Emerging Single Molecule Techniques II

3036-Pos

Extending the Nano-Positioning System (NPS)

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Single-Pair Fluorescence Resonance Energy Transfer (FRET) experiments reveal structural and dynamic information about macro-molecules by monitoring the change in FRET efficiency between fluorescent dyes attached to a macromolecule. The Nano-Positioning System (NPS) developed recently [1] uses data from several of such experiments to infer the position of a dye attached to protein sites unresolved by x-ray crystallography. Briefly, we perform probabilistic data analysis that allows us to calculate the distribution of possible dye positions in a simple and objective way without relying on ad-hoc procedures. Up to now NPS was limited to the triangulation of just one fluorescently labelled position based on FRET measurements to several other positions known from crystal structure [1,2]. Here, we discuss ways to extend the present model beyond this basic triangulation principle. In particular, we show how to gain three dimensional distance information by analysing triangulation networks where FRET is measured between arbitrary labelling sites in absence of other structural information.

[1] A. Muschielok, J. Andrecka, A. Jawhari, F. Brückner, P. Cramer & J. Michaelis, Nat. Meth. 5, 965-971 (2008)
[2] J. Andrecka, B. Treutlein, M.A. Izquierdo Arcusa, A. Muschielok, R. Lewis; A.C.M. Cheung, P. Cramer, and J. Michaelis, NAR doi:10.1093/nar/gkp601 (2009)

3037-Pos

Single Molecule Tracking Inside Individual Living Bacterial Cells

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We have developed a single-molecule fluorescence assay to directly observe the stringent response in individual living E. coli cells. For this purpose, we have created chromosomal fusions of both RelA and three ribosomal proteins with a photo-activatable fluorescent protein.

The stringent factor RelA binds to a small fraction of ribosomes, where it synthesizes the global transcriptional regulator ppGpp in response to amino acids deprivation. Our objective is to study the binding kinetics of individual RelA molecules to the ribosome in living cells and to observe how its kinetics changes during a nutritional down-shift.

While E. coli contains on average about 100 RelA molecules and 20000 ribosomes, using a photo-activatable fluorescent probe we can activate only a few fluorescent molecules per cell at any given time. We induce stringent response by rapid addition of amino acid hydroxamates. Since our fluorescent tag is photoconvertible, we can repeat tracking experiments many times in the same E. coli cell.

We record trajectories of individual RelA molecules diffusing in living E. coli cells with a laser exposure of 1 millisecond, a frame time of 5 milliseconds, and a spatial precision of 50 nanometers. The high resolution of the experiments makes it possible to characterize RelA binding kinetics under varying growth conditions. When the cell grows exponentially, RelA trajectories closely resemble trajectories of fluorescently tagged ribosomal proteins. After nutritional downshift, RelA binding kinetics changes rapidly. Results suggest that under amino-acid starvation, RelA is only transiently bound to the ribosome. The data is consistent with an order of magnitude drop in affinity to the ribosome.

3038-Pos

Development of a Plasmonic Nanoparticle-Based Assay to Observe Nanoscale Biological Dynamic Interactions

