Mechanical manipulation at the single molecule level of proteins exhibiting mechanical stability poses a technical challenge that has been almost exclusively approached by atomic force microscopy (AFM) techniques. However, due to mechanical drift limitations, AFM techniques are restricted to experimental recordings that last less than a minute in the high-force regime. Here we demonstrate a novel combination of electromagnetic tweezers and evanescent nanometry that readily captures the forced unfolding trajectories of protein L at pulling forces as low as 10~15 pN. Using this approach, we monitor unfolding and refolding cycles of the same polyprotein for a period of time longer than 30 minutes. From such long lasting recordings, we obtain ensemble averages of unfolding step sizes and rates that are consistent with single molecule AFM data obtained at higher stretching forces. The unfolding kinetics of protein L at low stretching forces confirms and extends the observations that the mechanical unfolding rate is exponentially dependent on the pulling force within a wide range of stretching forces spanning from 13 pN up to 120 pN, thereby excluding the presence of curvature in the rate-versus-force plot. Our experiments demonstrate a novel approach for the mechanical manipulation of single proteins for extended periods of time in the low-force regime, providing an ideal complement to force-clamp AFM, expanding the accessible regions of the unfolding energy landscape of a mechanically stable protein.

2860-Plat
Magnetic Tweezers Measurement of Single Molecule Torque
Alfredo Celedon[1], Sean Sun[2], Gregory Bowman[1], Denis Wirtz[1], Peter Searson[1].
[1]Johns Hopkins, Rockville, MD, USA, [2]Johns Hopkins, Baltimore, MD, USA.
Helical duplex DNA continually experiences torque from translocating macromolecular complexes and helix unwinding proteins. We have developed a magnetic tweezers methodology using a cylindrical magnet and magnetic nanorods to directly measure torsional stress, or resistive torque, as twists are introduced at low pulling forces. We demonstrate the utility of this method by measuring the resistive torque of single DNA molecules and, for the first time, single chromatin fibers.
Figure: New and conventional magnetic tweezers configurations. (a) In conventional magnetic tweezers, the field orients the induced dipole of the superparamagnetic bead horizontally, producing a strong horizontal angular trap that prevents angular fluctuations of the probe. (b) In the new configuration consisting of a vertical magnetic field and nanorod-bead construct, the magnetic field and the probe dipole align vertically, thus horizontal angular movements are not constrained. A weak horizontal force generates a weak horizontal angular trap allowing us to measure the torque applied to the molecule. (c) Scanning electron micrograph of magnetic Ni-Pt nanorods (bar = 1μm). (d) Bright-field image of nanorod-bead probe. Nanorod and bead self-assemble by magnetic attraction.

2861-Plat
A Novel Way To Combine Magnetic Tweezers and Fluorescence Microscopy For Single Molecule Studies
Ludovic Disseau[1], Judith Miné[2], Monique Dilhan[3], Henri Camon[3], Jean-Louis Viovy[1].
[1]Institut Curie, Paris, France, [2]Columbia University, New York City, NY, USA, [3]LAAS CNRS, Toulouse, France.
Single-molecule experiments make it possible to look at and manipulate individual molecular systems in real time and do not suffer from averaging over a large number of unsynchronised events. In magnetic tweezers, one traps a single DNA molecule between a glass surface and a magnetic bead, and exerts controlled force and topological constraint on the double helix. However, visualization of events occurring along the molecule is made difficult by the geometry of the device, in which the trapped molecule is pulled perpendicular to the observation plane. We developed a new generation of magnetic tweezers that addresses this problem, while keeping the mechanical control of the trapped molecule. The DNA-bead system is injected into a microfluidic channel placed on a home-made epifluorescence microscope. The channel's top surface is coated with a reflective metallic layer and is tilted so that the whole length of the stretched tether's reflection will be located at the focus of the objective. We applied this instrument to the study of homologous recombination, a DNA double-strand break repair pathway playing an essential role in vivo in genome maintenance and replication. The functional form of the central protein in this process, RecA in bacteria and Rad51 in eukaryotes, is a nucleoprotein complex in which protein monomers assemble into a helical filament on single-stranded DNA. This filament promotes homologous pairing and strand exchange with duplex DNA. Despite the extensive literature on homologous recombination, several key aspects of the detailed molecular mechanism remain quite controversial, especially concerning how the nucleoprotein filament interacts with homologous DNA and exchanges strands. For the first time, our instrument allowed us to observe individual nucleoprotein filaments, labeled with quantum dots, interacting with a double-stranded DNA molecule held by the tweezers.

