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Versatile Tools towards Real Time Single Molecule Biology
Jordi Cabanas-Danés, Rosalie P.C. Driessen, Avin Ramaiya, Philipp Rauch, Andrea Candelli.
LUMICKS, Amsterdam, Netherlands.
Biological processes performed by proteins interacting with and processing DNA and RNA are key to cell metabolism and life. Detailed insights into these processes provide essential information for understanding the molecular basis of life and the pathological conditions that develop when such processes go awry. The next scientific breakthrough consists in the actual, direct, real-time observations and measurements of the individual mechanisms involved, in order to validate and complete the current biological models. Single-molecule technologies offer an exciting opportunity to meet these challenges and to study protein function and activity in real-time and at the single-molecule level. Here, we present our efforts for further enabling discoveries in the field of biology and biophysics using both the combination of optical tweezers with single-molecule fluorescence microscopy (C-Trap^{TM}). We show the latest applications of these technologies that can enhance our understanding not only in the field of DNA/RNA-protein interactions but also in the fields of molecular motors, protein folding/unfolding, cell membranes and genome structure and organization. These experiments show that the technological advances in hybrid single-molecule methods can be turned into an easy-to-use and stable instrument that has the ability to open up new venues in many research areas.

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Fluorescence Correlation Spectroscopy with Nanowire Waveguide Illumination for High Concentration Conditions
Olaoluwalotobi Thomas, Arstanbek Tulekeyev, Justin Isaac, Huizhong Xu.
Dept of Physics and Astronomy, San Francisco State University, San Francisco, CA, USA.
Fluorescence correlation spectroscopy (FCS), a technique based on correlating the fluorescence signal from a small sample volume, has been commonly used to study biological processes such as photophysical phenomena, binding kinetics, and intracellular dynamics. In a conventional FCS setup, the sample volume is defined by a focused laser beam for excitation and a confocal pin hole for collection resulting in a size of about one femtoliter. Evanescent waves from total internal reflection have been used to obtain a smaller sample volume, as a result of the improved confinement along the longitudinal axis for the illumination. However, applying FCS under physiological conditions where the molecule concentration is typically in the μM range requires an even smaller sample volume. Here we propose to apply FCS with nanowire waveguide illumination with nanometer-sized lateral and longitudinal confinement. Nanowire waveguides, made of a dielectric nanowire with a diameter of ~50 nm embedded inside a metal film, allow for the efficient transmission of visible light by exploiting surface waves traveling along the dielectric-metal boundary. Such waveguides can generate a lateral illumination area of about 50 nanometers in diameter. In addition, a longitudinal confinement of around 20 nanometers is achieved with the rapidly-attenuating near fields exiting from the waveguide. The strong confinement in both lateral and longitudinal directions thus lead to a sample volume on the order of one zeptoliter, allowing for FCS measurements in the μM concentration range. By combining Brownian dynamics simulations with the illumination profile obtained from finite element method simulations of a zinc oxide nanowire waveguide in a silver metal film, we numerically calculate the correlation function to demonstrate the use of this method in the study of molecular dynamics under high concentration conditions.

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Anomalous Ultra-Fast Energy Transfer Suggests Coherent Energy Transfer between Fluorescence Proteins
Youngchan Kim[1], Grace H. Taumoefolau[1], Henry L. Puhl[1], Tuan A. Nguyen[1], Paul S. Blank[2], Steven S. Vogel[1].
[1]NIH/NIAAA, Rockville, MD, USA, [2]NIH/NICHD, Bethesda, MD, USA.
Two assumptions for FRET experiments using fluorescent proteins (FPs) are that FPs act independently (i.e. very weak coupling) and that they behave like classical organic fluorophores. When conventional fluorophores are in close-proximity (< 1 nm) and/or are cooled to temperatures approaching absolute zero, stronger coupling is possible. Under these conditions coherent energy transfer (CET) may enable multiple fluorophores to behave as a single quantum entity. CET is thought to play a key role in photosynthesis, and vis-à-vis technology, may enable quantum computing. CET manifests as ultra-fast long-distance energy transfer within fluorophore assemblies. Antibunching, a uniquely quantum mechanical behavior, is consistent with CET. Physiological temperatures extinguish CET by promoting rapid collisional dephasing of fluorophore vibrational modes. Moreover, because FP fluorophores are encased in a ß-barrel structure, proximities closer than 2 nm are not possible. Thus, CET between FPs at physiological temperatures is thought to be impossible. Nonetheless, using two-photon excitation and time-correlated single photon counting we have observed both anomalous ultra-fast energy transfer and strong antibunching behaviors in FP assemblies composed of mVenus, mClover, or mNeonGreen. Our experiments suggest stronger than expected coupling between FPs. Thus, we speculate that CET between FPs at physiological temperature may be possible.

