Two types of transcriptional repression in living cells of [i]Bacillus subtilis[/i] characterized by number and brightness analysis

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Conformational Dynamics of Mitochondrial Hsp70
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Recently, we have investigated the conformation of Mitochondrial Heat Shock Protein 70 (mtHsp70), a protein involved in the import of proteins into mitochondria and in protein folding in the mitochondrial matrix. To investigate the distribution of conformations in mtHsp70 molecules under different conditions, we performed single-pair Förster resonance energy transfer (spFRET) experiments using burst analysis and pulsed interleaved excitation (PIE). The FRET efficiency is a very sensitive measure for the distance between donor and acceptor on the scale of 2-10 nm and thus provides information over the conformation of mtHsp70 during its conformational cycle. To extract quantitative information about the homogeneity of the observed states, we used probability distribution analysis on the burst analysis data.
MtHsp70 consists of two functional domains: the N-terminal nucleotide binding domain and the C-terminal substrate binding domain with a lid to hold the substrate peptide. Experiments were performed using FRET sensors for the interdomain distance and for the lid conformation with different nucleotides as well as in the absence or presence of peptide substrates and cochaperons. Our results show a surprisingly heterogenous conformational distribution for ADP bound mtHsp70, opposed by a more homogenous state with an open lid and close domain in the ATP bound state. Substrate binding in the presence of the cochaperone Mdj1 results in the undocking of the domains and closure of the lid holding the peptide. To investigate the conformational dynamics on a longer timescale (10 ms to 10 s) , we have performed spFRET TIRF experiments on immobilized molecules. We utilized two techniques to avoid immobilization artifacts. Either the protein was encapsulated in lipid vesicles or the substrate peptide was immobilized. The dynamics for the two sensors in the different states were compared.

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A Microfluidic Cell Sorter for Directed Evolution of Fluorescent Proteins Based on Dark-State Conversion and Photobleaching
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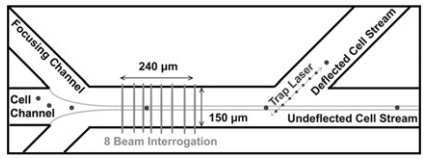
Fluorescent proteins (FPs) have revolutionized cellular imaging, however, they have a tendency to convert to non-radiative dark states and to undergo rapid, irreversible photobleaching. These effects limit their use in imaging applications involving low concentrations or long observation times. We demonstrate an innovative microfluidic cytometer for high-throughput sorting of FP-expressing mammalian cells based on the selective measurement of photostability, magnitude of dark-state conversion, or the combined effects of both. In the cytometer (shown schematically), eight elliptically shaped laser beams intersecting a hydrofocused stream in a microfluidic channel stroboscopically interrogate each cell. Subsequently, a piezo-steered infrared trapping beam deflects cells for sorting. The multi-beam sequence provides millisecond timescales of excitation and dark intervals to separate the effects of dark-state conversion and irreversible photobleaching. This technology is uniquely capable of sorting genetic libraries of cell-based FPs on the basis of photokinetics, independent of FP expression level. Furthermore, its ability to investigate two processes that limit total photon output of FPs make this device a versatile and powerful tool for characterizing FP photophysics and engineering a new generation of either brighter, more photostable FPs, or photoswitchable FPs.



965-Plat
Objective, Bayesian Analysis of Fluorescence Correlation Spectroscopy Data
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Fluctuations in fluorescence intensity provide quantitative insight into a range of molecular processes including diffusion, active transport, and binding kinetics in living cells and tissues. Classical fluorescence correlation spectroscopy (FCS) measures fluctuations in fluorescence intensity in a small detection volume to infer molecular transport properties from governing continuum diffusion-convection equations. Essential to the interpretation of FCS data is the use of an underlying mathematical model that governs the molecular process under study. Autocorrelations in fluctuations in fluorescence intensity may then be fit to the analytical solution of the model, and corresponding molecular properties may be inferred. While the choice of physical process may be unambiguous for special cases of free molecular diffusion or convective transport in solution, the correct choice of model becomes considerably less unambiguous in the application of FCS to complex biological processes in living cells or extracellular matrices. For this reason, an objective and unbiased approach to model selection and parameter estimation is of interest. The method of Bayesian inference provides such a framework. At the level of parameter estimation, Bayesian inference is similar to Maximum Likelihood Estimation when flat priors are chosen for initial parameter estimates. At the level of model selection, however, Bayesian inference assigns explicit model probabilities that are proportional to their marginal likelihoods by considering the full range of parameter values and their posterior probability distributions rather than only using point-wise, Maximum Likelihood estimates, thereby appropriately penalizing model complexity and preventing over-fitting of experimental data. Here we illustrate application of Bayesian inference to the problem of model selection and parameter estimation of molecular transport properties from FCS data, comparing it to traditional model selection approaches.