2862-Plat
First-principles Calculation Of DNA Looping In Tethered Particle Experiments
Kevin B. Towles[1], John F. Beausang[1], Hernan G. Garcia[2], Rob Phillips[2], **Philip Nelson**[1].
[1]Univ Pennsylvania, Philadelphia, PA, USA, [2]Caltech, Pasadena, CA, USA.
We show how to calculate the probability of DNA loop formation mediated by regulatory proteins such as Lac repressor, using a mathematical model of DNA elasticity. Our approach has new features enabling us to compute quantities directly observable in Tethered Particle Motion (TPM) experiments; e.g. it accounts for all the entropic forces present in such experiments. Our model has no free parameters; it characterizes DNA elasticity using information obtained in other kinds of experiments. It can compute both the ''looping J factor'' (or equivalently, looping free energy) for various DNA construct geometries and repressor concentrations, as well as the detailed probability density function of bead excursions. We also show how to extract the same quantities from recent experimental data on tethered particle motion, and compare to our model's predictions. In particular, we present a new method to correct observed data for finite camera shutter time.
The model successfully reproduces the detailed distributions of bead excursion, including their surprising three-peak structure, without any fit parameters and without invoking any alternative conformation of the repressor tetramer. However, for short DNA loops (around 95 bp) the experiments show more looping than is predicted by the linear-elasticity model, echoing other recent experimental results. Because the experiments we study are done in vitro, this anomalously high looping cannot be rationalized as resulting from the presence of DNA-bending proteins or other cellular machinery. We also show that it is unlikely to be the result of a hypothetical ''open'' conformation of the repressor.
Ref: KB Towles et al, accepted for publication in Physical Biology.

2863-Plat
Single Molecule Investigations of HSP70 Proteins
Koyeli Mapa[1], Martin Sikor[2], Karin Waegmann[1], Walter Neupert[1], Dejana Mokranjac[1], **Don C. Lamb**[2,3].
[1]Physiological Chemistry, LMU Munich, Munich, Germany, [2]Physical Chemistry, LMU Munich, Munich, Germany, [3]Department of Physics, University of Illinois at Urbana-Champaign, Urbana, IL, USA.
Heat Shock Proteins 70 (HSP70) are a class of proteins involved in protein folding and are highly upregulated when a cell is under stress. The protein consists of three functional domains: the N-terminal ATP binding domain where ATP and ADP can bind, the substrate binding domain which can bind small residues and the C-terminal domains which acts as a lid for the substrate binding domain. It is thought that the binding of ATP/ADP drives conformational changes in this protein.
Using burst analysis with pulsed interleaved excitation, we have performed single-pair Förster resonance energy transfer (FRET) experiments to investigated the distribution of conformations in HSP70 molecules under different conditions. The FRET efficiency is very sensitive to the distance between donor and acceptor on the scale of 2-10 nm and thus provides information over the conformation of the different HSP70 domains as they diffusion through the focus of our confocal microscope. As experiments are performed on single molecules, subpopulations can be directly observed. We have investigated the conformation of HSP70 by cloning a number of mutants that allowed specific labeling of the different domains. Experiments were performed with or without ATP, ADP, and nonhydrolysable ATP analogs as well as in the absence or presence of peptide substrates or cochaperons. We will present how the conformation and flexibility of HSP70 is influenced by these various interactions.

2864-Plat
Single-molecule Observations of Replisome Structure and Function
Joseph J. Loparo, Samir M. Hamdan, Charles C. Richardson, M. van Antoine Oijen.
Harvard Medical School, Boston, MA, USA.
DNA replication requires the coordinated activity of a large number of enzymes at the replication fork. Understanding the mechanisms controlling this organization requires a direct probing of the dynamics of fully functional replisomes during replication. Observations at the single-molecule level provide the most direct way to visualize the complex biochemistry of the replisome and to quantify the many transient intermediates essential to replication. We present a novel assay that combines the observation of individual fluorescently labeled proteins with the mechanical manipulation of DNA. Surface-tethered DNAs labeled

with quantum dots are hydrodynamically stretched and imaged with a TIRF microscope. Activity of the replisome is observed as a change in the DNA length due to the differing force-dependent extension of single- and double-stranded DNA at low pico-Newton forces. We employ a two-color imaging scheme to monitor DNA length in real-time and to stroboscopically image fluorescently labeled single proteins interacting with DNA. Observation of labeled proteins in an ongoing replication reaction allows us to pose structural questions about the stoichiometry and exchange of proteins at the prokaryotic replication fork. We will discuss preliminary results on primer extension by the T7 DNA polymerase and strand-displacement synthesis by the coupled activity of the T7 helicase and polymerase.

