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Minireview

Discovering the Power of Single Molecules

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Mechanical manipulations of single biological molecules have revealed highly dynamic and mechanical processes at the molecular level. Recent developments have permitted examination of the impact of torque on these processes and visualization of detailed molecular motions, enabling studies of increasingly complex systems. Here we highlight some recent important discoveries.

Introduction

Many cellular processes are carried out by DNA-based motor proteins (e.g., RNA and DNA polymerases) that translocate along, and rotate around, their DNA substrates. These motors, in turn, generate forces and torques on DNA. Because of the mechanical nature of these processes, single-molecule manipulation techniques are ideally suited to examine how motor proteins take steps and generate rotations and how mechanics in turn regulates the functional behavior of DNA and DNA-bound proteins including the motor proteins themselves. This approach has answered many fundamental questions in biology that are difficult or impossible to address with bulk biochemical studies. The hallmark of these manipulation studies has been the clarity and directness in data interpretation, making it possible to attain a wealth of information and provide important insights into many fundamental biological processes. Although initial manipulation techniques only permitted force measurements, more recent technical innovations have also enabled torque measurements. In addition, a number of novel combinations of manipulation and fluorescence techniques have been developed. These are all significant additions that have substantially extended the realm of problems that may be interrogated via mechanical manipulation. This Minireview will focus on some of these recent additions to the single-molecule manipulation arsenal and their impact on biology. Below, we highlight a few studies that focus on the role of torque on DNA and DNA-based processes and the fluoresecence visualization of molecular events such as target search, protein diffusion, and motor proteins overcoming obstacles. We will also provide our perspective on future challenges.

Torque in DNA

The physical properties of DNA are critical to cellular processes such as transcription, replication, and DNA repair, all of which involve many DNA-based motor proteins. Because of the helical nature of the DNA molecule, motor proteins that translocate along DNA will necessarily have to rotate relative to the DNA. Since neither a motor protein nor DNA is able to freely rotate due to viscosity and cellular constraints, this relative rotation instead subjects the DNA to local torsional stress. Early magnetic tweezers studies demonstrated that DNA would transition to new phases with distinct structures when subjected to excessive twist (Strick et al., 1996). These transitions should occur at specific torques; however, a new generation of single-molecule instruments had to be developed in order to directly measure these torques.

Bryant et al. (Bryant et al., 2003) pioneered the rotary bead assay in which the torque in a DNA molecule was measured by monitoring the angular velocity of a small bead attached to the side of the DNA (Figure 1A). After the DNA was extended under force, twist was added. Torque stored in the DNA induced rotation of the bead around the DNA axis, allowing the applied torque to be determined by calculating the viscous drag on the bead. The torsional stiffness of the canonical B-DNA was measured, as well as the torques required to induce phase transitions to various other DNA structures (such as L-DNA, S-DNA, and P-DNA). These represent the first direct torque measurements of DNA. Subsequent enhancement of the rotary bead assay allowed measurement of transitions to Z-DNA in response to (−) torque (Oberstrass et al., 2012).

Advancements have also enabled direct torque measurements in DNA using optical trapping (Forth et al., 2008; Sheinin et al., 2011). In an angular optical trap, a nanofabricated quartz cylinder is trapped and rotated via the rotation of the trapping laser polarization, and the torque on the cylinder is detected by the change in the polarization state of the transmitted beam. More recently, torque detection has also been enabled for magnetic tweezers (Lipfert et al., 2010). These techniques have also demonstrated the capability for torque measurements during DNA phase transitions.

