Manipulation of single molecules in biology

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The mechanical manipulation of single biological molecules is stimulating new and exciting research in many fields of study, including molecular motor mechanics, biopolymer properties, protein unfolding, receptor–ligand interactions, and more. Some recent highlights include the elucidation of the coupling ratios of myosin and kinesin, the demonstration of oscillatory forces in dynein arms, the determination of the force-velocity relation of RNA polymerase, and the direct mechanical observation of unfolding of single domains of titin and tenascin.

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Abbreviation
AFM atomic force microscopy

Introduction

Since the early part of this decade, the advent of biophysical techniques for the manipulation of single biological molecules has made possible a large number of significant breakthroughs in biology. The effectiveness of these techniques has perhaps been best demonstrated in the field of molecular motors, particularly with the use of optical trapping techniques in conjunction with nanometer-precision position detection schemes (optical trapping nanometry). An optical trap is produced by highly focused laser light, and can be used to grab, move, and exert measurable forces on micron-sized (and smaller) objects, such as dielectric microspheres (see Figure 1). A microsphere, chemically coupled to a molecule of interest, provides a means of measuring the molecule's position and the force that it exerts. About six years ago, when single kinesin molecules were observed to have a step-size of 8 nm [1], it became clear that optical trapping nanometry had great potential to probe the molecular mechanisms of motor proteins. Further demonstration came from the subsequent observation of forces and displacements produced by single myosin molecules using feedback-enhanced optical traps [2]. Since then, optical trapping nanometry has revolutionized the field of molecular motors, and has become the technique of choice for many researchers in this field.

Single-molecule mechanical manipulations complement conventional biochemical approaches and can often yield important new information. Firstly, motions of individual molecules can be monitored without the complications of population kinetics. Secondly, the progress of a reaction or process can be tracked continuously without suffering loss of temporal and spatial resolution. Thirdly, and most importantly, the mechanical properties of molecules can be studied through direct and well-controlled manipulation.

A number of single molecule manipulation techniques exist, including optical trapping nanometry, magnetic bead techniques, microneedle techniques, micropipette techniques, and some scanning probe microscopies. These techniques differ in their precision of position detection (~1 Å to 10s of nanometers) and force regimes (~0.1–10,000 pN).

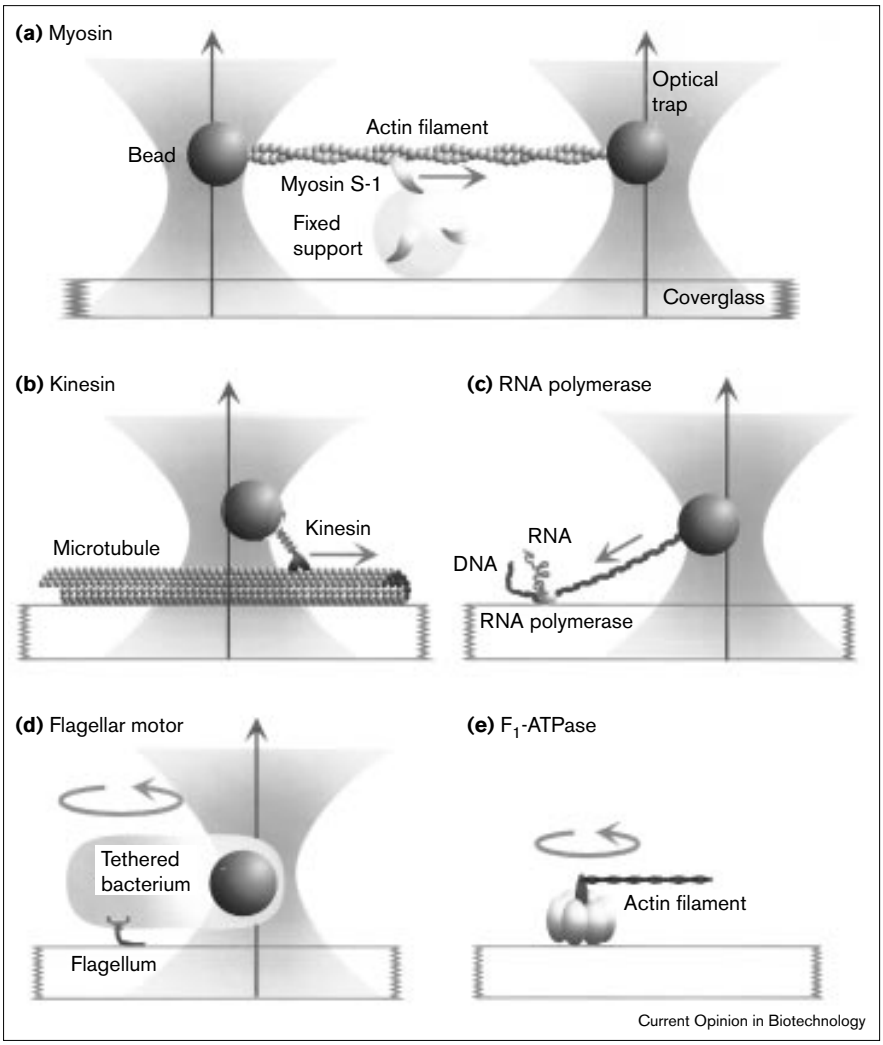
Besides the field of molecular motors, single molecule manipulation techniques have transformed many other fields of biology, such as biopolymer mechanical studies, protein unfolding, receptor–ligand interactions, and others. This review will highlight advances in these areas of study from 1997 to the present day, which were brought about by the development and use of single-molecule manipulation techniques.