966-Plat
Single and Two-Photon Sensitized Acceptor Emission and Anisotropy Studies of Protein-Protein Interactions
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Donor lifetime modification by Förster Resonance Energy Transfer (FRET) is widely used to detect protein-protein interactions and protein conformation change. Such measurements can be compromised by the presence of a significant non-interacting fraction of molecules. We have recently shown that FRET arising from homodimerisation in 3-phosphoinositide dependent protein kinase 1 (PDK1) can be measured for a small fraction of the total protein population from the rise-time in sensitized acceptor emission [1]. We present single and two-photon lifetime and anisotropy measurements of homodimerisation in EGFP and mCherry labelled PDK1 constructs. A computational model was employed to determine the limits and relative probabilities of the FRET orientation parameter using intrinsic donor and acceptor anisotropy decays together with that of sensitized acceptor emission. A consideration of the physically allowable minimum separation of the donor and acceptor fluorescent proteins allows physically unrealistic values of the orientation parameter to be removed. This was seen to lead to a reduction in the uncertainty of the mean donor-acceptor distance. Combined wavelength-resolved lifetime and anisotropy measurements show promise as a valuable tool for obtaining accurate angular and distance information from FRET in samples containing a small proportion of interacting molecules.
[1] Masters, T. J., V. Calleja, D. A. Armoogum, R. J. Marsh, C. J. Applebee, M. Laguerre, A. J. Bain and B. Larijani, 2010. ''In vivo regulation of 3-phosphoinositide dependent protein kinase 1 (PDK1) activity by homodimerisation''. Science Signalling (in press).

967-Plat
Two Types of Transcriptional Repression in Living Cells of Bacillus Subtilis Characterized by Number and Brightness Analysis
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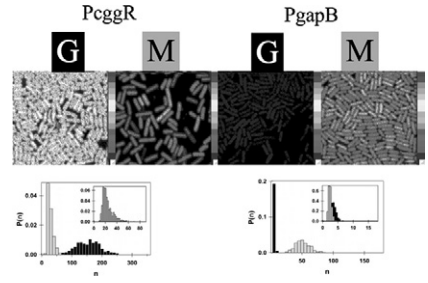
Number and brightness analysis (N&B) is a useful technique for characterizing the concentration and molecular brightness of fluorescent molecules in vivo. Here we investigate stochastic gene expression in Catabolite Repression in Bacillus subtilis. In particular the transcriptional activity of promoters implicated in the switch between glycolysis and gluconeogenesis was investigated. Promoter activity was measured using green fluorescent protein (GFP) promoter fusions.



Two photon laser scanning microscopy and true N&B analysis allowed determination of the absolute concentration of GFP molecules inside the bacterial cells. We collected data on hundreds of B. subtilis cells expressing GFP under control of the promoters of interest and grown under glycolytic or gluconeogenic conditions. Results showed no regulation of the promoter expressing the gluconeogenic repressor, strong repression of the gluconeogenic enzyme promoters and weak auto-repression of the glycolytic promoter, with a highly asymmetric distribution when repressed. All promoters showed strong evidence for transcriptional bursting. Analysis of the data using stochastic models of gene expression is currently underway. The figure shows number maps of bacterial cells grown on glucose(G) or Malate(M). Each change in color represents 10 molecules up to 180.

PLATFORM T: DNA, RNA Structure & Conformation/RNA Folding

968-Plat
Flexibilities of Single-Stranded Nucleic Acids Measured by Single Molecule FRET
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The flexibilities of single-stranded RNA has not been as well studied as double-stranded RNA, despite their significant biological importance. Their values for persistence lengths have often been inferred from that of single-stranded DNA. In this poster, we compare the persistence lengths of poly rU and poly-dT as measured by single molecule FRET experiments. We show differences in flexibilities when the nucleic acids are in the presence of monovalent and divalent salts.

969-Plat
Mechanical Unfolding of the Beet Western Yellow Virus −1 Frameshift Signal
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Using unfolding by optical tweezers and steered molecular dynamics (SMD) simulations we have demonstrated the critical importance of Mg^{2+} ions for the mechanical stability of the BWYV RNA pseudoknot. The optical tweezers experiments pointed to a critical role of stem 1 of the pseudoknot, a finding that was confirmed using the SMD simulations. These simulations supported the notion that the stability of stem 1 is critical for −1 frameshifting, a translational recoding event essential for replication of the BWYV. Furthermore, they clarified the precise role of two Mg^{2+} ions, Mg45 and Mg52, in −1 frameshifting. The ions were shown to play a critical role in stabilizing stem 1 by two possible mechanisms depending upon the hydration of the Mg^{2+} ions. Mg^{2+} ions were either directly forming a salt bridge between the strands of stem 1, or they stabilized parallel orientation of the strands in stem 1. Interestingly, these findings explain the drop in frameshifting efficiency, down to null levels, of the C8U mutant. The large effect of this mutant upon the frameshift efficiency seemed surprising as only a single hydrogen bond appeared to have been sacrificed. However, the SMD simulations clarify how the C8U mutation affects the Mg^{2+} coordination and destabilizes stem 1 of the pseudoknot.

