fluorescence assays and include the fingers-closing transition that has been characterized in structural studies. Using DNA polymerase I (Klenow fragment) labeled with both donor and acceptor fluorophores, we have employed single-molecule fluorescence resonance energy transfer (smFRET) to study the polymerase conformational transitions that precede nucleotide addition. Our experiments clearly distinguish the open and closed conformations that predominate in Pol-DNA and Pol-DNA-dNTP complexes, respectively; minor conformations (corresponding to the closed conformation in the Pol-DNA complex, and the open conformation in the Pol-DNA-dNTP) are also present. By contrast, the unliganded polymerase shows a broad distribution of FRET values, indicating a high degree of conformational flexibility in the protein in the absence of its substrates; such flexibility was not anticipated on the basis of the available crystallographic structures. Real-time observation of conformational dynamics showed that most of the unliganded polymerase molecules sample the open and closed conformations in the millisecond timescale. Ternary complexes formed in the presence of mismatched dNTPs or complementary ribonucleotides show novel FRET species, which we suggest are relevant to kinetic checkpoints that discriminate against these incorrect substrates. Our results advance the mechanistic understanding of the process of nucleotide addition by DNA polymerases and suggest ways to study conformational dynamics in other nucleic-acid polymerases.

2257-Plat

Single Molecule Studies of Eukaryotic Replisomes in Xenopus Egg Extracts

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In eukaryotes, two MCM2-7 helicases are assembled at each origin of replication in the G1 phase of the cell cycle. In S phase, the helicases are activated, leading to assembly of two sister replisomes that replicate DNA in opposite directions. At present, little is known about the spatial arrangement or molecular mechanism of MCM2-7 complexes that are engaged in DNA replication. One scenario is that the two sister MCM2-7 complexes dissociate during initiation and then travel away from one another. Alternatively, the sister helicases might remain physically coupled. To differentiate between such models, we have established a series of single-molecule visualization tools using a nucleus-free replication system of *Xenopus* egg extracts. We demonstrate that these extracts can replicate lambda phage DNA that is mechanically well-stretched and specifically tethered at both ends to a functionalized surface. Our observation of large replication bubbles from single origins on such doubly-tethered DNA argues that helicases located at sister forks can function independently during replication.

In addition, we aim to observe real-time dynamics of different replisome components on doubly-tethered DNAs. For this purpose, we have generated fluorescently tagged Cdc45, an MCM2-7 co-factor that travels with the MCM2-7 helicase. Since the high concentration of labeled Cdc45 needed to support replication causes a high fluorescence background, we tagged Cdc45 with the photoswitchable fluorescent protein mKikGR. The ability to switch on the fluorescence of only those mKikGR proteins that are bound to DNA via Cdc45 enables single-molecule imaging of active replisomes, even at high ambient concentrations of Cdc45-mKikGR. We present the results of initial experiments that prove the feasibility of these techniques as novel ways to study the activity of replication factors in a physiologically relevant environment.

2258-Plat

DNA Base Flipping: New-Found Insights into the DNA Mismatch Recognition Process in E. Coli Muts

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Detection of various base-base mismatches and small insertion/deletion loops in DNA is performed by the MutS mismatch recognition protein. This highly conserved process exists in both prokaryotes and eukaryotes and failure to recognize DNA damage can have a detrimental effect on the fidelity of the genome and, in humans, has been linked to numerous forms of cancer. Several crystal structures have emerged over the years capturing MutS bound to various mismatches. However, the events directly following mismatch recognition remain unclear. To shed light on this matter, we present nine sub-μs, all-atom molecular dynamics simulations of Escherichia coli MutS bound to a G•T mismatch with different nucleotide configurations. From these simulations, we identified significant instability in the adjacent base 5′ to the thymine mismatch. In one case, the 5′ adjacent base completely loses base stacking and flips out spontaneously via the minor groove. To the best of our knowledge, this rare event is the first ever documented case of DNA base flipping from any unrestrained protein-DNA simulation. In addition, these observations are in excellent agreement with a recent experimental study where it was suggested that the 5′ adjacent base could exist in an extrahelical state. To further understand the energetics of base flipping in MutS, we utilized the Hamiltonian replica-exchange molecular dynamics (HREMD) method simulating 44 independent replicas in an explicit water box. The free energy profile generated from HREMD shows two distinct minima, one for the stacked state and one for flipped out state, separated by a small energy barrier with the flipped out state being the more favorable of the two. Together, our results offer an unprecedented level of new insight into the mismatch recognition process and further our knowledge of this complex system.