Molecular motors

The field of molecular motors continues to benefit from the advancement of single-molecule manipulation techniques. Molecular motors are essential molecules of life, carrying out diverse functions in cells, such as muscle contraction, vesicle transport, chromosome separation, replication, transcription, translation, and so on. Two classes of molecular motors exist — those that move along a linear substrate (linear motors) and those that rotate (rotary motors). Examples of linear motors include the conventional molecular motors, such as actin-based myosin, and microtubule-based kinesin and dynein, as well as the not-so-conventional molecular motor RNA polymerase. Examples of rotary motors include the bacterial flagellar motor and F_1-ATPase. All molecular motors use available chemical energy to perform mechanical work.

During this decade, much progress has been made towards understanding the conventional motors myosin and kinesin (Figures 1a,b). Recently, more advanced physical instrumentation and analysis provided insights into a key aspect of these motors — the coupling of the mechanical cycle of a motor with its chemical cycle of ATP hydrolysis. By combining optical trapping nanometry with total internal reflection fluorescence microscopy, Ishijima *et al.* [3••] directly observed the interactions of a single myosin molecule with an actin filament while simultaneously detecting ATP binding to, and ADP release from, the myosin. Using optical trapping nanometry in conjunction with caged-ATP photolysis, Higuchi *et al.* [4•] examined the lag time between ATP binding to kinesin and kinesin force generation. Using optical trapping interferometry (which combines optical trapping

Figure 1



Cartoon (not drawn to scale) illustrating some typical experimental configurations for manipulating molecular motors. Note that vertical arrows indicate the direction of laser light propagation. **(a–d)** The force and displacement of the motor are detected via the trapped bead. **(a)** Myosin. An actin filament, suspended by two separate optical traps via two beads attached to its ends, is lowered over a third, fixed bead coated with myosin S-1 fragment (e.g. see [2]). **(b)** Kinesin (or dynein), coated onto a bead, moves along a microtubule which is attached to the surface of a microscope coverglass (e.g. see [1]). **(c)** RNA polymerase. A bead is attached to the transcriptional downstream end of DNA so that it becomes tethered to the surface of a microscope coverglass via the RNA polymerase fixed to the coverglass (e.g. see [19]). **(d)** Bacterial flagellar motor. An *E. coli* cell is anchored to the surface of a coverglass via its flagellum and rotates around the point of attachment. The cell body pushes against the trapped bead (e.g. see [21]). **(e)** The F_1-ATPase is anchored to the surface of a coverglass. The rotation of its γ-subunit (in the center of the molecule) relative to the rest of the molecule is visualized by an attached fluorescent actin filament (e.g. see [22•]).

with an interferometric position detection scheme) in conjunction with a statistical analysis method, Schnitzer and Block [5••] found that kinesin consumes one ATP per 8 nm step, consistent with results of a video-tracking method applied to the same question [6].

Progress has also been made towards understanding other aspects of these motors. For myosins, optical trapping nanometry and atomic force microscopy (AFM) have permitted investigation of individual myosin–actin interactions [7], the strength of these interactions [8], the orientation dependence of these interactions [9], the stiffness of actomyosin cross-bridges [10], and the forces and displacements of smooth, skeletal, and cardiac muscle myosins [11,12]. For kinesins, optical trapping nanometry has permitted further investigation of the kinesin velocity dependence on the applied load [13,14], the function of the neck domain of kinesin using recombinant kinesin [15], the velocity of a kinesin-like motor NCD [16], and the organization of microtubules by kinesin [17].

