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Single Molecule Techniques II

2638-Pos Board B657

Mechanisms in Co-Translational Protein Folding Elucidated using Single Molecule FRET

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In nature, protein folding mechanisms are determined by both amino acid sequence AND physiological context. Inside living cells the transient and vectorial nature of protein synthesis, the presence of molecular chaperones, and macromolecular crowding can each have a profound effect on the paths nascent chains take en route to their native states. This adds a daunting level of complexity to the study of *in-vivo* protein folding mechanisms. To begin to address these issues we have developed methods which enable the detection of ribosome-bound nascent chain (RNC) conformational distributions and dynamics using single molecule fluorescence resonance energy transfer. Our approach employs a purified and reconstituted *E. coliin-vitro* translation system to generate stalled RNCs, the site-specific incorporation of chemically-reactive alkyne-bearing unnatural amino acid tags into nascent chains, and post-translational bioconjugation of pairs of single-molecule donor and acceptor fluorophores to the RNCs using a highly-efficient ligand accelerated copper-click chemistry reaction. We apply our novel RNC labeling approach to explore the role of helix stabilization by and propagation from the exit tunnel of the ribosome as a general means by which the ribosome may direct the co-translational folding of nascent polypeptides.

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Learning Kinetic Models from Single-Molecule FRET Data using Bayesian Inference

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Single-molecule FRET studies have enabled real-time observation of conformational transitions in individual molecules, allowing targeted investigations into the mechanistic function of molecular machines such as the ribosome. Like in many single-molecule platforms, a fundamental problem with sm-FRET studies is that our noisy fluorescence signal does not unambiguously determine the underlying conformational state. Moreover, a single experiment often yields hundreds of time series, which report on the same underlying process, but exhibit significant variations in photophysical properties and kinetic rates. This combination of lots of data and lots of stochasticity means that interpretation of sm-FRET experiments often requires use of statistical inference techniques. Hidden Markov Models are a widely used tool for parameter estimation in time series data, and have been successfully applied to sm-FRET experiments by several groups. A fundamental limitation of existing approaches is that inference is only performed on one time series at a time, yielding a large number of parameter estimates of variable quality which must now be related to each other using ad-hoc experiment specific post-processing steps.
Here, we propose a technique known in the statistical community as Empirical Bayes estimation, to perform combined analysis on the entire collection of trajectories in an experiment. This allows straightforward and statistically principled learning of a consensus kinetic model from an ensemble of time series. Moreover, the method allows significantly better estimates of the kinetic rates associated with conformational transitions. Finally we demonstrate how inference results on models with varying kinetic structures can be compared to directly test detailed mechanistic hypotheses in a statistically principled, adaptable manner.

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Single-Molecule FRET Studies of a Y-family Polymerase Provide New Insights into Nucleotide Binding Mechanism

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High fidelity polymerases are normally blocked by damage in the DNA template. Stalled replisomes can lead to DNA double-strand breaks or other detrimental genotoxic effects, thereby increasing genome instability. A specialized variety of DNA-polymerases is mobilized when replication across damaged bases (translesion DNA synthesis) is required. Y-family polymerases perform the majority of translesion synthesis and are generally found to be specialized for specific types of lesions. These Y-family polymerases typically present a wide active site to accommodate distorted/bulky DNA.
Dpo4 is a Y-family polymerase that has been extensively characterized by ensemble experiments. However, the exact polymerization mechanism that occurs during the bypass of the DNA damage remains unclear. We have used single-molecule fluorescence resonance energy transfer (smFRET) to investigate the interactions between DNA and Dpo4. Our data show that Dpo4 binds DNA in two different conformations that interconvert reversibly. Experiments carried out in the presence of nucleotides using Ca^{2+} instead of Mg2+ to prevent DNA extension suggest that one of these conformations is preordered to accept the incoming nucleotide. We were able to characterize the binding and dissociation dynamics between nucleotides and the DNA/Dpo4 complex. Our results also suggest that Dpo4 undergoes a similar conformational rearrangement upon binding a correct or incorrect (mismatched) nucleotide.

2641-Pos Board B660

Internucleosomal Interactions Monitored at a Single Molecule Level

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We studied interactions between two nucleosomes at a single molecule level using FRET. According to the results, internucleosomal interactions strongly depend on various histone modifications. Our unique single molecule setup will facilitate investigations on the detailed structure of chromatin packaging affected by histone modifications.

