the glass. With the same technique we have quantified the reduction of binding by protein blocking of the surface. Our results show the feasibility of the rotating particles probing technique to study biomolecular interactions, which opens further applications such as the characterisation of ligand-receptor binding and torsion stiffness of biomolecular complexes.

949-Pos

On-Chip Single Molecule Detection of Unlabeled DNA Targets

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Ultrasensitive biological sensors for low-abundant DNA and protein detection have emerged as an important tool for improving biomedical diagnostics, drug discovery and advanced bioanalytical assays in fundamental research. We report on a single-molecule readout scheme based on total internal reflection fluorescence microscopy (TIRFM) demonstrating a detection limit in the low fM regime for short (30 mer) unlabeled DNA strands[1]. Detection of target-DNA is accomplished by mediating the binding of suspended fluorescently labeled DNA-modified small unilamellar vesicles (Ø~100) to a DNA-modified substrate by unlabeled complementary single-stranded DNA. On top of rapid and sensitive detection, the technique is also shown capable of extracting kinetic data from statistics of the residence time of the binding reaction in equilibrium, i.e. without following neither the rate of binding upon injection nor release upon rinsing. The potential of this feature is demonstrated by discriminating a single mismatch from a fully complementary 30-mer DNA target[2], an important capability for single nucleotide polymorphism (SNP) diagnostics. Furthermore, means of using lipid vesicles as barcodes, utilizing the potential of time-of-flight secondary ion mass spectrometry (TOF-SIMS) to discriminate different lipid compositions, will be disclosed.

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[2] Gunnarsson, A. et al. Nucleic Acids Res. 2009 37(14):e99

950-Pos

Integrating a High-Force Optical Trap with Gold Nanoposts and a Robust Gold-DNA Bond

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Gold-thiol chemistry is widely used in nanotechnology but has not been exploited in optical-trapping experiments due to laser-induced ablation of gold. We circumvented this problem by using an array of gold nanoposts ($r = 50$-250 nm, $h \sim 20$ nm) that allowed for quantitative optical-trapping assays without direct irradiation of the gold. DNA was covalently attached to the gold via dithiol phosphoramidite (DTPA). By using three DTPAs, the gold-DNA bond was not cleaved in the presence of excess thiolated compounds. This chemical robustness allowed us to reduce nonspecific sticking by passivating the unreacted gold with methoxy-(polyethylene glycol)-thiol. We routinely achieved single beads anchored to the nanoposts by single DNA molecules. We measured DNA's elasticity and its overstretching transition, demonstrating moderate- and high-force optical-trapping assays using gold-thiol chemistry. Force spectroscopy measurements were consistent with the rupture of the strepavidin-biotin bond between the bead and the DNA. This implied that the DNA remained anchored to the surface due to the strong gold-thiol bond. Consistent with this conclusion, we repeatedly reattached the trapped bead to the same individual DNA molecule. Thus, surface conjugation of biomolecules onto

an array of gold nanostructures by chemically and mechanically robust bonds provides a unique way to carry out spatially controlled, repeatable measurements of single molecules.

951-Pos

Model for Harnessing the Device Stiffness in Dynamic Single-Molecule Force Spectroscopy

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Single-molecule force spectroscopy methods use optical traps or tiny cantilevers to impose controlled forces to individual molecules for studying their mechanical behavior and transitions along specific paths called reaction coordinates. A powerful application of these sophisticated approaches is the extraction of activation energy barriers and intrinsic rates of transition from measurements of the rupture force, i.e., when the molecules stretched at a speed undergoes sudden unfolding.

Existing analyses of force measurements relies heavily on theoretical models for reliable extraction of kinetics and energetic properties. Despite significant advances, there remain large gaps in fully exploiting the experiments and their analyses. Specifically, the effect of pulling device stiffness or compliance has not been comprehensively captured. Hence, the best models for extracting molecular parameters can only be applied to measurements obtained from soft pulling devices (e.g., optical tweezers) and result in well-documented discrepancies when applied to stiff devices (e.g., AFM). This restriction makes pulling speed the only control parameter in the experiments, making reliable extraction of molecular properties problematic and prone to error.

Here we present an analytical model derived from physical principles for extracting the intrinsic rates and activation free energies from rupture force measurements that is applicable to the entire range of pulling speeds and device stiffnesses. The model therefore is not restricted to the analyses of force measurements performed with soft pulling devices only. On the contrary, the model allows better design of experiments that specifically exploit device stiffness as a control parameter in addition to pulling speed for a more reliable estimation of energetic and kinetic parameters. The model also helps explain previous discrepancies noted in rupture forces measured with devices of different effective stiffnesses and provides a framework for modeling other stiffness-related issues in single-molecule force spectroscopy.