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Single Molecule Study of ATAD5-Induced Unloading of PCNA
SeungWon Lee, Eunji Ryu, Sukhyun Kang, HaJin Kim.
UNIST, Ulsan, Republic of Korea.
The loading and unloading steps of the DNA clamp, proliferating cell nuclear antigen (PCNA), which acts as a processivity factor for DNA polymerase, are crucial for the precise control of DNA replication. The underlying molecular mechanisms for these processes are yet to be well understood. Recently it was discovered that ATAD5 protein is involved in the unloading of PCNA from the chromatin. We studied the molecular mechanism of this process from single molecule approaches. By site-specific fluorescent labeling of PCNA proteins, which form trimeric rings around DNAs by the clamp loader, replication factor C (RFC) complex, we could directly observe their loading and unloading dynamics at the ssDNA-dsDNA junction, together with their diffusion along the DNA. Upon loading on the DNA junction, PCNA was stably bound at the preferred 3'-end ssDNA-dsDNA junction and also made transient diffusion dynamics away from this site, as observed from our single molecule fluorescence resonance energy transfer (smFRET) measurements. By substituting the first subunit of five-subunit RFC complex with ATAD5, its function can be switched to the unloading of PCNA. Interestingly, PCNA trimer was unloaded by ATAD5 complex not all at the same time but one-by-one in concentration-dependent manner. This suggests an intriguing mechanism of PCNA unloading, in which the incomplete PCNA complex can be held on the DNA while being serially detached. By additionally labeling ATAD5 protein, we could reveal more precise mechanism from three-color smFRET measurements. We present our preliminary results on these attempts.

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Single-Molecule DNA Unzipping Reveals Asymmetric Modulation of the Transcription Factor EGR-1 by its Binding Site Sequence and Context
Hadeel Khamis[1], Sergei Rudnizky[1,2], Omri Malik[1], Allison Squires[3], Amit Meller[4], Melamed Philippa[2], Ariel Kaplan[2].
[1]Physics, Technion, Haifa, Israel, [2]Biology, Technion, Haifa, Israel, [3]Chemistry, Stanford University, Stanford, CA, USA, [4]Biomedical Engineering, Technion, Haifa, Israel.
Binding of transcription factors to regulatory elements is a central step in the complex regulation of gene expression, and its perturbation is linked to numerous disease states. Egr-1 is an inducible transcription factor that binds to 9-bp response elements via three zinc finger domains in response to a variety of stimuli, such as hormonal signals and stress. In our work, we use single-molecule DNA unzipping with optical tweezers to study the binding properties of Egr-1 to its binding sites, using as a model the promoter of the *Lhb* gene. We find that both the core 9 base pairs bound to Egr-1 in each of the binding sites on *Lhb*, and the base pairs flanking these sites, modulate the affinity and structure of the protein-DNA complex. The effect of the flanking sequences is asymmetric, with a stronger effect for the sequence flanking the triplet in contact with zinc finger 3. Next, using a novel method to characterize the dissociation time of Egr-1 at the single molecule level, we show that a local, mechanical perturbation of the interactions of zinc finger 3 is able to destabilize the complex more effectively than a similar perturbation acting on the interactions of ZF1. Taken together, our findings suggest a novel functional role for ZF3 in the interaction of Egr-1 with other proteins.

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Cholesterol Promotes Cytolysin a Activity by Stabilizing the Intermediates during Pore Formation
Pradeep Sathyanarayana[1], Satyaghosh Maurya[2], Monisha Ravichandran[2], Ganapathy K. Ayappa[3], Sandhya S. Visweswariah[4], Rahul Roy[3].
[1]Center for Biosystems Science and Engineering, Indian Institute of Science, Bangalore, India, [2]Indian Institute of Science, Bangalore, India, [3]Chemical Engineering, Indian Institute of Science, Bangalore, India, [4]Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, India.
Pore-forming proteins (PFP) are cell membrane rupturing proteins and form the largest class of proteins that mediate bacterial virulence. PFPs are secreted as water-soluble monomers that bind strongly to the lipid membrane of eukaryotic cells. They do not contain a signature transmembrane motif, but nevertheless adopt structures that traverse the membrane, thereby allowing the passage of