2642-Pos Board B661

Simple Autofocusing System for Single-Molecule FRET Experiment Based on Single-Molecule Image Analysis

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Single-molecule FRET has greatly contributed to our detailed mechanistic understanding of many bio-molecular systems. While reactions occurring in the range of several minutes can be readily studied, data acquisition for longer time scales is hindered by accumulated focal drift of a high numerical aperture objective, which should be corrected in real time. Here, we develop an autofocusing system based on the analysis of optical astigmatism of single-fluorophore images. Compared to the other autofocusing methods, our approach has a merit of simplicity that neither fiduciary makers nor additional light sources and detectors are required. As a demonstration of the new autofocusing system, we observed slow B-Z transition dynamics occurring in several hours using single molecule FRET.

2643-Pos Board B662

Unfolding and Degradation of Proteins by ClpXP Monitored with Single Molecule FRET

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Proteins, the working machineries inside a cell, are tightly regulated from birth through maturation to death. ClpXP is an ATP dependent protease that is involved in protein quality control and regulation. ClpX is a ring-shaped ATPase that recognizes, denatures and translocates target substrates into ClpP. ClpP is a barrel shaped protease that degrades polypeptide substrates. Despite extensive biochemical studies over the last two decades, much remains unknown about the molecular mechanism of ClpXP. We will visualize the whole process of substrate recognition, denaturation, translocation, and degradation using multicolor single-molecule FRET. This single-molecule study will reveal the mechanism of degradation and product release by ClpXP.

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High Speed Magnetic Tweezers at 100KHz with Superluminescent Diode Illumination

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Magnetic tweezers apply force to single DNA molecules to measure changes in DNA length as a function of time. The technique is ideally suited to explore the dynamics of biophysical processes such as DNA unzipping by helicase motor proteins due to the constant force applied to the DNA. However, instrumental improvements are needed to resolve the length and time scales of single enzymatic steps. Here, we introduce a superluminescent diode as the illumination source and a high-speed CMOS camera as the optical detector to achieve magnetic tweezing of DNA at a bandwidth of 100 kHz. We show how GPU-accelerated video processing can be used to determine three-dimensional particle positions from a series of video frames with a spatial resolution below 1 Angstrom. We demonstrate the quantitative capabilities of this instrument by measuring the drag coefficient of a magnetic bead and the stiffness of a tethered DNA molecule at a corner-frequency of

1 kHz, and by measuring DNA hairpin dynamics that were previously characterized in optical tweezer experiments.

2645-Pos Board B664

Force and Fluorescence Experiments in a Hybrid smFRET Magnetic Tweezer

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Single-molecule FRET has become a powerful tool to study conformational changes in cellular machines and many other biomolecules. Labeled with fluorophores at specific positions, distance changes can be tracked with high temporal and spatial resolution. Correspondingly, magnetic tweezers have evolved into an important force-based single-molecule technique that allows the manipulation of molecules through piconewton forces in combination with torques. The control over molecular twist is particularly interesting for the study of chiral biomolecules such as double-stranded DNA (dsDNA).
Most single-molecule experiments, however, are limited to one of these dimensions of molecular characteristics at a time, leaving some questions unanswered. Simultaneously controlling force and distance in a molecular frame promises to provide a more complete picture of biomolecular interactions.
Here, we present a hybrid instrument, combining these two techniques, smFRET and magnetic tweezers, and its application to model systems, such as dsDNA and DNA hairpins. Our experiments demonstrate a parallel force manipulation of several individual biomolecules with forces ranging from 100 fN to 20 pN, while simultaneously recording single fluorophores, leading to an expansion of the available information space.

2646-Pos Board B665

Ultrafast Force-Clamp Spectroscopy of Single Molecular Motors and DNA Binding Proteins

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Force plays a fundamental role in a wide array of biological processes, regulating, for example, enzymatic activity, kinetics of molecular bonds, and molecular motors mechanics. Single molecule force spectroscopy techniques have enabled the investigation of such processes, but they are inadequate to probe short-lived (millisecond and sub-millisecond) molecular complexes. Here, we present a novel force-clamp laser trap that is capable of applying constant loads to molecular complexes with lifetimes above ~10 μs [Capitanio *et al.*, Nature Methods 9, 1013-1019 (2012)]. Such capability is enhanced by a detection strategy allowing the investigation of very short interactions (~100μs). Moreover, the high temporal and spatial resolution of the method enables us to probe sub-nanometer conformational changes with a time resolution of few tens of microseconds. We tested our method on molecular motors and DNA-binding proteins. We could apply constant loads to a single motor domain of myosin before its working stroke was initiated (0.2–1 ms), thus directly measuring its load dependence. We found that, depending on the applied load, myosin weakly interacted (<1 ms) with actin without production of movement, fully developed its working stroke or prematurely detached (<5 ms), thus reducing the working stroke size with load. These results resolve the molecular mechanism underlying regulation of muscle contraction by force. Our technique could be straightforwardly applied to a wide variety of non-processive molecular motors, single domains of processive motors, protein-DNA and protein-RNA interactions, and conceivably to any short-lived protein-protein interaction, opening new avenues for investigating the effects of forces on biological processes.