Of the DNA phase transitions, the melting transition upon (−) DNA supercoiling is of particular interest because of its importance in the initiation of transcription and replication. (−) DNA supercoiling is known to promote DNA melting. The torque of the melting transition was measured by several groups and was consistently found to be approximately −10 pN•nm (Bryant et al., 2003; Lipfert et al., 2010; Oberstrass et al., 2012; Sheinin et al., 2011). Torsionally melted DNA was long thought to be equivalent to a thermally melted DNA bubble. Interestingly, recent studies using the angular optical trap and the rotary

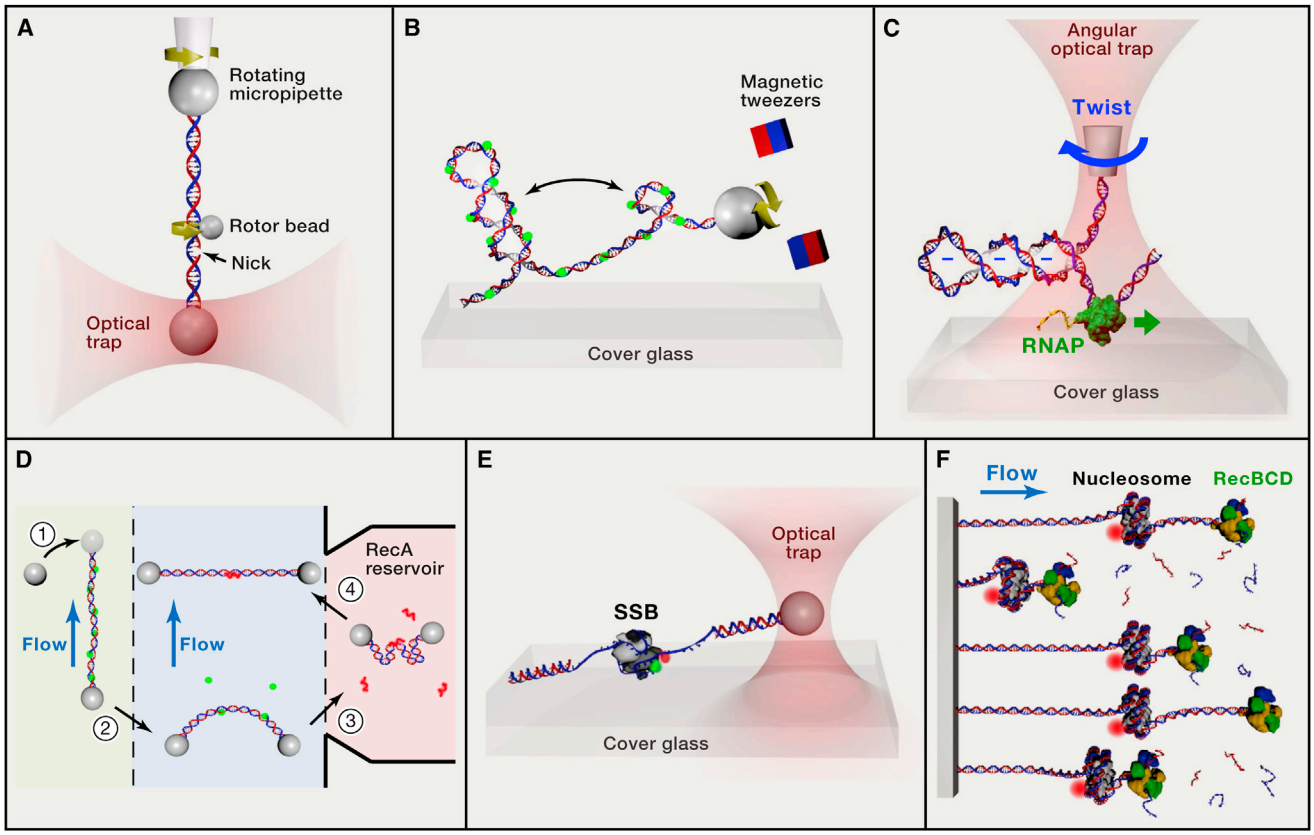

Figure 1. Some Novel Enhancements of Single-Molecule Manipulation Techniques

(A) Torque measurement using the rotary bead assay (Bryant et al., 2003). One end of a DNA molecule was attached to a bead held in an optical trap, and the other end to a bead which was rotated by a micropipette. A smaller bead was attached internally to the DNA, just above a nick in the DNA backbone, which allowed this bead to rotate freely. The torque applied to the DNA was inferred by how much the rotation of this smaller bead, which experiences viscous drag, lagged behind the rotation of the micropipette.