molecules from within the cell to the exterior, resulting in host cell lysis. The conformational transitions of a PFP from a water-soluble structure to a distinct membrane-associated form are not understood in mechanistic detail. In this study, we use single molecule tracking and spectroscopy to understand the dynamics of Cytolysin A (ClyA), a prototypical α-PFP from E. coli, on bilayer lipid membranes. Binding of ClyA to PEG-cushioned supported bilayer was rapid and reached saturation within a few seconds. Diffusional analysis of particle trajectories showed existence of mobility states that could be assigned to two distinct structural states that match structures of the protein in the monomer form and the protomer state. In the presence of membrane cholesterol, the population of the slower moving species increased, with the concomitant decrease in the fast mobility population. Analysis of transition probabilities by Hidden Markov Model revealed that the conversion from fast to slower mobility state was due to the conformational transition from a peripherally-associated protein conformation to a membrane-inserted conformation respectively. Furthermore, a hitherto unknown cholesterol interaction domain was discovered in the transmembrane helix of ClyA and validated by mutational studies. Therefore, we propose a molecular mechanism for selective pore formation in eukaryotic membranes which is driven by conformational selectivity in the presence of cholesterol.

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DNA Stability after Oxidative Damage

Micah J. McCauley[1], Leah Furman[2], Catherine A. Dietrich[3], Caitlin J. Cain[2], Diana Seminario[3], Ioulia Rouzina[4], **Megan E. Nunez**[2], Mark C. Williams[1].
[1]Department of Physics, Northeastern University, Boston, MA, USA,
[2]Department of Chemistry, Wellesley College, Wellesley, MA, USA,
[3]Department of Chemistry, Mount Holyoke College, South Hadley, MA, USA, [4]Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH, USA.

One of the most common DNA lesions is created when reactive oxygen species modify guanine bases to create 8-oxoguanine. 8-oxoguanine may bind to the opposing cytosine in the *anti* conformation or to an opposing adenine in the *syn* conformation. To elucidate the free energy of 8-oxoguanine-containing base pairs, DNA hairpin stability was quantified using optical tweezers and compared to a predictive model of base-pair energies. In contrast to either a canonical guanine-cytosine or adenine-thymine pair, an 8-oxoguanine-cytosine base pair shows modest destabilization of several k_BT. The amount of destabilization is comparable to the destabilization induced by a thymine-guanine mispair but less than a thymine-cytosine mispair. Furthermore, while the 8-oxoguanine-cytosine base pair is not predicted by the model, the energies of the guanine-thymine 'wobble' and thymine-cytosine mismatch base pairs match their expected values. Notably, the measured energy of 8-oxoguanine-adenine matches the model for the guanine-adenine mispair, indicating that oxidative damage does not further destabilize this mismatch. These single molecule results support earlier findings that oxidative damage changes the local backbone conformation, altering the direction of the guanine dipole and disrupting stacking interactions, while leaving base pairing intact.

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Quantification of Single-Molecule FRET between Quantum Dots and Organic Dyes

Nooshin Shatery Nejad, Candice M. Etson.
Physics, Wesleyan University, Middletown, CT, USA.

Single-molecule Förster resonance energy transfer (FRET) is a useful technique for studying inter- and intra-molecular dynamics in biophysics. FRET efficiency is highly sensitive to distance, with half-maximal energy transfer occurring at an inter-dye distance on the order of 5 nm, which makes it possible to quantify distances changes on the molecular scale. FRET efficiency also depends on the spectral overlap and quantum yield of the donor and acceptor molecules, and therefore the choice of dye pair is critical to the success of any experiment. Most organic dyes that are used for single-molecule imaging have bleaching lifetimes in the tens of seconds at most, which limits their use to relatively fast processes. Quantum dot nanocrystals can emit for thousands of seconds without entering a long-lived dark state, which should make them good candidates for use as a FRET donor for longer single-molecule experiments, but there has been little quantitative study of the energy transfer efficiency at the single-molecule level. In this work, we use dsDNA to couple individual quantum dot donors to fluorescent dye acceptors. We then immobilize these FRET pairs on a functionalized glass coverslip and image them using total internal fluorescence (TIRF) microscopy with additional optics that allow us to image the donor and acceptor simultaneously. The length of the DNA between the pair can be varied from 11 to 32 base pairs, allowing us to observe and quantify the energy transfer efficiency over a range of distances on the nanometer scale. Our goal is to develop the use of quantum dots as donors for quantitative single-molecule FRET experiments.

