E. coli attC sites from dsDNA and their interaction with integrase hinting at possible molecular mechanisms underlying the function of this system in bacteria.

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Investigating the Dynamics of the β_2 Sliding Clamp in Escherichia Coli at the Single-Cell Level Utilizing Single-Molecule Fluorescence Microscopy

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An *E.coli* cell contains a single circular chromosomal DNA, and recent studies have shown that this chromosome is duplicated in an independent bidirectional manner by two individual replisomes prior to cell division. Here, we investigate the dynamics of the replisome complex, specifically the β_2 sliding clamp, in living cells. We use a combination of microfluidics and single-molecule fluorescence microscopy to track the replisomes in individual *E.coli* cells, whereby the native β_2 clamp proteins are labeled with a fluorophore (mYpet) by chromosomal fusion. To carry out these experiments, we have reliably quantified the fluorescence signal generated by the β-mYpet fusion inside a growing cell by comparing numerous *in vitro* and *in vivo* calibration standards. Additionally, our use of microfluidics has increased the yield of the measurement with respect to the number of analyzed cells and the measurement time, as well as facilitated automated analysis using customized MATLAB scripts. The results of our experiments clearly demonstrate that the β_2 proteins are temporally and spatially dynamic during a cell cycle: following initiation, there is a gradual increase in the number of β_2 clamps bound to the DNA until a steady state is reached. This steady state, which surprisingly involves approximately 50% of the β_2 proteins inside the cell, is maintained for most of the cell cycle until a gradual decrease is observed around 15 minutes before termination of replication. We conclude that there is a clear accumulation of β_2 clamps along the two replication forks, and we propose a model that the β_2 clamps are left behind on the lagging strand upon synthesis of each Okazaki fragment as the replication fork progresses.

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SV40 Helicase Unwinds DNA against Force

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Double stranded DNA must be distorted and melted for replication. The large tumor antigen (LTag) of the Simian Virus 40 (SV40) is a simple but robust motor enzyme that opens the DNA duplex, functionally mimicking the eukaryotic replication helicase MCM (MiniChromosome Maintenance). Six monomers of SV40 LTag assemble into a ring around the origin of replication (oriDNA), followed by the formation of a second hexamer. In the presence of ATP and SSB, the two hexamers unwind duplex DNA into single strands. However, the enzyme dynamics and mechanisms largely remain elusive. We use single-molecule magnetic tweezers to investigate the SV40 LTag helicase activity on DNA as a function of force. Magnetic tweezers unwind a torsionally constrained DNA like a helicase, which provides us a convenient way to characterize the DNA response upon unwinding in a broad force range. DNA length changes when unwinding, indicating enzyme activity. From these experiments, we observe that LTag helicase processivity differs between low and high force regimes. We have evaluated the unwinding rate, enzyme pausing, and the effect of SSB and ATP. Our results shed light on the molecular mechanism utilized by the SV40 LTag to open the DNA duplex. Furthermore, our force dependency study indicates how the hexamer-hexamer interactions break during DNA unwinding.

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Simultaneous Imaging of Leading- and Lagging-Strand Synthesis Reveals Distinct Operational Modes of Single Replication Machines

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The proliferation of all organisms depends on the precise coordination of a large number of enzymatic events within multiprotein replisomes to ensure that chromosome duplication occurs in a timely and faithful manner. While the core architectural characteristics of replisomes are known, the inner workings of these sophisticated molecular machines remain poorly understood. In particular, it remains unclear how discontinuous synthesis on the lagging strand, which requires several slow enzymatic steps, can occur without causing loss of coordination with continuous synthesis of the leading strand. To study the coordination of enzymatic events during replication, we have developed an assay to simultaneously monitor the synthesis of each daughter strand by single replisomes. Using ultra-low-magnification single-molecule microscopy, we image up to 30,000 individual surface-immobilized DNA molecules simultaneously. Beads attached at either end of these molecules reveal the rates of synthesis on the leading- and lagging-strand independently. To derive detailed kinetic information from these complex multistate observations, we have developed a novel change-point algorithm for identifying and fitting distinct kinetic regimes in an unbiased manner. We have applied our single-molecule assay and analysis method to study the coordination of daughter stand synthesis by the bacteriophage T7 replisome. Using inhibitors and a variety of different enzymatic conditions, we have selectively altered the rates of helicase unwinding, priming and polymerase loading. These studies have allowed us to deconstruct the relative timing of events on the leading- and lagging-strands, resulting in a revised understanding of how enzymatic events are coordinated at the replication fork.

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Domain Architecture of RecQ Helicase Defines Mechanochemical Linkage via Multipartite Interactions with DNA Substrate during Unwinding Activity

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RecQ helicases are ubiquitous enzymes that safeguard the genome by playing multiple roles in DNA repair, replication and recombination. They are unique in their capability to process a wide range of non-canonical structures associated with DNA metabolic intermediates. DNA-binding domains linked to the RecQ helicase core, including the winged-helix (WHD) and helicase-and-RnaseD-C-terminal (HRDC) domains, are thought to confer substrate specificity and modulate enzymatic activities. We combined ensemble biophysical and single-molecule magnetic tweezers assays to determine the mechanochemical linkage between ATP hydrolytic and DNA-restructuring activities of E. coli RecQ constructs of varying domain architecture. We compared the activities of wild-type, HRDC point mutant and deletion mutant, and WHD-HRDC deletion mutant constructs using several defined DNA structures and experimental geometries. We show that the WHD enhances unwinding processivity by stabilizing enzyme-DNA interactions, whereas the HRDC domain increases the overall DNA affinity but hinders the unwinding and ATPase activities of the helicase through interactions with single-stranded (ss) DNA regions. Intriguingly, HRDC-ssDNA interactions stabilize the pausing of the helicase during unwinding of a DNA hairpin in which both nascent ssDNA strands are mechanically strained, but not that of gapped duplex DNA in which one of the liberated ssDNA strands is mechanically unconstrained. Our study reveals that both the DNA substrate geometry and the contribution of auxiliary DNA-binding domains greatly influence the mechanochemical behavior of the helicase and the outcome of DNA-processing reactions, bearing consequences on the diverse in vivo actions of RecQ enzymes.

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Vizualizing Repliication Restart Process In Vivo with Single-Molecule Sensitivity

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The rapid and faithful replication of the genome is of central importance to cell proliferation. During this process, the replication machinery (replisome) frequently encounters obstacles, including DNA damage, concurrent DNA transcription, and DNA-bound protein complexes, resulting in conflicts that stall the replication process. The completion of the replication process depends on the efficient resolution of these conflicts. How this is accomplished in the face of different obstacles and the impact of replication conflicts on the core components of the replisome remains unclear. To quantitatively analyze the restart process in single cells, we have built a model system in the gram-positive bacterium Bacillus subtilis. We describe the visualization of replication conflicts with single-molecule resolution. We probe the protein composition and stoichiometry of active and stalled replisomes and the dynamics of replication restart.