surface-tethered vesicles with up to 3–4 consecutive injections per reactor. Development of ultra-small-volume fluidic platforms will enable novel ways to implement simultaneous screening of biochemical properties, molecular function or confined reactions over millions of samples while consuming total reactant volumes of few picoliters.

References:

3053-Plat
Single Molecule Fluorescence Imaging at Micromolar Concentrations
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The conventional total internal reflection (TIR) microscopy or confocal microscopy renders a detection volume of 20-200 attoliters, limiting single molecule experiments to nanomolar concentrations of fluorescently labeled reagents. However, many biological processes require higher concentrations of proteins or substrates. Here, we present a novel combination of existing techniques with confocal microscopy to reduce the detection volume to below 300 zeptoliters, which represents a three orders of magnitude reduction compared to the confocal case. The sub-diffraction focal spot of stimulated emission depletion microscopy accounts for >20-fold reduction of volume in lateral direction and a simple convex lens provides >40-fold confinement in axial direction. This method should allow single molecule studies of complex processes that require transient interactions between multiple components.

Platform: Protein–Nucleic Acid Interactions

3054-Plat
Structural Basis of RNA Recognition and Activation by Innate Immune Receptor RIG-I
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RIG-I-like receptors (RLRs) of the innate immune system are the cell’s principal detector of viral RNA. These proteins distinguish between cellular and viral RNAs by recognition of Pathogen Associated Molecular Pattern (PAMP) motifs that are associated with viral RNAs. RIG-I (Retinoic acid Inducible Gene - I) is a cytosolic pattern recognition receptor that recognizes viral RNA motifs and triggers an immune signaling cascade resulting in type-I interferon induction. RIG-I consists of three domains: the N-terminal Caspase Recruitment Domain (CARD), the central helicase domain and the C-terminal repressor domain (RD). The helicase and RD of RIG-I recognize double-stranded (ds) RNA and 5’-triphosphate RNA as foreign and activate the RIG-I CARD for signaling. However, the nature of RIG-I-RNA interaction remains unclear. To understand how the RIG-I helicase binds RNA and leads to activation, we have determined the structure of the human RIG-I helicase-RD domain bound to dsRNA and ADP•BeF3. The structure of the ternary complex reveals a major contribution of the helicase to RNA binding and a synergy between the helicase and RD in the recognition of blunt-ended dsRNA. Helicase-RD organizes into a ring with the helicase utilizing previously uncharacterized motifs to specifically recognize dsRNA. Additional biochemical and chemical results demonstrate that RIG-I, in absence of RNA is flexible and becomes more compact upon RNA binding. These results provide a greater understanding of the cellular response and immune activation to viral infection. However, the role of ATPase/helicase function of RIG-I remains elusive. The RIG-I helicase-RD represents the first structure of an RNA helicase bound to dsRNA and provides a new perspective in understanding how other homologous RNA helicases may engage their targets.

3055-Plat
Oligomerization of HIV-1 Restriction Factor APOBEC3G Transforms it from a Fast Enzyme to a Slow Nucleic Acid Binding Protein
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Human APOBEC3G (A3G) is a cellular protein that inhibits reverse transcription and replication of human immunodeficiency virus type-1 (HIV-1) in the absence of the viral protein Vif. A3G impairs viral replication by two different mechanisms, which both rely on its ability to bind single-stranded nucleic acids. First, A3G deaminates cytidine bases of viral single-stranded DNA (ssDNA). Secondly, A3G blocks DNA synthesis by reverse transcriptase (RT), the viral DNA polymerase, by a mechanism independent of catalytic activity. Seven A3G proteins are packaged per HIV-1 virion, requiring that each molecule rapidly locate deamination sites on viral ssDNA, which is a transient intermediate during reverse transcription. In contrast, the roadblock mechanism, a model in which A3G oligomerizes on the viral template strand and blocks RT-catalyzed DNA elongation, requires an extremely slow off-rate from single-stranded nucleic acids. We hypothesize that A3G exhibits fast binding kinetics as a dimer, enabling rapid deamination activity, and slow kinetics as an oligomer, preventing RT from elongating viral DNA. We use optical tweezers, in combination with fluorescence anisotropy and surface plasmon resonance, to quantify both types of binding kinetics. DNA stretching experiments reveal that the time constant for oligomerization, ranging from 200 to 1000 s, is inversely dependent on protein concentration. The apparent dissociation constant of A3G oligomerization decreases exponentially with ssDNA incubation time, dropping by an order of magnitude in 1000 s, which suggests that fast binding of catalytically active A3G converts to oligomerization on this timescale. These are slow association and dissociation of A3G oligomers, which is consistent with ensemble methods, supports the roadblock hypothesis. Collectively, our measurements quantitatively characterize the complex, highly unusual nucleic acid binding kinetics of A3G responsible for its dual mechanism for inhibiting viral replication.
probing cooperative interactions between distant DNA regulatory sites that would have been difficult to test otherwise.

3058-Plat
The Primary DNA-Binding Subsite of the Rat Pol Beta. Energetics of Interactions of the 8-kDa Domain of the Enzyme with the ssDNA
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Interactions of the 8-kDa domain of the rat pol β and the intact enzyme with the ssDNA have been studied, using the quantitative fluorescence titration technique. The 8-kDa domain induces large topological changes in the bound DNA structure and engages much larger fragments of the DNA than when embedded in the intact enzyme. The DNA affinity of the domain is predominantly driven by entropy changes, dominated by the water release from the protein. The thermodynamic characteristics dramatically change when the domain is embedded in the intact polymerase, indicating the presence of significant communication between the 8-kDa domain and the catalytic 31-kDa domain. The diminished water release from the 31-kDa domain strongly contributes to its dramatically lower DNA affinity, as compared to the 8-kDa domain. Unlike the 8-kDa domain, the DNA binding of the intact pol β is driven by entropy changes, originating from the structural changes of the complex.