952-Pos

Extracting Complex Network and Effective Free Energy Landscape of Protein Fluctuation from Single-Molecule Time Series

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The complexity in kinetics observed in single molecule measurements arises from the morphological feature inherent to the underlying multidimensional energy landscape or, in general, state space. However, how can one extract such dynamic information from a set of single molecule time series? Recently we developed two methodologies for extracting an effective free energy landscape composed of local equilibrium states [1,2] and a multiscale state space network (SSN) from single molecule time series [3,4]. Both are designed as free from a priori assumption such as local equilibration. The state is defined not by the value of the observable at each time but by a set of subsequences of the observable. These methods enable us to lift degeneracy_different physical states having the same value for a measured observable_as much as possible under the limitation of scalar quantity. The morphological feature of the free energy landscape and the SSN naturally depends on the time scale of observation. The length of the subsequence constructing the states in the multiscale SSN can tell us the extent to which the memory of the system can predict the next state. We present the brief overview with some examples such as strange diffusion kinetics in conformation fluctuation of Flavin-Enzyme System (H. Yang *et al Science*, **302**, 262 (2003)) and show the multiscale SSN buried in the observation.

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953-Pos

Influence of the Experimental Set-Up on Single Molecule DNA Dynamics When Analyzed by Tethered Particle Motion

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Among the various experimental techniques now available to explore the interaction between a chosen protein and a double stranded DNA, the single molecule technique of Tethered Particle Motion (TPM) is the only one to permit the observation of the dynamics of the DNA polymer at mechanical equilibrium as it consists in tracking the movement of a bead tethered to the glass surface by a DNA molecule. Relatively easy to implement on a microscope, the details of the experimental set-up might nevertheless have some strong influence on the collected data. Considering that the DNA molecules under studies have a size varying between a few hundred to a few thousand base pairs, they are assimilated to semi-flexible polymers. With the help of both experiments and simulations, we show that the beads used for the labelling, whose diameters are usually about a few hundred of nanometres, can slow down the observed dynamics due to their own drag force. We also quantify the bias resulting from the detector averaging effects in this case of TPM experiments.

954-Pos

DNA Origami as a Nanoscopic Ruler For Super-Resolution Microscopy

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The possibility of highly parallel formation of nanostructures using self-assembly of DNA molecules provides a powerful tool for bottom-up fabrication. The DNA origami technique, involves folding a long single-stranded DNA scaffold using short DNA staple strands that can only bind at particular points along this scaffold. With this technique large numbers of identical structures can be assembled simultaneously in a single experiment. One of the most attractive features of the origami technique is the precise addressability of the DNA structures formed. Each staple strand can serve as an attachment point for many different kinds of molecules or other objects.

Due to their small size, DNA nanostructures are commonly imaged using atomic force microscopy or electron microscopy, but with recent advances in far-field fluorescence microscopy beyond the diffraction limit (super-resolution microscopy), structures in the sub-200 nm regime become amenable also to optical analysis.

We here show that the distance between fluorescently labeled staple strands bound on specific positions of rectangular DNA origami structures can be accurately determined using a variety of super-resolution techniques such as single-molecule high-resolution imaging with photo-bleaching (SHRImP), direct stochastic optical reconstruction microscopy (dSTORM), and Blink-Microscopy.

955-Pos

Near-Field Fluorescence Correlation Spectroscopy Approach to the Study of Living Cell Membrane Dynamics

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We developed a near-field (NF) microscopy-based fluorescent correlation spectroscopy (FCS) approach that allows to measure protein and lipid mobility on living cells plasma membrane at sub-diffraction scale. The near-field excitation is obtained by means of an aluminum-coated optical fiber with sub-wavelength aperture (<100 nm in diameter), effectively reducing the illumination area of about one order of magnitude compared to standard confocal FCS. The use of this kind of probe also provides capability for dual-color FCS and fluorescence cross-correlation spectroscopy (FCCS) and guarantees the overlap of the excitation areas. The optical fiber is attached to an oscillating tuning fork and a shear force based feedback keeps the probe in the close proximity of the cell, preventing the membrane from fluctuating outside the evanescent field volume while minimizing probe-membrane interactions.

We demonstrated the feasibility of the dual-color NF-FCS approach by measuring the diffusion of phosphoethanolamine or sphingomyelin simultaneously with GPI-anchored protein on the plasma membrane of living CHO cells. The comparison of these results with those obtained by confocal FCS highlights the advantages of the reduced illumination area in detecting anomalous or heterogeneous diffusion.

Although other techniques have been shown to provide comparable illumination sizes, the NF-FCS approach offers the further advantage of dual-color FCS and FCCS and therefore represents a powerful tool to unravel the details of a variety of membrane processes occurring at the nanometric scale.