Thus, exposure to a high stretching force will result in unraveling of these proteins and lead to a loss of their functionality. To overcome this challenge, we combine protein engineering with single molecule force spectroscopy to demonstrate that domain insertion is an effective strategy to control the mechanical unfolding hierarchy of multi-domain proteins and effectively protect mechanically labile domains. As a proof-of-principle experiment, we spliced a mechanically labile T4 lysozyme (T4L) into a flexible loop of a mechanically stronger host domain GL5 to create a domain insertion protein. Using single molecule force spectroscopy, we showed that the mechanically labile T4L domain unfolds only after the mechanically stronger host domain GL5 has unfolded. Such a reverse mechanical unfolding hierarchy effectively protects the mechanically labile T4L domain from applied stretching force and significantly increased the lifetime of T4L. The approach demonstrated here opens the possibility to incorporate labile proteins into elastomeric proteins for engineering novel multifunctional elastomeric proteins.

**3204-Plat**
**Paleoenzymology at the Single-Molecule Level: Probing the Chemistry of Resurrected Enzymes with Force-Clamp Spectroscopy**
**Raul Perez-Jimenez**[1], Alvaro Inglés-Prieto[2], Inmaculada Sanchez-Romero[2], Jorge Alegre-Cebollada[1], Pallav Kosuri[1], Sergi Garcia-Mañes[1], Eric Gaucher[3], Jose M. Sanchez-Ruiz[2], Julio M. Fernandez[1].
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A journey back in time is possible at the molecular level by resurrecting proteins from extinct organisms. In the last two decades several methods based on statistical theory have been developed to computationally reconstruct ancestral protein sequences. Laboratory resurrection of these ancestral proteins provides an excellent opportunity to explore aspects of ancient life that cannot be inferred from fossil records. Here we report the resurrection of ancestral thioredoxin enzymes (Trx) from the Precambrian era, dating back between 1.5 to 4 billion years (Gyr). Using single molecule force-clamp spectroscopy we demonstrate that all ancestral enzymes efficiently reduce disulfide bonds. From the force-dependency of the rate of reduction of an engineered substrate, we conclude that the Precambrian enzymes have similar chemical mechanisms of reduction that the extant enzymes. By contrast, the resurrected enzymes show thermal stabilities 20 to 30 °C higher than those of modern E. coli and human Trx as revealed by Differential Scanning Calorimetry (DSC). This high thermostability illustrate that ancient organisms lived in hot environments that have progressively cooled. Trx enzymes adapted to these environmental temperatures with similar chemical mechanisms than those observed in extant Trxs. Our work demonstrates that the combination of single molecule force spectroscopy together with the resurrection of ancestral proteins is a powerful new approach to study molecular evolution and the sequence-chemistry relationship in enzymes.

**3205-Plat**
**The Unfolding Behavior of RNase H Under Force**
**Jesse Dill**, Carlos Bustamante, Susan Marqusee.
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We have used optical tweezers to revisit the energy landscape of E. coli RNase H under mechanical force. This protein's equilibrium energetics and folding pathway have been studied in bulk and in single-molecule mechanical denaturation experiments, which showed the presence of a collapsed folding intermediate that is on-pathway to the native state (1).
Taking advantage of improvements in our optical tweezers instrumentation, we have now revealed the existence of a short-lived high-energy unfolding intermediate. Our data suggest that this intermediate is obligatory in the mechanical unfolding pathway for this protein. This unfolding intermediate appears to be a local, partial denaturation of the C-terminus of the protein's structure. We explore the energetics of this transient state, and characterize how it defines the unfolding kinetics of RNase H.
1) Cecconi, Shank, Bustamante, Marqusee. Science 2005

**3206-Plat**
**Analysis of Reversible Two-State Systems Under Force**
**Raymond W. Friddle**[1], Peter Talkner[2], James J. De Yoreo[1].
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The dissociation of inter- and intra-molecular bonds by force is a process that occurs regularly in biological machinery and many cellular events. Forcing such transitions in a controlled environment has also emerged as a modern practice in the laboratory for studies of the physical principles of bond lifetimes and protein unfolding. It is commonly assumed that force-driven dissociation is irreversible, which leads to the analysis of first-passage statistics and results in simple analytical results for the distribution and moments of the transition force. However, the irreversible model is a first-order approximation which is only valid very far from equilibrium, or under specific irreversible circumstances. Furthermore, the irreversible model has led many to conclude that force spectra that deviate from linearity unequivocally represent multiple energy barriers along the intermolecular reaction coordinate.
We show that irreversible first-passage analysis, which fails for two-state systems, can be replaced by analyzing the conditional single-passage time between the two states. We find simple solutions for the forward and time-reversed distributions of the transition force, and the isothermal work, which analytically satisfy the fluctuation theorem. We also define how stochastic force trajectories should be measured when multiple forward-reverse events occur. By accounting for reversibility, we show that both the distribution and the first moment of the rupture force significantly differ from the irreversible model and clearly connect with the equilibrium regime. We find that the resulting spectrum of rupture forces is not monotonic with log of the loading rate, but follows at least two major regimes - a linear-response and a dynamic response - with the linear regime tending to the equilibrium free energy change. We validate our analytical results with simulations and experimental data on bond rupture and protein unfolding.