2259-Plat

DNA Conformational Dynamics in Mismatch Recognition

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DNA mismatch recognition is done by the homodimer MutS in prokaryotes and by its homologues: heterodimers Msh2-Msh3 and Msh2-Msh6 in eukaryotes. Msh2-Msh6 binds preferentially to single insertion/deletions. Msh2-Msh3 has been shown to bind to DNA hairpins. It has been suggested that the conformational dynamics of the DNA substrate (bending and unbending) plays a fundamental role in the recognition process. Mismatch recognition allows identifying a single mismatched DNA pair among thousands of matched basepairs. The process is ATP dependent and different models for DNA discrimination have been proposed based in biochemical evidence as well as AFM studies. In this work we study the conformational dynamics of several DNA substrates and its complexes with the human MutS homologs. The DNA substrates were labeled with fluorescent dyes that constitute a fluorescence resonant energy transfer (FRET) pair. Experiments at the single molecule level allow us to follow the conformational dynamics of the substrates by determining the substrate's end to end distance. We were able to determine the binding and dissociation rates of the proteins from the substrates as well as the conformational state of the substrates under different conditions, including studies with ATP and ADP under both hydrolytic and non-hydrolytic conditions. In particular we discuss the role of the substrate's intrinsic dynamics for binding of hMsh2-hMsh3 to DNA hairpins and DNA 3-way junctions.

2260-Plat

The Dance of Chromosomes during DNA Repair

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DNA repair is an essential process for preserving genome integrity. Among the various forms of DNA damage, double-strand breaks (DSBs) are the most cytotoxic and genotoxic. To repair them, eukaryotic organisms use homologous recombination (HR): it consists of exchanging DNA strands between the broken DNA and an intact homologous DNA and it is choreographed by multi-protein complexes (1). During HR, the search for an intact homologous sequence among the whole genome is the most enigmatic stage (2). How can two homologous needles find each other in the genomic haystack? Is search the result of diffusion and chance encounters, or is there a search apparatus dedicated to bringing the homologous sequences together?

To explore the choreography of the DNA and the recombination proteins during homology search, we developed an *in vivo* 3-colors assay in diploid yeast cells where 2 homologous chromosomes are fluorescently marked at the same locus (with GFP-Lac and RFP-Tet arrays), as well as recombination factors (CFP-tagged proteins). Using deconvolution microscopy, we tracked the movement of the two chromosomes in 3-dimensions in the absence and in the presence of a unique DSB induced near one of the marked chromosome. In the absence of DSB, we found that homologous chromosomes undergo a constrain Brownian motion with a diffusion coefficient of 4.10^{-4} $\mu m^2/s$ inside a small region of 300 nm. When a DSB is induced, the two homologous DNA become highly dynamic and homologous pairing occurs within one hour. This work is the first attempt to visualize simultaneously the movement of two homologous sequences *in vivo* into and out of repair centres.

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2261-Plat

Single-Molecule Measurements of Synthesis by DNA Polymerase with Base-Pair Resolution

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The catalytic mechanism of DNA polymerases has been shown to involve multiple steps that precede and follow the transfer of a nucleotide to the 3′-hydroxyl of the growing DNA chain. Here we report a single-molecule approach to monitor the movement of E. coli DNA polymerase I (Klenow fragment) on a DNA template during DNA synthesis with single base-pair resolution. As each nucleotide is incorporated, the single-molecule Förster resonance energy transfer (smFRET) intensity drops in discrete steps to values consistent with single nucleotide additions to the primer terminus. Purines and pyrimidines are incorporated with comparable rates. When a mismatched primer-template junction is used, smFRET is observed consistent with the primer moving into the exonuclease domain. This analysis was used to determine the fraction of primer-termini bound to the exonuclease and polymerase sites. Most interestingly, we observe a structural change following the incorporation of a correctly-paired nucleotide, consistent with transient movement of the polymerase past the preinsertion site or a conformational change in the polymerase. This may represent a previously unobserved step in the mechanism of DNA synthesis that could be part of the proofreading process.

2262-Plat

Single-DNA Detection of Stepwise DNA Compaction by Cohesin SMC Protein Complexes

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Structural Maintenance of Chromosomes (SMC) protein complexes are essential for the precise folding and segregation of chromosomes during cell proliferation in both prokaryotes and eukaryotes. Cohesin SMC complexes are believed to hold sister chromatids together during chromosome segregation. However, how cohesins interact with DNA at the molecular level is still poorly understood. In our current study, we report that yeast cohesin SMC heterodimers condense single-DNA molecules by distinct steps in a force-dependent manner in the absence of ATP. The rate of condensation is supercoiling-dependent, and positive supercoiling of the DNA accelerates the condensation. We also observed stepwise DNA condensation by cohesin SMC complexes (with Scc1 kleisin subunits). Moreover, the DNA compaction by cohesin complexes is regulated by ATP, and requires the torsional rigidity of dsDNA. Our results suggest that cohesins may play a broader role as organizers of chromatin, possibly by defining ''cross-linkers'' or ''loops'' through the whole cell cycle.

2263-Plat

Torsional Regulation of hRPA-induced Unwinding of Double Stranded DNA

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Replication Protein A (RPA) is the main eukaryotic single-stranded binding protein and is essential for most aspects of cellular DNA metabolism. RPA is able to unwind the dsDNA helix by binding to transiently forming single-stranded DNA bubbles [1]. We present single-molecule measurements of the dynamics of human RPA (hRPA) activity on dsDNA and ssDNA obtained with a magnetic tweezers-based assay. We show that hRPA-induced dsDNA-helix unwinding is strongly promoted by unwinding torque and can be reversed by rewinding torque exerted on the DNA. Torque thus provides a means for tight mechanical regulation. The torque generated by the application of a modest stretching force (~0.5 pN) on supercoiled DNA is sufficient to overcome the previously reported inhibitory effects of high salt. We propose a torque-based model that explains the dynamics of hRPA duplex (un-)binding and provide mechanistic insight. The mechanochemical regulation of hRPA binding to dsDNA is likely important for replication initiation and for various DNA repair pathways.