2865-Plat

Real-time DNA Synthesis Dynamics Of Single f29 DNA Polymerase Molecules With Base Pair Resolution

Jonas Korlach.
Pacific Biosciences, Menlo Park, CA, USA.

Real-time observation of uninterrupted DNA synthesis by individual f29 DNA polymerase molecules is presented. Four spectrally distinct fluorophores were used to label the four nucleotides via the terminal phosphate moiety, enabling identification of nucleotide incorporation events into the growing DNA strand with base-pair resolution, and correlating these events to the DNA template sequence. Immobilization of polymerase molecules inside zero-mode waveguide nanostructures allowed detection of incorporation events at 100-500 nM nucleotide concentrations, resulting in polymerization rates of ~1-5 bases/s, and lasting for thousands of bases synthesized. Many aspects of the underlying DNA polymerase dynamics are directly observable, such as nucleotide binding, catalysis, pausing, persistence of distinct kinetic states, DNA template sequence context effects, and switching between polymerization and exonuclease activities.

2866-Plat

Anchoring, Sliding, And Rolling: Visualizing The Three-dimensional Nano-motion And Orientation Of A Single Virus As It Diffuses On A Flat Membrane

Philipp Kukura, Helge Ewers, Alois Renn, Ari Helenius, Vahid Sandoghdar.
ETH Zurich, Zurich, Switzerland.

The localization of objects within the cell and the accurate measurement of relative positions on the molecular level are essential to an understanding of the function of macromolecular complexes. As a consequence, much effort has been directed towards developing imaging techniques that allow the temporal and spatial resolution of events on the nanoscopic scale. Due to their non-invasive nature, optical techniques are particularly suited for studying live samples and several methods have recently demonstrated subdiffraction resolution. However, all these novel methods are based on detecting fluorescence and thereby face strict limitations in accuracy, time-resolution and dimensionality. Here, we show how the combination of label-free detection of nano-objects and single molecule fluorescence detection allows one to map the center of mass motion and the absolute orientation of a single virus with nanometer resolution in real time. We use interferometric scattering detection to resolve the position of individual virions of Simian Virus 40 (a 45 nm DNA tumor virus) with 2 nm accuracy while bound to its cellular receptor GM1 in supported membrane bilayers. At the same time, we detect the fluorescence of a single fluorescent quantum dot attached to the virus via streptavidin-biotin linkage and determine its position with 4 nm accuracy. By overlapping the fluorescence and scattering trajectories, we can resolve the absolute three-dimensional nano-motion of the virus as it diffuses on a two-dimensional membrane. We find that membrane-bound virions exhibit different modes of motion that are strongly influenced by the concentration of the GM1 receptor in the membrane. Besides Brownian motion in the plane of the membrane, we also observe rolling motion on the sub-20 nm scale and periods of apparent standstill in both two and three dimensions.

Platform AZ: Heme Proteins

2867-Plat

The Single Domain Hemoglobin From Campylobactor Jejuni: The Unique Structural Features Underlying Its NO Dioxygenase Activity

Syun-Ru Yeh[1], Changyuan Lu[1], Tsuyoshi Egawa[1], Yu Lin[1], Masahiro Mukai[2], Guanghui Wu[3], Mark Shepherd[3], Robert K. Poole[3].
[1]Albert Einstein College of Medicine, Bronx, NY, USA, [2]Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan, [3]University of Sheffield, Sheffield, United Kingdom.