Another microtubule-based molecular motor, dynein, has not been left out of the discovery-fest. Using optical trapping nanometry combined with photolysis of caged-ATP, Shingyoji *et al.* [18•] recently found that single dynein arms produced oscillatory forces as they moved along a microtubule. Their discovery suggests that these oscillatory forces of dynein may be the key to the rhythmic beating motions of eukaryotic flagella.

Many DNA-based enzymes are also molecular motors. For example, RNA polymerase is a highly processive molecular motor, capable of moving through thousands of basepairs without detaching from the DNA template. Using optical trapping interferometry, Yin *et al.* [19] demonstrated that RNA polymerase is capable of generating at least 14 pN of force (Figure 1c). Recently, with a high precision, feedback-enhanced optical trapping interferometer, Wang *et al.* [20••] revised this figure up to 25 pN, and determined the speed of transcription as a function of applied force. These studies demonstrate the

potential of optical trapping nanometry for the study of DNA-based mechanoenzymes.

Besides the aforementioned molecular motors that move along a linear substrate, rotary motors have also been studied using single molecule techniques (Figures 1d,e). By using optical trapping nanometry, Berry and Berg [21] demonstrated that a bacterial flagellar motor is capable of generating a torque of ~4500 pN nm. A single molecule *in vitro* motility assay has already been established for another rotary motor — F_1-ATPase, which is a part of ATP synthase. By attaching a fluorescent actin filament to the γ-subunit of the F_1-ATPase, Noji *et al.* [22•] directly demonstrated that the γ-subunit of F_1-ATPase rotates relative to the rest of the molecule in the presence of ATP. The torque that the motor can generate was estimated to be ~40 pN nm based on viscous drag. Further studies showed that the γ-subunit rotates in discrete 120^o increments [23]. More elaborate and controlled biophysical manipulation techniques should help to further elucidate the molecular mechanism of the rotation.

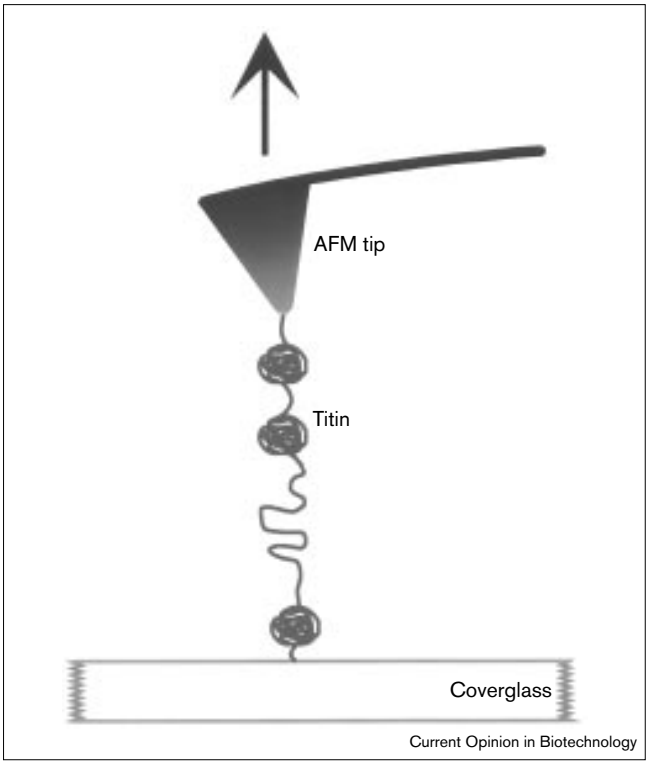
Biopolymer mechanics

Single-molecule manipulation techniques are ideally suited to the study of both molecular motors and the substrates along which they move. In fact, these techniques can be readily adapted to investigate the physical properties of single biopolymers (or single polymers in general). Mechanical properties of these substrate molecules feed back to the functions of their motors, and also determine the structural rigidity of cellular components.

The flexural rigidity of an actin filament (the substrate for myosin) has been measured to be $\sim 2 \times 10^4$ pN nm^2 using optical trapping nanometry [24]. These results are in the same ballpark as earlier single-molecule mechanical measurements on the torsional rigidity, flexural rigidity, and axial stiffness, of actin filaments [25–27]. Using optical tweezers, flexural rigidity values have also been determined for single microtubules (the substrate of kinesin and dynein) without ($\sim 4 \times 10^6$ pN nm^2) and with microtubule-associated proteins [28]. These measurements supplement somewhat similar earlier studies [29,30]. In addition to being the substrate for molecular motors, actin and microtubules have been speculated to drive various cellular motility activities (e.g. motions of chromosomes during mitosis) via their polymerization and depolymerization reactions. Significant progress towards single-molecule investigations of these possibilities has already begun [31,32].

