Optimizing single-molecule experimental approaches for the study of complex protein assemblies on DNA

Dekker, Nynke H.

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1591-Wkshp
Characterizing protein conformational ensembles and dynamics with 2D IR spectroscopy
Andrei Tokmakoff.
University of Chicago, Chicago, IL, USA.
Advances in protein two-dimensional infrared spectroscopy and computational spectroscopy of amide I vibrations have provided a way to characterize the conformational variation and disorder in protein structure. These techniques use isotope-edited amide I spectra in combination with spectra computed from Markov state models built from molecular dynamics simulations to infer variation in local configuration and to refine ensembles for peptides and proteins. This talk will describe these tools drawing on examples from studies of insulin monomer and dimer structure and the dynamic interactions in the monomer-dimer equilibrium.

Workshop: High Throughput Single Molecule Spectroscopy

1592-Wkshp
Optimizing single-molecule experimental approaches for the study of complex protein assemblies on DNA
Nynke H. Dekker.
Department of Bionanosciences, Delft University of Technology, Delft, Netherlands.
Over the past few decades, together with biochemical approaches single-molecule biophysics has made substantial contributions to our understanding of molecular machines. This has led to our understanding of their mechano-chemical cycles, their different types of behavior (e.g. depending on substrate or cellular conditions), their interplay with other proteins, etc. etc. An ongoing challenge is to probe more complex molecular machines from a biophysical perspective and how to do so while maintaining an acceptable degree of experimental efficiency.
I will discuss our recent efforts along these lines and in particular how we have endeavored to extract information from the eukaryotic replisome at the single-molecule level by finding an optimum between factors such as sample preparation, throughput, automation, and the number of biophysical parameters read out.

1593-Wkshp
Single molecule manipulation and imaging of complex DNA-protein interactions
Gijs Wuite.
Vrije Universiteit Amsterdam, Amsterdam, Netherlands.
The genetic information of an organism is encoded in the base pair sequence of its DNA. Many specialized proteins are involved in organizing, preserving and processing the vast amounts of information on the DNA. In order to do this swiftly and correctly these proteins have to move quickly and accurately along and/or around the DNA constantly rearranging it. In this presentation I will show (Super-resolution) Correlative Tweezers-Fluorescence Microscopy (CTFM), a single-molecule approach capable of visualizing individual DNA-binding proteins on densely covered DNA and in presence of high protein concentrations. Moreover, proteins on DNA can be visualized on multiple DNA strand. Next, using this instrument we have investigated human non-homologous end joining (NHEJ). NHEJ is the primary pathway for repairing DNA double-strand breaks (DSBs) in mammalian cells. Here we show that the XRCC4-XLF complexes robustly bridge two independent DNA molecules and that these bridges are able to slide along the DNA. These observations suggest that XRCC4-XLF complexes form mobile sleeve-like structures around DNA that can reconnect the broken ends very rapidly and hold them together. I will also show how we can use this instrument for the study of mitotic chromosomes. These structures are highly dynamic throughout the cell cycle, and undergo compaction during mitosis to adopt the characteristic ''X-shape''. Here I introduce a workflow to interrogate the organization of human chromosomes based on optical trapping and manipulation. This allows high-resolution force measurements and fluorescence visualization of native metaphase chromosomes to be conducted under tightly controlled experimental conditions. The methods described here open the door to a wide array of investigations into the structure and dynamics of both normal and disease-associated chromosomes.

1594-Wkshp
RNA structure directs the targeting specificity of Cas13d
Ilya Finkelstein[1,2].
[1]Department of Molecular Biosciences and Institute for Cellular and Molecular Biology, University of Texas Austin, Austin, TX, USA, [2]Center for Systems and Synthetic Biology, University of Texas Austin, Austin, TX, USA.
Type VI CRISPR enzymes exclusively bind and cleave target RNAs, and are widely used for gene regulation, RNA tracking, and diagnostics. However, a systematic understanding of their RNA binding specificity is lacking. Here, we describe RNA-CHAMP, a massively parallel platform that repurposes used next-generation DNA sequencing chips to measures the binding affinity for >10,000 RNA targets containing structural perturbations, mismatches, insertions, and deletions relative to the guide RNA. We profile the RNA binding specificity of Cas13d, a compact and widely used RNA nuclease. Contrary to other type VI CRISPR enzymes, Cas13d does not have a protospacer flanking sequence (PFS) preference. Cas13d tolerates mismatches, insertions, and deletions, but is exquisitely sensitive to secondary structure within the target RNA. Basepairing in the distal region of the target RNA strongly decreases Cas13d binding, whereas structuring the first six nucleotides inhibits nuclease activity without impacting binding. A biophysical model built from these data reveals that target recognition begins at the distal end of unstructured target RNAs and proceeds to the proximal end with limited RNA melting. Using this model, we design a series of partially mismatched guide RNAs that modulate nuclease activity to detect single nucleotide polymorphisms (SNPs) in circulating SARS-CoV-2 variants. This work describes the key determinants of RNA targeting by a type VI CRISPR enzyme to improve RNA targeting and CRISPR diagnostics. More broadly, RNA-CHAMP provides a quantitative platform for systematically measuring protein-RNA interactions.

1595-Wkshp
Single-molecule investigation of ligand-activation mechanisms for membrane proteins in cell-derived nanovesicles
Karleen Wu, **Marcel P. Goldschen-Ohm**.
Department of Neuroscience, University of Texas Austin, Austin, TX, USA.
Molecular recognition of ligands for specific binding sites in membrane proteins is a cornerstone of biological signaling processes. Despite structural and functional evidence often painting a relatively clear picture of the binding sites, the mechanism by which ligand binding to multiple active sites confers a change in protein activity is in many cases still poorly understood. This is due in part to the fact that individual binding events are rarely directly observed, and their asynchronous dynamics are occluded in ensemble-averaged measures. For membrane proteins, single-molecule approaches that resolve these dynamics are challenged by dysfunction in non-native lipid environments, lack of access to intracellular sites, and costly sample preparation. Here, we describe an approach for resolving individual binding events of fluorescently labeled ligands at single membrane proteins in cell-derived nanovesicles. The approach involves simple transient transfection, does not use detergent solubilization so that proteins are maintained throughout in their native lipid bilayer, and provides solution access to either intracellular or extracellular binding domains. Furthermore, we describe a fully automated approach to idealization of fluorescence time series describing the binding dynamics at many molecules imaged simultaneously. As an exemplar, we apply these approaches to cyclic nucleotide binding to TAX-4 ion channels critical for sensory transduction and discuss mechanistic insights from kinetic modeling of their binding dynamics. This approach is broadly applicable to studies of binding dynamics for membrane proteins with extracellular or intracellular domains in native cell membrane.