Recently three groups of hemoglobins (Hbs) have been identified in unicellular organisms: (1) the truncated Hbs (trHb) that display a novel two-over-two alpha-helical structure, (2) the flavohemoglobins (FHb) that comprise a Hb domain with a classical 3-over-3 alpha-helical structure and a covalently attached flavin-containing reductase domain, and (3) the single-domain Hbs (sdHb) that exhibit high sequence homology and structural similarity to the Hb domain of FHb. On the basis of phylogenetic analysis, the trHbs can be further divided into three subgroups: TrHb-I, TrHb-II, and TrHb-III. C. jejuni contains two globins, a single domain hemoglobin, Cgb, and a truncated hemoglobin, Ctb. Neither Cgb nor Ctb are required for the survival of the bacterim in air. Cgb knock-out mutant cells are hypersensitive to reactive nitrogen species, while Ctb knock-out cells do not display any sensitivity to nitrosative stress. As the expression of Cgb is induced by nitrosative stress, it is believed to function as a NO dioxygenase to protect C. Jejuni against the toxic effects of NO. In contrast, Ctb is thought to be involved in regulating O2 flux into and within the cell. To study the structural and functional properties of Cgb, we have purified the recombinant Cgb protein expressed in E. coli. Due to its high affinity towards cyanide, Cgb is isolated in the cyande-bound ferric state. By using resonance Raman scattering and fast kinetic techniques, we have studied the structural, functional and ligand binding properties of Cgb, with respect to Ctb as well as other globins. The implications of these data will be discussed in the context of the NO dioxygenase chemistry carried out by this fascinating globin.

2868-Plat

Two distinct functional globin classes in *Caenorhabditis elegans*

Eva Geuens[1], David Hoogewijs[2], Evi Vinck[1], Sabine Van Doorslaer[1], Jacques Vanfleteren[2], Luc Moens[1], **Sylvia Dewilde**[1].
[1]University of Antwerp, Wilrijk, Belgium, [2]Ghent University, Ghent, Belgium..

The nematode *Caenorhabditis elegans* expresses 33 different globin genes. We studied the expression of two of them, *glb-1* and *glb-26* in more detail using green fluorescence protein technology and found that both are expressed in distinct subsets of cells. GLB-1::GFP is mainly observed in head and tail muscular or hypodermal tissue and in a subset of nerve cells. GLB-26::GFP is seen in the head mesodermal cell and in stomato-intestinal muscle. Hypoxia causes upregulation of the expression of *glb-1,* but not *glb-26.* After expression in an *E. coli* system purified GLB-1 and GLB-26 were spectroscopically (UV/VIS, resonance Raman, electron paramagnetic resonance) and kinetically characterized. The 3D structure of GLB-1 was determined.

GLB-1 is pentacoordinated and exhibits high affinities for O_2 and CO. The bound O_2 is stabilized through hydrogen bonding interactions involving a B10 tyrosine and an E7 glutamine. In contrast, in absence of ligands, GLB-26 is strongly hexacoordinated with E7 distal histidine as sixth ligand. In the presence of O_2, this globin is instantly oxidized to the ferric form and is therefore incapable of reversible oxygen binding. Most likely this molecule will function as an electron transfer protein.

Taken together these data strongly suggest that GLB-1 and GLB-26 belong to two globin classes with totally different functions. Potential functions will be discussed.

2869-Plat

Peroxidase Activity Of Respiratory Proteins. The Role Of Protein Bound Free Radicals

Dimitri Svistunenko.
University of Essex, Colchester, United Kingdom.

Peroxidase activity of respiratory haem proteins is associated with formation of two active species on the protein when it reacts with peroxides: the oxoferryl state of the haem and a protein bound free radical. Both the free radical and the oxoferryl haem are capable of oxidising a range of substrates. General principles of formation of these two species and of their further reactions will be considered. Experimental confirmation of the proposed view involves a complex analysis of diverse pathways by which electrons can be passed between different parts of the protein. Associated with this electron transfer, the process of free radical character transfer around the protein as well as between different protein molecules can be monitored by the EPR spectroscopy. If a protein contains tyrosine residues (which is often but not always the case), the chance of observing a tyrosyl radical is high. EPR data will be presented on formation, transformation and transfer of protein bound radicals in different respiratory proteins. A method will be described that allows very accurate determination of the tyrosyl radical parameters. By using the method, it is possible to determine the three principal g-values of the radical solely from an X-band EPR spectrum. (And if a high field EPR spectrum of the radical is available, and the g-values are measured directly, the other radical parameters, e.g. the hyperfine interaction constants, can be determined much more accurately.) The radicals parameters extracted from experimental EPR spectra are then compared with either some