2647-Pos Board B666

The Role of H2A-H2B Dimers in the Mechanical Response of Single Nucleosomes

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During transcription RNA polymerase frequently evicts the H2A-H2B dimers from nucleosomes. This might be necessary to access the DNA but is also believed to mediate a feedback mechanism by which transcription of a gene facilitates the exchange of epigenetic marks and histone variants. This in turn can then immediately influence gene expression by consecutive RNA polymerases. Little is known about the role that H2A-H2B dimers play in the response of chromatin to mechanical stresses during transcription.
In order to elucidate the fate of dimers during transcription, we studied DNA unwrapping from single nucleosomes in the presence of an external force using magnetic tweezers. We subjected reconstituted nucleosomal arrays, containing up to 48 nucleosomes, to tension and monitored the DNA length in relation to force. We find significant differences in the DNA unwrapping forces between nucleosomes containing canonical histones only, the H2AvD histone variant and H3-H4 tetramers. This reveals an important role of the H2A-H2B dimer during mechanical DNA disruption from the nucleosome, as might occur during transcription.

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Simultaneous High-Resolution Optical Trapping and Single Molecule Fluorescence Measurements of the UvrD Helicase: DNA Motor Protein Structure and Function Observed Simultaneously

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We present single-molecule in vitro measurements of the DNA unwinding activity of the Superfamily 1 UvrD helicase that reveal directly how helicase stoichiometry and conformation correspond to motor activity. Although a single UvrD molecule can translocate along ssDNA, it has been postulated that only a dimer is capable of unwinding duplex DNA. UvrD is known to exhibit two different conformations: the ‘closed’ and ‘open’ orientation of the 2B regulatory subdomain. The function of these conformations has remained elusive. using a new instrument combining high-resolution optical tweezers with simultaneous single-molecule fluorescence microscopy (Comstock, Ha, and Chemla, Nature Methods 2011), we obtain an unprecedented detailed view of helicase motor dynamics by recording single base pair-scale DNA unwinding activity (optical tweezers) simultaneously with helicase stoichiometry and conformation (fluorescence). We observe two classes of DNA unwinding by UvrD: long distance continuous unwinding (~100 bp) and repetitive, nonprocessive (~12 bp) unwinding. We determine directly the stoichiometry of UvrD molecules participating in unwinding by quantifying the fluorescence intensity using singly-labeled UvrD molecules. We observe that pairs of UvrD molecules are required for long distance unwinding but individual molecules exhibit limited non-processive unwinding activity. Furthermore, we observe directly the conformation of the 2B subdomain of individual UvrD molecules during unwinding by measuring the fluorescence of FRET-labeled proteins. We observe that UvrD is in the ‘closed’ conformation during DNA unwinding but surprisingly switches to the ‘open’ conformation when UvrD translocates on the opposing ssDNA strand away from the junction with the DNA junction re-annealing behind it. These measurements may explain UvrD helicase’s remarkable ability to switch translocation strands. We hypothesize that the UvrD 2B subdomain acts as a switch that controls DNA unwinding vs. re-annealing.

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Using Single-Molecule Approches to Study the Alba1’s Function in Thermalphilic Archaea

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All thermophilic and hyperthermophilic archaea have one or more copies of the Alba (Sac10b or Sso10b) gene, which is an abundant, dimeric and highly basic protein with cooperatively binding ability to DNA. It has been speculated to play an important role in DNA packaging in the Archaea. Now single molecule approaches, tethered particle motion (TPM) and magnetic tweezers (MT) were adapted to study the interactions between DNA molecules and Alba1. It was found that Alba1 will bind to dsDNA cooperatively once the concentration above 10 μM. Instead of condensation, the extension in the DNA length for DNA molecules in response to Alba1 was observed. Moreover, the extension rate and extension ratio are length dependent, indicating that the initiation binding sites are increased as DNA becomes longer. The length dependent extension ratio, similar to that of RecA filament, indicated that not only the length is changed after Alba1 binding but also the persistence length of the DNA molecule. Last, it was found that Alba1 possesses dimer-dimer contacts between two fibers, which bring two dsDNA strands together, suggesting the roles of Alba1 on the DNA packaging.