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To measure nanoscale distances relevant to quantifying bio-molecular dynamics, a widely used technique is fluorescence resonance energy transfer (FRET). While FRET is sensitive to distances between donor and acceptor fluorophores below 10nm, the technique suffers from a low signal-to-noise ratio, limited range of distance sensitivity, and rapid photobleaching of the fluorescent dyes. These unavoidable experimental shortcomings greatly hinder the robustness of the technique in measurements characterizing dynamics at the single-molecule level. A new approach that exploits the plasmon coupling of gold nanoparticles has been introduced, which has a high signal-to-noise ratio, distance ranges from sub-nanometer to hundreds of nanometers (depending on particle size), and superior photostability. Previous work has demonstrated the viability of plasmon coupling to report the distance between two gold nanoparticles conjugated to the ends of DNA. We have extended the technique such that the distance between the gold nanoparticles and the plasmon coupling spectral response are independently determined through the use of image analysis and spectrophotometry. In this manner, this technique can be extended to observe the dynamics of any protein interaction where gold nanoparticles may be conjugated. We have designed and implemented a custom spectrometer with high spectral resolution (0.015nm) and low integration times (millisecond), surpassing commercially available instruments, by using a sensitive CCD array and off-axis parabolic mirrors. By simultaneously analyzing the collected spectra of single gold nanoparticle pairs and images of their diffraction-limited spots using a 2-D Gaussian fitting algorithm, the spectra are correlated to the measured distance between the gold nanoparticles. As a proof-of-concept, we are now creating calibrations between the plasmon coupling spectra and the distance between dually-labeled DNA molecules. This experimental technique has the potential to study DNA-protein and protein-protein interactions with high spatial (sub-nanometer) and temporal (millisecond) resolution.

3039-Pos

Advanced Multidimensional Optics to Investigate Biological Complexity at the Single Molecule Level in Living, Functional Cells

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Fluorescence microscopy offers a minimally-perturbative, non-invasive means to probe functional living cells. This project aims to develop super-resolution, multi-colour, multi-polarization fluorescence microscopy capable of simultaneous imaging of multiple bio-molecules, such as genomically encoded fluorescent protein fusion constructs. It will enable dynamically-modulated high-contrast imaging to a bandwidth of ~1kHz, facilitate ~millisecond tracking of single protein molecules in a single cell to ~10nm spatial precision, and permit nanoscopic length scale conformational change to be measured. It will be constructed to permit future addition of laser-tweezers and will be used to perform investigations on single living cells. Under investigation will be the cellular processes that bring about assembly of individual proteins into functional biological machines. The microscope will allow precise determination of how many components such machines have, dynamics of assembly and disassembly processes, mechanistic interactions, molecular stability and how machines of different biological processes may co-operate to produce compounded effects across the whole cell.

3040-Pos

Detection of Rare Interaction Events Via Combined Photobleaching and Single Molecule Microscopy

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Gathering information on the interplay between different membrane components and the characteristics of protein interactions in live cells requires the design of sensitive methods that allow for the simultaneous study of different probe molecules. Especially the aim to single out rare interaction events under a vast excess of non-interacting molecules remains challenging.

The two-color single molecule imaging technique presented here is the advancement of a recently presented approach to virtually dilute fluorescently labeled membrane constituents by photobleaching [1]. Using this technique, single molecule microscopy can be performed at almost arbitrarily high surface densities of fluorescent probe molecules. The method yields information on the fraction of colocalized particles and their position within 40nm accuracy. Supplemental data on the mobility and stoichiometry of the labeled molecule species can also be deduced. The sensitivity of our two-color single molecule imaging technique is significantly increased by tracking colocalized spots over consecutive images. We present a detailed statistical description of false positives and false negatives and quantify the sensitivity of our method.

Proof of principle experiments were performed by measuring the interaction between Alexa647-labeled Cholera Toxin B (CTX-B-Alexa647) and Bodipy-labeled GM1 (Bodipy-GM1) diffusing in a fluid supported lipid bilayer. We directly observed single Cholera Toxin molecules bound to Bodipy-GM1 and quantified their occupancy via brightness analysis. We demonstrate that extremely low interaction probabilities of only 2.5% can be unambiguously identified by tracking colocalized spots.

We also present preliminary results on the application to live cells investigating the interaction between Bodipy-GM1 and Ab-Alexa647 labeled CD59.

[1] Moertelmair et al., Appl. Phys Lett. 87, 263903, 2005.

3041-Pos

Single Molecule Chemical Reactions within Femtoliter Volume Containers

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We create and observe controlled single molecule chemical reactions within femtoliter containers called hydrosomes. Hydrosomes are stable aqueous

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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