970-Plat
Two Distinct Overstretched DNA States
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Double-stranded DNA can undergo an ''overstretching'' transition within a narrow force range when the external force reaches around 60 pN. The basic question of the overstretched DNA is whether the strands are separated or not after transition. Despite numbers of studies, this question still remains controversial. In this research, we directly show that DNA overstretching transition actually involves two distinct types of double-helix reorganization: slow hysteretic ''unpeeling'' of one strand off the other; and a fast, non-hysteretic transition to an elongated double stranded form. The competing between these two overstretched forms is sensitive to factors that affect DNA base pair stability: DNA sequence, salt concentration, and temperature. The balance between the two forms shifts near physiological solution conditions. This result clearly demonstrates that an overstretched double stranded state does exit. It also shows that DNA double helix physical properties, unpeeling or overextension, can be selected via small changes in molecule environment.

971-Plat
Magnetic Torque Tweezers and Their Application in Probing the Torsional Properties of DNA, RNA, and DNA Filaments
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The double-stranded nature of DNA links processes such as replication, transcription, and repair, to rotational motion and torsional strains. Magnetic tweezers (MT) are a powerful single-molecule technique to apply both forces and torques to individual molecules. However, while the forces applied in conventional MT can be calibrated from thermal fluctuations or computed from first principles [1], direct measurement of the torque is challenging.
Here we present the magnetic torque tweezers (MTT), which enable the direct measurement of torque [2] based on a tracking protocol that monitors x, y, z, and angle and on a redesigned magnet configuration. We have applied the MTT to dsDNA, dsRNA, and RecA-DNA heteroduplex filaments. Our measurements of the effective torsional stiffness C of dsDNA indicate a significant force dependence, with $C \approx 40$ nm at low forces up to $C \approx 100$ nm at high forces, reconciling previous partially conflicting measurements. Torque measurements on RecA-DNA heteroduplex filaments reveal an initial torsional stiffness about two-fold higher than that of DNA. However, at relatively moderate torques further build-up of torsional strain is prevented by partial RecA disassembly and structural transitions in the filament. Preliminary results on the torsional properties of fully dsRNA indicate static properties overall similar to dsDNA, but significantly different dynamics of supercoil formation.
Finally, we compare the MTT configuration with recently developed strategies to measure torque using optical tweezers [3] and with a related MT approach that allows straight-forward measurements of free rotation termed freely-orbiting magnetic tweezers [4].
[1] Lipfert, Hao & Dekker, *Biophys. J.* (2009).
[2] Lipfert, Kerssemakers, Jager & Dekker, *Nature Methods, in press* (2010).
[3] Forth, *et al. Phys. Rev. Lett.* (2008).
[4] Lipfert, Wiggin, Kerssemakers & Dekker, *submitted* (2010).

972-Plat
Structural Characterization of Torsional Destabilization in DNA
Troy A. Lionberger[1], Davide Demurtas[2], Todd Lillian[3], Julien Dorier[4], Noel Perkins[1], Andrzej Stasiak[4], Edgar Meyhofer[1].
[1]Univ. of Michigan, Ann Arbor, MI, USA, [2]Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, [3]Texas Tech. University, Lubbock, TX, USA, [4]University of Lausanne, Lausanne, Switzerland.
Within cells, supercoiled DNA is inherently subjected to various degrees of bending and twisting, and many DNA-binding proteins are now known to be sensitive to these mechanical features of DNA. Under significant levels of bending and/or torsional stress, DNA has been shown to depart from native B-form structure and adopt a number of more energetically favorable alternate conformations. However, very little is known about the structural details of these stress-induced structures. Recently, the DNA nuclease BAL 31 was used to assay for helical destabilizations in small DNA minicircles sustaining fixed amounts of bending and torsional stresses (Nucl. Acids Res., 36(4), 2008). Here, we seek to determine if the helical destabilizations that accompany elevated levels of negative torsional stress in tightly looped minicircles produce localized structures that confer enhanced bending flexibility to the DNA structure, such as would occur in kinked DNA. Towards this end, we synthesized untwisted, 100-bp minicircles that are recognized and digested by BAL 31 and overtwisted 106-bp minicircles that are resistant to degradation by BAL 31. Using cryo-electron microscopy, we then generated three-dimensional image reconstructions of these two minicircle species. From these quantitative descriptions of the minicircle geometries, we observe no evidence of DNA kinking in either the BAL 31-sensitive, 100-bp minicircles or the BAL 31-insensitive, 106-bp minicircles. Since the torsional destabilizations that are recognized by BAL 31 were not observed to confer significant enhancements in bending flexibility, we propose that the torsional destabilizations appearing with the 100-bp minicircle relieve negative torsional stress through the formation of a left-handed dinucleotide stack with no interruption in base-stacking at the right-to-left transition, thus minimally affecting the local bending stiffness. Our observations are consistent with the formation of Z(WC)-DNA, which has been previously theorized to be the predominate form of left-handed DNA appearing in nature.