(B) Visualizing supercoil migration using magnetic tweezers and fluorescence (van Loenhout et al., 2012). A long DNA molecule was twisted by rotating the magnetic tweezers, and then extended laterally. The DNA was labeled with fluorescent dyes. Plectonemes were revealed as bright fluorescent spots.

(C) Measurement of the torque generated by RNA polymerase (RNAP) using an angular optical trap (Ma et al., 2013). An RNAP was torsionally constrained to a coverslip surface, and the upstream DNA was torsionally constrained to the bottom of a nanofabricated quartz cylinder held in an angular optical trap. As the RNAP translocated along the DNA, RNAP generated (−) supercoils in the upstream DNA and eventually reached a stall. The angular optical trap monitored the transcription and the torque that RNAP generated in real time.

(D) Abbreviated procedure for the measurement of the RecA homology search using a multichannel flow cell (Forget and Kowalczykowski, 2012). Step 1: A DNA molecule, labeled with YOYO1 for visualization, was tethered at both ends to beads held in optical traps. Step 2: the DNA was moved to a different channel of the flow cell to remove the YOYO1. Step 3: the DNA was moved to a reservoir containing fluorescently labeled RecA filaments and incubated at a fixed extension without flow. Step 4: the DNA was returned to the flow cell and extended to visualize RecA binding.

(E) Characterization of single-stranded binding protein (SSB) diffusion using optical trapping and FRET (Zhou et al., 2011). SSB was bound to a segment of ssDNA held between two dsDNA anchors, with one anchor attached to a coverslip surface and the other attached to a bead held in an optical trap. Force applied by the optical trap was used to control the degree of ssDNA unwrapping from the SSB. A donor dye (green) was attached to SSB, and an acceptor dye (red) to the ssDNA, allowing motion of the ssDNA relative to SSB to be monitored using FRET.

(F) A DNA curtain experiment used to observe collisions between RecBCD and a nucleosome (Finkelstein et al., 2010). An array of DNA molecules, each attached at one end to a fabricated surface, was fluorescently labeled and extended using flow. This allowed the progress of a RecBCD enzyme, attached to the free end of the DNA, to be monitored as it displaced the fluorophores attached to the DNA. The nucleosome was fluorescently labeled with a different dye. When RecBCD encountered a nucleosome, both the fates of the RecBCD and the nucleosome were monitored.

bead assay determined that melted DNA, when held under slight tension, actually exists in a unique left-handed configuration, termed L-DNA (Oberstrass et al., 2012; Sheinin et al., 2011).

The existence of these phase transitions demonstrates that DNA is not merely a static carrier of information, but rather a complex biological machine, capable of radically altering its structure in response to torsional stress. A complete understanding of the response of DNA to biologically achievable forces and torques provides the framework necessary for measuring the mechanical properties of proteins which interact with DNA.

Visualizing DNA Supercoiling Dynamics

The initial dynamic torsional manipulation of DNA was achieved using magnetic tweezers: as twist was added to a DNA molecule under a small tension, the supercoiled DNA eventually buckled to form a plectoneme (Strick et al., 1996). Subsequent experiments showed that prior to buckling, DNA behaved like a simple

torsional spring, with torque increasing linearly with an increase in twist (Bryant et al., 2003; Forth et al., 2008). Therefore, buckling may occur when it becomes more energetically favorable for DNA to form a plectoneme, leading to a torque plateau, as has been experimentally demonstrated (Forth et al., 2008). Although the buckling transition was extensively investigated experimentally and theoretically, the number and locations of the plectonemes and the kinetics of plectoneme formation remained elusive.