We conclude the presentation with single-molecule measurements on the HepA-related protein (HARP), which recently was proposed to act as an annealing helicase that is able to rewind DNA bubbles that are stably bound by RPA [2]. We demonstrate that HARP is able to rapidly re-anneal hRPA-stabilized ssDNA bubbles.

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Platform AS: Exocytosis & Endocytosis

2264-Plat

Localized Plasma Membrane Topological Changes upon Exocytosis Visualized by Polarized-TIRFM

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Total internal reflection fluorescence (TIRF) microscopy images the plasma membrane-cytosol interface and has allowed insights into the behavior of individual secretory granules before and during exocytosis. Much less is known about the dynamics of the other partner in exocytosis, the plasma membrane. Here we report the implementation of a TIRFM-based polarization technique to detect rapid submicron changes in plasma membrane topology as a result of exocytosis. A theoretical analysis of the technique is presented taking into account the high numerical aperture lenses that are used in through-the-lens TIRFM, the point spread function, and pixilation on the CCD camera. Image simulations are presented for predicted topologies of the post-fusion granule membrane/plasma membrane complex. Experimental results on dil-stained bovine adrenal chromaffin cells using polarized TIRFM demonstrate rapid and varied submicron changes in plasma membrane topology at sites of exocytosis. They occur immediately upon fusion in at least 80% of the fusion events and decay with varying speeds from as fast as 100 ms to tens of seconds. Endocytosis, also imaged by optical techniques, revealed that less than 10% of the fusion events stimulated with elevated K^+ result in rapid endocytosis. Thus, most of these topological changes are kinetically distinct from endocytosis and reflect a varying time course for the flattening of fused granule membranes into the plasma membrane.

2265-Plat

Compound Exocytosis in Rat Beta-Cells Triggered by Global Elevation of Cytosolic Calcium

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Exocytosis in β-cells is traditionally thought to involve fusion of individual insulin granules. It has been proposed, however, that granules may prefuse with each other inside the cell and that these aggregates subsequently undergo compound exocytosis. ATP is stored in insulin granules and co-released with insulin. At low $[Ca^{2+}]_i$ (~0.2 micromol/l), most exocytotic events (detected as ATP release in rat beta-cells expressing ATP-sensitive $P2X_2Rs$) were small. However, at 2 micromol/l $[Ca^{2+}]_i$, 20% of the events became 5- to 10-fold larger than those seen at low $[Ca^{2+}]_i$. The small events were associated with capacitance increases of ~3 fF, close to that expected for 300-nm secretory vesicles. Occasionally, we observed much larger stepwise capacitance increases (20-40 fF) indicating that 5-10 secretory granules fused simultaneously. The ATP-induced currents associated with the large capacitance steps were correspondingly increased and rose monotonically (i.e. without signs of superimpositions of several smaller events). This argues that they reflect the emptying of prefused granule aggregates via single fusion pores. Two photon imaging of exocytosis using the fluorescent polar tracer sulforhodamine B (SRB) confirmed that compound exocytosis does not contribute to glucose-stimulated insulin secretion (average diameter: 400 nm). However, in the presence of carbachol (20 micromol/l), exocytosis of large structures corresponding to 4-5 secretory vesicles were observed and accounted for 20% of the events. These large events attained their maximum size within the temporal resolution of the imaging system (0.7 s) and were not slower than the ordinary (small) events. Using 3-dimensional scanning electron microscopy we obtained ultrastructural evidence for the formation of multivesicular structures in beta-cells in islets exposed to carbachol for 5 min. We conclude that compound exocytosis becomes quantitatively significant in response to global elevations of $[Ca^{2+}]_i$ such as those elicited during muscarinic stimulation.

2266-Plat

Exocytosis, Dependent on Calcium Release from Calcium Stores, is Regulated by Calcium Microdomains

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The relationship between the cellular Ca^{2+} signal and exocytic vesicle fusion is a key determinant of the regulation of the kinetics and magnitude of the secretory response. Here, we have investigated secretion in epithelial cells where the exocytic response is controlled by Ca^{2+} release from intracellular Ca^{2+} stores (Ueda & Petersen 1977). Using live-cell 2-photon microscopy, identifying each individual exocytic fusion event (Thorn *et al.,* 2004) and recording cytosolic Ca^{2+} signals with Fura-2 (Nemoto *et al.,* 2001), we show no evidence for spatial clustering of exocytosis; indeed exocytosis is actually specifically excluded from sites of Ca^{2+} release hot-spots. Consistent with this data, loading the cells with EGTA potently blocks exocytosis. These results indicate that the control of exocytosis, triggered by Ca^{2+} release from stores, is through the regulation of cytosolic Ca^{2+} concentrations within large volume microdomains.

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