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.

[1] Gunnarsson, A. et al. Nano Lett. 8(1): p. 183-188, 2008.
[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