**3207-Plat**
**Multi-dimensionality of Proteins' Free-Energy Landscapes Revealed by Mechanical Probes**
**Emanuele Paci**[1], Zu Thur Yew[1], Michael Schlierf[2], Matthias Rief[3].
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The study of mechanical unfolding, through the combined efforts of atomic force microscopy and simulation, is yielding fresh insights into the free-energy landscapes of proteins. One-dimensional models of the free-energy landscape have been widely used to analyze experiments. We show that as the two ends of a protein are pulled apart at a speed tending to zero, the measured mechanical strength of filamin plateaus at about 30 pN instead of going towards zero. Simulations reproduce this phenomenon and indicate that it can be explained by a switch between parallel pathways. Insightful analysis of mechanical unfolding kinetics needs to account for the multi-dimensionality of the free-energy landscapes of proteins, which are crucial for understanding the behavior of proteins under the small forces experienced in vivo.

**3208-Plat**
**Noise Induced Regulation of DNA Loops**
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Protein-mediated DNA loop formation is a ubiquitous means of regulating gene transcription. Loop formation in ds-DNA is driven by tiny forces on the order of fN arising from thermal fluctuations within the intracellular environment. Surprisingly, these forces are much smaller than the typical piconewton forces that arise from various intracellular processes, such as the procession of molecular motors or DNA-cytoskeletal attachments. This has led to theoretical predictions, and a recent experimental confirmation by our lab, that forces as small as a few hundred femtonewtons can severely reduce the rate of loop formation. We further explore the utility of using tension as a regulatory mechanism by asking how this mechanism is effected by noise. From empirical data that we have collected on loop formation rate as a function of substrate tension, we develop an effective potential that reproduces the loop transition rates given by the mean-first passage time of escape from the potential. We next incorporate this effective potential into a stochastic model of DNA subjected to an applied, fluctuating force.
The theory predicts a strong enhancement in the rate of loop formation under increasing levels of noise and, when normalized to the noise free rate, displays a universal behavior relatively independent of the mean force. This suggests that applying a varying level of tension to the DNA may be a robust method for regulating transcription. We then compare the theory with an experiment performed in our lab where we have subjected DNA, capable of forming LacI mediated loops, to a fluctuating force by means of axial optical tweezers.

**3209-Plat**
**Single Molecule Force Spectroscopy of Peptide Aptamers**
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Aptamers have broad applications in sensing, diagnostic, therapy and in the design of novel materials via molecular assembly. Peptide aptamers engineered to

have high affinity for specific materials (carbon nanotubes, glass, polystyrene) were studied for the first time at the single molecule level with optical tweezers. One-micron DNA tethers are used to connect the peptide to the trapped bead. Single-molecule force spectroscopy studies revealed similar rupture forces between the aptamers of ~20pN at loading rates in the range of 1-10pN/s. Optical tweezers were found to be a powerful tool to probe this type of non-covalent biomolecular interactions.

The single molecule rupture force probabilities are fitted to force spectroscopy models [1] in order to extract information such as the lifetime of the aptamer-material bond as a function of force, the distance along the pulling direction between the free-energy minimum and the transition state, and the free energy of activation.

In order to demonstrate the generality of our single molecule assay for several types of biomolecular interactions, antibody-antigen rupture forces were also measured following the same procedure and kinetic information was obtained from the fits. Extracting off-rates from single-molecule techniques can aid ligand optimization, receptor design, and screening processes. We include a comparison study of the interaction between the antigen fluorescein and its murine monoclonal antibody, clone 4-4-20 using this tethered bead assay.

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**3210-Plat**

**Strength of Non-Covalent Biomolecular Interactions Probed at the Microsecond Timescale**

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We have measured strength of non-covalent interactions at the microsecond timescale by using a recently developed high-speed force spectroscopy technique. The resulting loading rates narrow the gap between time scales of experimental methods and molecular dynamics simulations substantially. Measurements on biotin-streptavidin complexes provide direct experimental verification of forces predicted by steered molecular dynamics simulations [1]. This technique uses a T-shaped atomic force microscope cantilever with its tip placed offset from the longitudinal axis [2,3]. When this cantilever vibrates at its vertical resonance, instantaneous forces acting on the tip are detected by the twisting motion due to its large mechanical bandwidth. As a result, force-distance curves are generated at every cycle of the vertical oscillations. Approximately ten thousand force curves are generated every second and analyzed in real time. The dramatic enhancement in measurement speed also enables a chemically specific imaging technique based on single molecule force spectroscopy.