3059-Plat
Turning on the Spliceosome
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The spliceosome is the complex macromolecular machine responsible for removing introns from pre-mRNAs. The processes of spliceosome assembly and activation rely on the coordinated interactions of many dozens of spliceosome components to identify splice sites in a pre-mRNA, build a spliceosome, and activate the spliceosome for catalysis by formation of an active site prior to transesterification. The activation step itself likely involves many intermediates. It results in loss of the U1 and U4 snRNPs from the spliceosome, removal of SF3 from the branchesite, and several conformational rearrangements of the snRNAs and pre-mRNA prior to lariat formation. These assembly and activation events are best studied using endogenous spliceosome components found in whole or nuclear cell extracts. We recently demonstrated that a single molecule technique (CoSMoS: Co-localization Single Molecule Spectroscopy) in combination with yeast genetic engineering and chemical biology provides a powerful method for studying spliceosome assembly in S. cerevisiae whole cell lysate (Hoskins et al., Science, v331, pg. 1289-95 (2011)). That study provided significant novel insight into the kinetics of the assembly reaction. We are now extending these results to spliceosome activation. By monitoring the relative association and dissociation kinetics of the U1, U4, U5, NTC, and SF3b spliceosome components on single pre-mRNAs, we are able to define the order of the snRNP association and dissociation events involved in spliceosome activation.

3060-Plat
Deletion of Ribosomal Cofactor Rimp Disrupts Late Stage 30S Subunit Assembly
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The bacterial 70S ribosome, consisting of the 30S and 50S subunits, facilitates protein synthesis and is a target for antibiotics. During 30S biogenesis, 16S ribosomal ribonucleic acid (rRNA) is co-transcriptionally processed by ribonucleases, and bound by ribosomal proteins (RPs) and cofactors. However, the coordination between rRNA processing, and cofactor and RP binding is unclear. Here, we reveal that deletion of the ribosomal cofactor gene, rimpP, disrupts binding of specific RPs (S2, S12, S21) during the late stages of 30S assembly in Escherichia coli. We use a stable isotope labeling/mass spectrometry approach to show that the rimpP deletion strain accumulates 30S assembly intermediates lacking the late assembly binders, S2, S12 and S21, with a marked delay in 30S assembly relative to 50S assembly. Further studies will determine the extent of rRNA processing in 30S assembly intermediates in the rimpP deletion strain, towards elucidating the coordination between rRNA processing, RP binding and cofactor function.

3061-Plat
Real Time Monitoring of DNA Bending and Unbending by E. Coli Integration Host Factor
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Integration Host Factor (IHF) is an abundant nucleoid associated protein in E.Coli that binds to and contributes to organizing the chromosomal DNA by non-specific protein-DNA interaction. In addition to the role in chromosomal DNA organization, IHF is also an indispensable part of site-specific integration of bacteriophage lambda into the host genome. This function of IHF largely originates from its ability to bind DNA with high specificity and bend the DNA about 160° at its binding sites. Using a novel magnetic tweezers instrument that allows manipulation of very short DNA tethers, we studied the interaction of one IHF with a specific binding sequence (H’ sequence) inserted into the middle of a 534-bp DNA fragment. Fluctuation between two distinct DNA extensions is observed in real-time at < 1 pN forces, which corresponds to the bending conformation and unbending conformation of an IHF/DNA complex (shown in figure below). Effects of environmental factors, such as osmolality, temperature, and IHF concentration are investigated in details. Our results have shed a light on understanding the properties of the IHF-H’ complex, which may improve our understanding of its role in the λ-integration process.

3062-Plat
Calcium Dysregulation of Voltage-Gated Sodium Channels Harboring LQT3 Mutations
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Intracellular calcium ions modulate sodium channel inactivation by producing a depolarizing shift the steady-state inactivation equilibrium. We have recently proposed a mechanism for this effect by which direct Ca2+/calmodulin (CaM) binding to the inactivation gate increases the transient availability of channels in the action potential by shifting the steady-state inactivation. Interestingly, a crystal structure of Ca2+/CaM bound to the inactivation gate of the sodium channel pinpoints the position of four mutations (M1498T, K1500Δ, L1501V, G1502S) shown previously to underlie long QT3 syndrome, leading to calcium regulation of Nav1.5. This possibility was first tested directly with Isothermal Titration Calorimetry (ITC) to determine the binding parameters of purified proteins and then by patch-clamp electrophysiology of expressed wild-type and mutant channels. Interestingly, ITC experiments demonstrated that the mutations impacted Ca2+/CaM binding by altering the affinity of Ca2+/CaM for the inactivation gate. Channels carrying inherited mutation showed robust expression in HEK-293 cells with either modest or severe effects on channel gating, as expected for LQT3 mutations. However, in terms of Ca2+ regulation, the LQT3 mutations significantly altered the calcium-induced shift in steady-state inactivation compared to wild-type channels. The data suggest that calcium dysregulation of the voltage-gated sodium channel may contribute to the pathogenesis of LQT3 syndrome.

3063-Plat
Single Channel Studies and Kinetic Modeling of an Inactivation Deficient Voltage-Gated Sodium Channel
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Rapid inactivation is a hallmark of voltage-gated sodium channels critical for regulating the rate of electrical signaling between excitable cells. However, activation and inactivation processes can overlap making it difficult to determine which process is altered during a given perturbation. Moreover, at the single channel level rapid entry into inactivated states occludes less frequent channel activity. Thus, removing inactivation should simplify the interpretation of macroscopic effects and reveal the intrinsic gating behavior associated with channel