van Loenhout et al. recently addressed these questions by achieving plectoneme visualization on a single DNA molecule (van Loenhout et al., 2012) (Figure 1B). A dye-labeled DNA molecule was supercoiled with magnetic tweezers and then imaged by epifluorescence. Bright spots on the DNA were detected, indicating high DNA density, which was interpreted as the existence of plectonemes. The authors found that multiple plectonemes could exist on a long DNA molecule and the number of plectonemes depended on the tension in the DNA as well as the salt concentration. They also observed that a plectoneme diffused along the DNA, and in a matter of milliseconds could hop from one location to another across thousands of base pairs. These findings have important implications for processes that take place over long distances on DNA. Plectoneme hopping allows for dramatic long-range rearrangement of DNA conformation and may facilitate fast searching during DNA recombination or enhancer-activated gene expression.

Torque Generated during Transcription

DNA supercoiling has been recognized as an important regulator of gene expression in vitro and in vivo (Kouzine et al., 2008; Revyakin et al., 2006). As RNA polymerase (RNAP) tracks the helical groove of DNA, it generates (−) DNA supercoiling behind and (+) DNA supercoiling ahead, as described by the "twin supercoiled domain model" (Liu and Wang, 1987). Supercoiled DNA experiences a torque, and the amount of the torque determines whether the DNA is denatured and whether the transcription rate is altered. However, experimental determination of the torque generated by RNAP is difficult and was only recently realized.

Ma et al. employed an angular optical trap to measure the torque an *E. coli* RNAP can generate during transcription (Ma et al., 2013) (Figure 1C). In this experiment, transcription was monitored in real time, as RNAP-generated supercoiling accumulated in the DNA until the polymerase stalled. RNAP was found to have a mean stall torque magnitude of 11 pN·nm as it worked against either upstream (−) supercoiling or downstream (+) supercoiling. For (−) supercoiling, this stall torque is sufficient to melt DNA of an arbitrary sequence (not just AT rich regions prone to melting), suggesting that RNAP has the capability to reliably melt DNA. Such melting could serve an important regulatory role in facilitating the initiation of transcription and replication at upstream sequences. For (+) supercoiling, the stall torque may impact bound proteins ahead of the polymerase. In eukaryotes, such a torque could impact downstream bound proteins. In fact, a recent study using the angular optical trap showed that nucleosomes are significantly more likely to lose H2A/2B dimers under a positive torque comparable to the RNAP stall torque than under zero or negative torque (Sheinin et al., 2013). These results suggest that the torque-generating capacity of RNAP may have been tuned to important transitions in DNA or chromatin structure.

These studies help to establish relevant torque scales for processes taking place over DNA. The framework established to measure torque generated by RNAP may be extended to study other DNA-based motors. Further direct measurements such as these will help us to gain insights into the role of torsional mechanics in all fundamental processes on DNA.

Target Searching on DNA

DNA processes, such as transcription, replication, DNA repair, and recombination, all require proteins to interact with specific DNA sequences. This interaction necessitates that a protein locates its DNA-binding target efficiently and accurately. These search processes are highly kinetic and are ideally suited for single-molecule investigations.

Recently, a single-molecule study has answered a long-standing question of how RecA proteins find DNA sequence homology, which is an important step during homologous recombination (Forget and Kowalczykowski, 2012). RecA is known to assemble on ssDNA to form a filament and then search for sequences in dsDNA that are homologous to the ssDNA. How this homology search occurs had been investigated by various groups for decades, but remained unresolved until recently. Forget et al. tackled this problem using an instrument that combined a dual optical trap, a novel microfluidic device, and multicolor fluorescence (Figure 1D). This instrument, a powerful way to visualize individual protein molecules on DNA, was an extension of an earlier invention from the group (Bianco et al., 2001). Here, the microfluidic device allowed for rapid exchange of chemical environments and thus precise timing of the initiation of the RecA-ssDNA search reaction, and the use of a dual trap gave full control of the tension in the DNA. They found that RecA-ssDNA complex failed to efficiently locate DNA sequence homology as the tension in the DNA increased. This was an important clue that eventually led them to propose an "intersegmental contact sampling" mechanism, in which the random coil structure adopted by unstretched DNA makes multiple weak contacts with the RecA nucleoprotein filament, allowing a rapid three-dimensional search of the DNA sequence space.