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Conformations and Single-Molecule Dynamics of Nitric Oxide Synthase

Carey K. Johnson[1], David C. Arnett[2], Brian C. Smith[3].
[1]Chemistry, University of Kansas, Lawrence, KS, USA, [2]Chemistry, Northwestern College, Orange City, IA, USA, [3]Biochemistry, Medical College of Wisconsin, Milwaukee, WI, USA.

Nitric oxide synthase (NOS) functions by transfer of electrons sequentially from FAD to FMN in the reductase domain of the enzyme and then from FMN to the heme in the oxygenase domain of the opposing member of a homodimeric complex. Efficient electron transfer is activated by the calcium signaling protein calmodulin (CaM) and requires close proximity of electron-transfer donors and acceptors. The sequence of electron transfers therefore necessitates multiple conformational states of the enzyme, suggesting that the activity of the enzyme is conformationally gated. We have detected the presence of multiple conformational states of NOS by time-resolved detection of fluorescence from a fluorophore attached to CaM. Fluorescence is quenched by FRET to the heme groups of the enzyme, and the extent of quenching depends on the conformational state of the enzyme. Single-molecule intensity trajectories reveal multiple fluorescence states with dynamics on the millisecond to second time scales. Analysis suggests sequential conformational interchange, with the longest-lived state being highly quenched, consistent with a conformation in which CaM is in close proximity with the heme groups. Analysis of conformational dynamics is underway.

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The Other Histone: Probing the Role of Linker Histone in a Chromatosome

Madhura De, Kathrin Lehmann, Katalin Tóth.
Biophysics of Macromolecules, Deutsches Krebsforschungszentrum, Heidelberg, Germany.

The chromatosome, or the smallest unit of chromatin, comprises of the nucleosome (core histone octamer + 146 bp DNA surrounding it in 1.65 turns) with additional stretches of linker DNA associated with the linker histone protein (LH). Although recent cryo-EM and X-Ray crystallographic studies have shed some light on the localization of the LH, the linker DNA specificity of LH still remains unclear. In this study, we have employed single molecule FRET spectroscopy to observe the disassembly of the LH induced by increasing salt concentration. Core histone octamers were reconstituted on 210 bp Widom 601 DNA labelled at the linker arms with the fluorophores Alexa488 and Alexa594. We measured FRET between the fluorophores on the two linker DNA arms in the presence and absence of LH and observed that the LH compacts the chromatosome by bringing the two linker arms closer together in a salt dependent manner. Further analysis by salt-dependent electrophoretic mobility shift assays of the chromatosome in the presence and absence of LH showed faster migration of the chromatosome in the presence of LH, supporting the hypothesis that the LH is compacting the chromatosome. This compaction is profound in the salt range 5 to 150mM, as seen by both FRET and EMSA. Moreover, both single molecule FRET experiments and EMSA revealed that presence of LH increases heterogeneity of the chromatosome population. By labelling the chromatosome at different positions we are currently addressing certain key issues: the linker DNA specificity of the LH, salt induced dissociation of the LH, and possible influence of the LH on chromatosomes reconstituted using mutated core histone octamers. We hope that this system will help pin-point the localization and role of the LH in freely diffusing mononucleosomes.

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Energy Landscape Analysis of the Full-Length SAM-I Riboswitch using Single-Molecule FRET Spectroscopy

Christoph Manz[1], Andrei Yu Kobitski[1], Ayan Samanta[2], Bettina G. Keller[3], Andres Jäschke[2], **G. Ulrich Nienhaus**[1].
[1]Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany, [2]Heidelberg University, Heidelberg, Germany, [3]Freie Universität Berlin, Berlin, Germany.

Binding of the ligand *S*-adenosyl-L-methionine (SAM) produces major structural changes in the SAM-I riboswitch (RS) and thereby regulates gene expression via transcription termination. As yet, the conformations and motions governing the function of the full-length *Bacillus subtilis yitJ* SAM-I RS have not been deeply investigated. We have studied its conformational energy landscape as a function of Mg^{2+} and SAM ligand concentrations using single-molecule Förster resonance energy transfer (smFRET) microscopy. smFRET histograms of differently FRET-labeled constructs were so complicated that they could only be resolved with the help of kinetic experiments on immobilized riboswitches and hidden Markov modeling (HMM) analysis. At least four conformational states were identified, both in the presence and the absence