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Studies of DNA Breathing and Helicase Mechanisms by Single Molecule (SM) Fret Between 6-MI and Cy3 in DNA Replication Fork Constructs

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6-methyl isoxanthopterin (6-MI) is a fluorescent nucleotide base analog of guanine (G) that can be site-specifically substituted for G at key positions within a DNA construct, with little or no disruption of the normal nucleic acid structure. DNA constructs fluorescently labeled with 6-MI have been useful in probing local base conformational changes in bulk solution experiments, but to date no experiments with 6-MI have been undertaken at the single molecule level. Major challenges towards achieving this goal include working with fluorophores with small extinction coefficients (ε350 nm ~ 12000 M-1cm-1) in the UV, and the necessity to separate weak fluorescence from scattered laser excitation light.

We here report sm experiments performed on 6-MI-labeled DNA constructs that were resonantly excited using the 351 nm line of an argon ion laser in combination with a home-built total internal reflection fluorescence (TIRF) microscope and split-screen CCD camera. To investigate the feasibility of such sm fluorescence experiments we used a long biotinylated DNA strand (91-mer) that provided binding sites for a short DNA molecule (23-mer) containing three 6-MI residues. From experiments on this substrate we were able to monitor three successive photo-bleaching steps, indicating the presence of the three 6-MI chromophores. We next developed a new Förster resonance energy transfer sm(FRET) system between 6-MI and the carbocyanine dye Cy3 site-specifically inserted into the DNA backbone of a fork construct. By performing experiments in bulk solution and at the sm level, we were able to demonstrate smFRET between a 6-MI probe at the fork junction and a Cy3 backbone probe within the dsDNA of the fork. We are now using this approach to study DNA unwinding mechanisms by the bacteriophage T4 helicase−primase (primosome) complex.

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Determining the Quantitative Dynamics of Nucleic Acids in Live Cells through Rics and iMSD Approaches

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Addressing the mobility and dynamics of DNA within live cells requires the characterization of individual particles in real time. Two such techniques enabling the quantification of the dynamics and means of motion of fluorescently labeled particles include the Raster Image Correlation Spectroscopy (RICS) and the image-based Mean Square Displacement (iMSD) approaches. In this study, the RICS and iMSD approaches were applied in order to elucidate the mobility and means of motion of DNA lipoplexes formed from varying sized DNA (20bp-5.5kbp). The RICS approach demonstrated that two species of mobility were present within the cytoplasm. Across the DNA fragment sizes, the slower species was not size dependent and were statistically the same. Whereas, faster moving particles which demonstrated a size dependent mobility. Nuclear localized DNA demonstrated to be significantly dynamic compared to other previously published work, ranging from 1.22 μm2/s (5.5kbp) to 3.67 μm2/s (21bp), consistent to nuclear proteins and RNA. The iMSD approach applied enabled the discrimination between different means of motion including random diffusion (RD), active transport (AT), confined diffusion (CD), anomalous subdiffusion (AS) and transient confinement (TC). Motion through RD and TC had demonstrated a size dependency across the DNA sizes assessed. Whereas, motion through AT, CD and AS did not demonstrate size dependency additionally these other means of motion had a higher mobility rate than that of RD.

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Photophysical and Dynamical Properties of Doubly Linked Cy3 - DNA Constructs

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Photophysical measurements are reported for Cy3 - DNA constructs in which both Cy3 nitrogen atoms are attached to the DNA backbone by short linkers. While this linking was thought to rigidify the orientation of the dye and hinder cis-isomerization, the relatively low fluorescence quantum yield and the presence of a short component in the time-resolved fluorescence decay of the dye indicated that cis-isomerization remained possible. Fluorescence correlation spectroscopy and transient absorption experiments showed that photoisomerization occurred with high efficiency. Molecular dynamics simulations of trans dye system indicated the presence of stacked and unstacked states, and free energy simulations showed that the barriers for stacking/unstacking were low. In addition, simulations showed that the ground cis state was feasible without DNA distortions. Based on these observations, a model is put forward in which the doubly linked dye can photoisomerize in the unstacked state.

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Associated Anisotropy Decays of Ethidium Bromide Interacting with DNA

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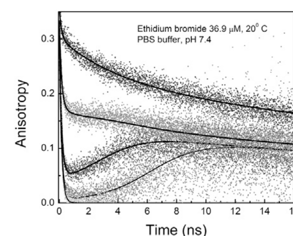
Ethidium Bromide (EB) is a commonly used dye in a deoxyribonucleic acid (DNA) study. Upon an intercalation, this dye significantly increases its brightness and lifetime. In this report we studied time-resolved fluorescence properties of EB fluorophore existing simultaneously in free and bound forms in the solution. Fluorescence intensity decays were fitted globally to a double exponential model with lifetimes corresponding to free (1.6ns) and bound (22ns) forms, and molar fractions were determined for all used solutions. Anisotropy decays displayed characteristic time dependence with an initial rapid decline followed by an increase and a slow decay. This is because two existing fractions contributing to a total anisotropy change in time. The short-lived fraction associated with free molecules decreases faster than long-lived fraction associated with bound EB. In a consequence, the contribution of fast rotation (free EB) to total anisotropy decreases in time. The effect of associated anisotropy decays in systems EB-DNA is clearly visible in a wide range of concentrations and should be taken into account in polarization assays.



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Comparing Optical and Magnetic Tweezers for Studying the RecQ Helicase

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Helicases are molecular motors that use the energy from ATP hydrolysis to translocate and unwind dsDNA, providing the ssDNA substrate needed in many cellular processes such as DNA replication, recombination and repair. Among DNA helicases, the RecQ family (conserved from bacteria to human) is essential for the maintenance of the genomic integrity. In this work we use optical and magnetic tweezers to study the E. Coli RecQ helicase. These single-molecule techniques allow to manipulate a DNA substrate (DNA hairpin) and follow in real-time the activity of a single RecQ motor. Mechanical force is applied at the ends of the DNA molecule, assisting the unwinding catalyzed by the helicase. We measure the unwinding velocity under different applied forces and different ATP conditions. Interestingly, we find that the unwinding rate depends only weakly on the force applied, revealing that RecQ behaves as an active helicase. Whereas magnetic tweezers allow us to perform several experiments simultaneously, optical tweezers offer data with a higher time and spatial resolution from which we are able to observe pauses and backward steps of the motor.

The quantitative understanding of the differences between both methods is a fundamental step towards the combined use of these techniques for future projects.

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Single Molecule Observation of Cyclization of Short DNA Duplex

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In the presented work, a single molecule DNA cyclization assay was used to follow the looping kinetics of single DNA 83 bp molecules, utilizing single