This single-molecule study has provided unprecedented visual detail about a homologous search mechanism. It also highlights the importance of a mechanical approach in gaining mechanistic insights at the molecular level. This general approach may be adapted to visualize target locations and mechanisms for a range of processes in which conformations of nucleic acids are important.

Protein Diffusion on DNA

The ability of a protein to diffuse along DNA not only provides a mechanism for a target sequence search, but also allows for coordination with other proteins, especially when the diffusing protein has little sequence specificity during diffusion. It has been a challenge to understand mechanically how a protein diffuses along DNA because the diffusion process is expected to involve minute conformational changes of the protein.

Zhou et al. took on this challenge (Zhou et al., 2011) and provided important insights. They investigated the mechanism by which ssDNA-binding protein (SSB) diffuses along ssDNA (Figure 1E). This was achieved by using optical trapping in conjunction with single-molecule fluorescence resonance energy transfer (FRET), a combination of techniques that was pioneered by the same group (Hohng et al., 2007). Using this approach, the authors monitored tension-dependent conformational transitions of the DNA/protein complexes at nanometer resolution. Their results are consistent with a diffusion mechanism in which SSB slides along ssDNA. The transition state for this sliding mechanism was found to be the formation of a DNA bulge which then propagates around the SSB.

The combination of FRET with mechanical manipulation allows detailed mapping of conformational dynamics in response to mechanical perturbations. This technique may be used to study reaction mechanisms, including transition state configurations, in many biomolecular systems.

Motor Proteins Encountering Obstacles

Motor proteins that translocate along DNA must deal with other DNA-bound proteins during many cellular processes. It is thus an intriguing question as to what happens to both the motor and the obstacle during their collision. These are highly kinetic and stochastic processes, so it would be ideal to visualize the entire distribution rather than just the mean. This calls for a single-molecule method with high throughput.

Finkelstein et al. (2010) achieved this experimentally by the use of a technique called "DNA curtains" that the group had previously developed (Fazio et al., 2008) (Figure 1F). To form a DNA curtain, an array of DNA molecules was anchored at one end via a nanofabricated structure and extended via flow to allow for fluorescence visualization of events occurring on the DNA. They used RecBCD as a model motor protein and investigated how it interacted with various bound proteins, such as paused RNA polymerases or nucleosomes. An intriguing observation was that RecBCD often did not pause upon collision with an obstacle and was frequently observed to push the obstacle thousands of base pairs before evicting it from the DNA.

The parallel nature of the measurements was essential in making it possible to collect a large amount of data that efficiently revealed the distribution of obstacle fates. This method clearly demonstrates the power of parallel measurements to rapidly examine the interaction between many different proteins.

Outlook

This review has highlighted the power of single-molecule manipulation to enable discoveries in molecular biology. However, considerable challenges still lie ahead. Mechanical manipulation experiments are carried out one molecule at a time, making data collection a time-consuming process. Partial relief has come from the DNA curtain method, which provides a means for parallel processing and thus high throughput. However, this method lacks the ability for full manipulation and control. In order to make manipulation techniques broadly accessible and more efficient, the future generation of manipulation tools will have to resolve these limitations. In addition, many complex molecular events involve multiple proteins working together within nanometers or tenths of nanometers. Detailed investigations of these events will require higher spatial resolution for visualization. An initial effort in this direction is demonstrated in a recent work by Heller et al. who combined optical trapping with stimulated emission depletion (STED) microscopy (Heller et al., 2013). Other state-of-the-art superresolution imaging methods, such as stochastic optical reconstruction microscopy (STORM) (Huang et al., 2010), should also be compatible with manipulation techniques. The ability to manipulate single biological molecules has brought about a remarkable revolution in biology in the past three decades. We anticipate that future innovations will continue to enable even more unique and important scientific discoveries.

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