Few polymers have captured as much fascination from researchers as has DNA. This is, at least in part, due to the importance of its physical properties in regulating genetic information storage and expression. Since the initial mechanical studies on single molecules of DNA [33], research in this area has expanded dramatically. Some representative recent work includes DNA elasticity studies using optical tweezers, micropipette, or AFM [34–36], DNA supercoiling and its effects on homologous pairing using a magnetic bead [37,38], effects of DNA-binding proteins on DNA elasticity using optical tweezers or micropipette [39,40], DNA duplex separation using AFM or microneedle [41,42], and hydrodynamics of DNA molecules using a flow field [43–46].

Figure 2



Cartoon illustrating a typical experimental configuration for unfolding single molecules of titin (or tenascin). Titin stretched by an AFM tip is used as an example here. One end of a titin molecule is anchored on the surface of a coverslip, while the other end is attached to the tip of an AFM tip. As the AFM tip is pulled away, the molecule unfolds (e.g. see [48••]).

Protein unfolding

Although proteins comprise a subset of biopolymers, the importance of recent protein unfolding studies using single-molecule manipulation techniques deserves more emphasis.

Conventionally, when a single protein molecule was manipulated (e.g. in molecular motor studies), it was typically studied as a whole when exploring its mechanics. Recently, several research groups have undertaken the task of unraveling the internal mechanical properties of single protein molecules (Figure 2). Among the most notable examples are studies of the proteins titin (important in maintaining sarcomere structural integrity and generating passive force in muscle), and tenascin (an extracellular matrix protein thought to provide a rigid mechanical anchor that supports and guides migrating and rolling cells). Individual protein molecules were stretched out from end-to-end at speeds of 0.01–10 μm s^{-1}, taking

advantage of several different biophysical manipulation techniques, including AFM [47••,48••], optical trapping nanometry [49•], and optical trapping techniques in combination with micropipette [50•]. One experimental configuration is shown in Figure 2. Both titin and tenascin contain repeating domains, which were expected to unfold under sufficient tension. Indeed, the force-extension curves of the stretching experiments showed repeating stick-slip patterns as a protein molecule was stretched out [47••,48••]. During stretching, the force increases monotonically, with extension to some value, but then dropped suddenly. Each of these drops in force (slip events) was speculated to correspond to the unfolding of one of the protein domains. The stick-slip peaks typically are 150–300 pN, spaced at 25–30 nm, over the range of the stretching speed. Upon relaxing the stretched molecule back to its natural equilibrium position, the molecule refolds. So, the same molecules can be repetitively stretched out and relaxed back reversibly. These types of studies lay the cornerstones for new approaches to the protein folding problem — the energy landscapes of individual proteins may be directly explored by improving the physical instrumentation to cover both smaller and larger time scales.

Receptor–ligand interactions

Receptor–ligand interactions, which are important in a wide range of signal transduction pathways and cellular adhesion processes, are also ideally suited for single-molecule manipulation techniques. The effectiveness of mechanical manipulation for the study of receptor–ligand interactions was first demonstrated using several physical techniques, including surface force apparatus [51,52], AFM [53,54], and micropipette suction apparatus [55]. Some representative recent studies have revealed the interaction forces for P-selectin–ligand complexes [56] and for antigen–antibody binding [57,58] using AFM.

New techniques for the future?

The advent of new physical instrumentation continues to redefine the limits of precision of single-molecule measurements and manipulations. For example, a single molecule of oxygen has been induced to dissociate, or to rotate forward and backward [59,60•]. These events were also simultaneously visualized using scanning tunneling microscopy (STM). High-precision and well-controlled manipulation techniques, such as STM, hold promise for addressing biological questions at the sub-macromolecular level.

Conclusion

The important contributions to biology by single-molecule manipulation techniques have marked the beginning of an exciting era for such studies. Motions of individual biomolecules can now be monitored with high temporal and spatial resolution. More importantly, the mechanical properties and behaviors of these molecules can now be directly examined at the single-molecule level and correlated with their native functions. The combination of advanced biophysical manipulation techniques with biochemical and structural studies will yield a complete picture of biological systems and processes in the years to come.

Acknowledgements

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