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# Workshop 4: Membrane Zoology: Model Membranes of Increasing Complexity

**3211-Wkshp**

**Measurements of Reductionist Membranes that Beautifully Fit Physics Theories**

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Micron-scale liquid domains appear in lipid membranes containing three lipid types (lipids with high melting temperature, lipids with low melting temperature, and cholesterol or a similar sterol) when the membrane is below a miscibility transition. When this transition occurs at a critical point, large fluctuations appear within the membranes. The fluctuations are described by beautiful physics: the critical exponents for correlation length and for the difference in composition between the two phases are consistent with the universality class of the 2-dimensional Ising model (Honerkamp-Smith et al., BJ, 2008). Complex mixtures of lipids and proteins derived from cell membranes in GPMVs (giant plasma membrane vesicles) exhibit the same critical behavior (Veatch et al., ACS Chem. Biol., 2008). Recently, we measured the effective dynamic critical exponent relating the decay time of membrane composition fluctuations to the wavenumber (an inverse length). We find that at temperatures far from the critical point, the exponent is 2, as expected from diffusion. As the temperature approaches the critical point, the exponent increases. We find that submicron membrane fluctuations corresponding to a wavenumber of 1/(50nm) persist for at least $0.8 \pm 0.3$ms, on the order of times required for changes in protein configuration (e.g. 1ms). Therefore, similar and longer-lived fluctuations in cell membranes can potentially alter protein function.

**3212-Wkshp**

**Membrane Interactions Mediated by DNA Hybridization**

**Steven Boxer**.
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Our lab has been involved for some time in the development of strategies for assembling and partitioning model membranes on solid supports. While ideally suited for analysis by surface-sensitive methods, the close proximity of the lower leaflet of the supported bilayer to the solid support limits its application, especially for transmembrane proteins. In order circumvent this limitation, we have developed three model membrane architectures in which the bilayer is separated from the support: tethered vesicles using DNA-lipid conjugates which can be used to study vesicle-vesicle interactions and fusion; structures that position a black lipid membrane in close proximity to a highly reflective mirror for interferometry in combination with electrical measurements; and membrane patches tethered to solid supports or to fluid supported bilayers using DNA-lipid conjugates. Each architecture offers specific advantages and opportunities, and recent results will be described.

**3213-Wkshp**

**The Language of Shape: Biological Reactions are Dramatically Affected by the Shape of Lipid Membranes**

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To date the fields of biophysics, biochemistry, molecular and cellular biology and have established exhaustive correlations between the lipid composition of membranes and its impact on membrane properties and protein function. However, in addition to composition the shape of cellular membranes appears to be a well-conserved phenotype in evolution. The characteristic membrane topology of organelles e.g. the folded structure of the endoplasmatic reticulum, is strictly retained in most types of cells. Nevertheless we largely ignore what are the consequences of membrane shape/curvature to biological functions that make it so critical for sustaining life. The lack of information on the significance of membrane shape has predominantly been due to the absence of reliable assays that allow us to perform systematic experiments as a function of membrane shape/curvature. We have recently demonstrated the possibility to construct a high throughput array of unique nanoscale membrane curvatures. The assay is based on unilamellar liposomes of different diameters (30 nm to 700 nm), and therefore curvature, that are immobilized on a surface at dilute densities allowing for imaging of single liposomes with fluorescence microscopy.

Here I will discuss published and unpublished data on two important classes of biomolecular interactions that exhibited dramatic curvature dependence: i) SNARE-mediated docking of single lipid vesicles and ii) membrane anchoring of lipidated proteins, and reveal previously unsuspected consequences of membrane curvature to biological function.

References: Biophys. J. 2008. 95 (3): p. 1176; PNAS. 2009, 106 (30): p. 12341; Angew. Chem. Int. Ed. 2003, 42, p. 5580; Nat. Chem. Biol., doi:10.1038/nchembio.213; EMBO J., in press; Methods in Enzymol., in press.

**3214-Wkshp**

**Sorting of Proteins and Lipids in Membrane Curvature and Composition Gradients**

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The sorting of lipids and proteins in cellular membrane sorting centers such as the trans-Golgi network, the plasma membrane, and the endocytic recycling compartment, lies at the heart of fundamental biological phenomena such as organelle homeostasis, membrane signaling, and trafficking. Our research is directed at understanding biophysical contributions to the sorting of membrane components, using experimental lipid model membranes, and analytical thermodynamic and membrane elasticity theory.

We will present measurements of thermodynamically reversible membrane curvature sensing for several peripherally binding membrane proteins, including toxins, endocytic accessory proteins, as well as naturally unfolded proteins. For example, whereas the cholera toxin subunit B is observed to partition away from regions of high positive membrane curvature, we show that the Epsin N-terminal homology domain enriches in such regions.

Our recent research has furthermore shown that ideally diluted lipids are not significantly sorted in curvature gradients presented by a cylindrical membrane