127-Plat
Multiple Lac-Mediated Loops Revealed by Bayesian Statistics and Tethered Particle Motion
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The bacterial transcription factor LacI loops DNA by binding to two distinct binding sites on DNA. Despite being one of the most well-studied model systems for transcriptional regulation, the number of possible loops, and their structures, are unclear. To clarify this question, we have developed a new analysis method for tethered particle motion (TPM), a versatile single-molecule technique commonly used to study Lac-mediated looping and other DNA-protein interactions in vitro. The method, called vbTPM, is based on a variational Bayes treatment of hidden Markov models. It learns the number of distinct states (e.g., DNA-protein conformations) in a TPM trajectory directly from the data with better resolution than existing methods, and also corrects for common experimental artifacts. Studying short (about 100 bp) LacI-mediated loops, we are able to resolve states and interconversion patterns that are best explained in terms of at least three distinct loop structures. These results indicate that changes in Lac conformation and DNA binding orientation both contribute to the repertoire of LacI-mediated loops, and provides new input for quantitative models of looping and transcriptional regulation.

128-Plat
Substrate Recognition and Specificity of Double-Stranded RNA Binding Proteins
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Recognition of double stranded (ds) RNA fragments is an important part of many cellular pathways, including RNA silencing, viral recognition, RNA editing, processing and transport. dsRNA recognition is often achieved by dsRNA binding domains (dsRBDs). We use atomistic molecular dynamics simulations to explain the binding of transactivation response RNA binding protein (TRBP) dsRBDs to dsRNA substrates, and their lack of binding to dsDNA and DNA-RNA hybrids. Our simulations confirm that dsRBDs bind more favorably to dsRNA than to DNA-RNA and dsDNA duplexes, in agreement with experimental observations. The dsRNA is recognized by dsRBDs through the A-form of both major and minor grooves and by the chemical properties of RNA bases, which have 2'-hydroxyl groups on their sugar rings. Our simulations show that the form of the DNA-RNA duplex can be modulated by dsRBD proteins, potentially explaining how some dsRBD proteins can bind weakly to DNA-RNA hybrids.

129-Plat
Molecular Mechanism of Inhibition of the PKR-RNA Interaction by the Influenza a Virus NS1 Protein - A Three Colour Based Flim-Fret Approach in Living Cells
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Influenza A Virus (IAV) protein NS1 is the major antagonist of the host cellular innate immune response. NS1 has a N-terminal RNA binding domain and a C-terminal effector domain for interaction with immune effector proteins, like RIG-I or PKR. Activation of the double stranded (ds)RNA-dependent protein kinase (PKR) by binding dsRNA induces a global block in protein translation. Hence, inhibition of PKR activation is an important function of NS1.
Here, we study PKR inhibition in a cell-based system with living cells. Therefore we use IAV NS1 protein tagged with a YFP or Turquoise fluorophore in combination with GFP-tagged PKR. For PKR activation we used dsRNA labelled with Rhodamine (Rhd). This combination of fluorophores allows us to detect interaction of all three components at the same time using fluorescence lifetime imaging with Förster resonance energy transfer (FLIM-FRET). The method allows us to detect an interaction in cell culture based systems with laser microscopy.
We show that co-transfection of PKR-GFP, dsRNA-Rhd and NS1-Turquoise leads to a reduction of the FRET efficiency between PKR and dsRNA. That indicates an increase of distance between PKR and dsRNA due to NS1. Furthermore, we could show an enhanced interaction between PKR and NS1 by adding dsRNA leading to the hypothesis that dsRNA is necessary for the interaction between NS1 and PKR. In addition, reduction of FRET suggests that NS1 prevents binding of PKR to dsRNA.
Our study identifies for the first time in a cell-based FLIM-FRET system the changes in interaction by analysing different combinations of PKR, NS1 and dsRNA. In upcoming experiments we will analyse NS1 Mutants with our system to check changes in FRET reduction of NS1 and PKR.

130-Plat
Elucidating Restriction Endonucleases Reaction Mechanisms *via* Dwell-Time Distribution Analysis
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We have developed a Total Internal Reflection Fluorescence (TIRF) microscopy based assay that allows us to simultaneously measure the length of the catalytic cycle for hundreds of restriction endonuclease molecules in one experiment. We stably attach thousands of short duplex DNA molecules, each labeled with a single quantum dot semiconductor nanocrystal, to a passivated glass surface within a flow channel. The disappearance of a quantum dot indicates that its DNA tether has been cleaved. We introduce restriction endonuclease molecules into the channel in the absence of magnesium, which permits binding to, but not cleavage of the surface immobilized DNA substrate. When buffer containing magnesium is introduced into the flow channel, DNA cleavage by the pre-bound restriction endonuclease molecules is initiated. This synchronization allows us to measure the lag time between the introduction of magnesium and the completion of DNA cleavage for the entire population of enzymes. Analysis of the dwell-time distributions can provide insights into the DNA cleavage mechanism. Our observations suggest that EcoRV, a dimeric Type II restriction endonuclease that cleaves the palindromic sequence GAT↓ATC (where ↓ is the cut site), requires two kinetic steps to complete duplex cleavage after prebinding. However, dwell-time distributions suggest that BcnI, which is active as a monomer and cleaves the pseudopalindromic sequence 5'-CC↓SGG-5' (where S stands for C or G), requires more than four kinetic steps to complete duplex cleavage. Furthermore, experiments performed with strand-specific DNA substrates suggest that the number of steps indicated by the dwell-time distribution depends on which strand of the recognition site must be cleaved to result in quantum dot release. By designing additional substrates that mimic the various intermediate states, we plan to dissect the mechanism by which BcnI cleaves each strand of the intact restriction site.

131-Plat
Defense Against Viral Attack: Single-Molecule View on a Bacterial Adaptive Immune System
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Escherichia coli maintain different strategies to protect the cell against invading foreign DNA. In a recently discovered adaptive immune system, fragments of foreign DNA are integrated into specific loci on the bacterial genome, known as clustered regularly interspaced short palindromic repeats (CRISPR). Short CRISPR-derived RNAs (crRNAs) are incorporated into the CRISPR-associated complex for antiviral defence (Cascade) and guide the complex's search for the DNA of returning invaders, which is targeted for destruction upon binding. Recent studies have shown that Cascade must recognize both a target sequence and an immediately adjacent PAM (protospacer adjacent motif) sequence in order for successful targeting of the foreign DNA. The mechanism and structural dynamics of this target recognition and binding process, however, are not well understood. Here we report a single-molecule FRET (Förster resonance energy transfer) based assay to monitor in real time the target recognition process of Cascade. We observe that Cascade binds target DNAs that vary widely in sequence. Notably, a complementary target sequence with a correct PAM will be completely opened up by Cascade, whereas an identical sequence with a mutated PAM will be only partially opened. In addition, the latter exhibits a weaker affinity to Cascade. A sequence that contains a PAM but lacks a correct target sequence, still exhibits binding, but is not opened. Taken together, these results suggest that the discrimination of self from non-self occurs after promiscuous target binding. Our single-molecule study promises to reveal further mechanistic details of target DNA identification by the CRISPR immune system.