Manipulation of Single Molecules in Living Bacteria

Laser tweezers are versatile for studying protein folding as well as molecular motors in living cells

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Starting in the 1980s, researchers began to realize the long-standing dream of studying individual molecules, particularly proteins. With a growing number of specialized techniques to use, researchers can now manipulate and visualize individual molecules and thus study mechanical unfolding and refolding of proteins or nucleic acids, the strength of receptor-ligand bonding interactions, and the nanomechanics of biological motors.

Optical tweezers provide a mechanical approach to studying protein folding that involves no denaturing chemicals, meaning structures can be probed at close to natural conditions. Moreover, optical tweezers are well suited for studying molecular motors that use adenosine triphosphate (ATP) molecules or ion gradients across membranes to drive motion. This approach enables researchers to ask important questions, such as how fast does a particular motor move, does it perform work against external forces, and how many ATP molecules or ions are required to generate each cycle. In vitro experiments provide a huge step towards understanding and modeling how molecular motors convert chemical energy into motion. For instance, researchers are studying proteins such as kinesin, myosin, polymerases, or helicases to measure their particular speeds, maximum forces, processivities, working stroke distances, and mechano-chemical coupling ratios. They find that such isolated molecular motors generate maximum forces within a range of 1 picoNewtons (pN) to 50 pN, and several such motors show considerable slippage and backward steps.

It is a big challenge to use these techniques on living cells; molecular motors are typically part of molecular complexes whose protein components are subject to the physiological state of the cell and environmental signals. Howard Berg and collaborators at Harvard University, Cambridge, Mass., proved that laser tweezers can be used to investigate rotating flagella in live Escherichia coli. George Whitesides, also of Harvard, and his collaborators use laser tweezers to measure the adhesion force between type I pili on bacterial cells and mannose-coated surfaces.

How Do Laser Tweezers Work?

Optical tweezers are built into optical microscopes in which the objective is used to focus a laser beam to a diffraction-limited spot in the image plane. A dielectric, nonabsorbing particle such as a glass bead or a bacterium in that plane will experience a force that tends to move it to the region of highest light intensity, namely the focal point of the laser.

Summary

- With laser tweezers, investigators can measure forces that molecular machines generate.
- Cell appendages can be used as extensions or lever arms to manipulate single molecules in living bacteria.
- Single P-pili can be mechanically unfolded and refolded by applying mechanical tension.
- Molecular machines prove considerably more efficient and powerful in situ than when isolated.
Objects smaller than the wavelength of the laser light develop an electric dipole in response to the electric field associated with that light beam, and that dipole is drawn up intensity gradients towards the focus. Larger objects act as lenses, refracting the rays of light and redirecting the momentum of their photons. The resulting recoil draws them toward the higher flux of photons near the focus. This recoil is imperceptible for macroscopic lenses but can have substantial influence on mesoscopic objects. Moreover, a small scattering force, the magnitude of which is directly proportional to the light intensity, pushes the particle beyond the focal point of the laser.

Such laser tweezers can trap objects as small as 5 nm and exert forces exceeding 100 pN ($10^{-10}$ N). Fast photodiodes allow investigators to detect the position of such objects at rates exceeding 10 kHz. By combining those analytic capabilities with particle-tracking techniques, they can measure displacements below 1 nm and forces as small as femtoNewtons. For instance, Steven Block and coworkers at Stanford University in Stanford, Calif., developed a setup for following DNA translocation by RNA polymerase at a resolution below 0.1 nm, which corresponds to the dimension of a single hydrogen atom.

When a molecular machine exerts force on the object in the laser trap, the particle is displaced from focus in a manner similar to an object that is attached to a mechanical spring. At small displacements, the restoring force increases linearly with the deflection (Fig. 1). In other words, the laser tweezers exert a force proportional to the deflection, and, therefore, the setup allows investigators to determine the force generated by the molecular machine. Moreover, when force is applied to the object in the trap—for example, by moving the microscope stage relative to the trap center—it is possible to measure the elastic response of a polymer to externally applied forces.

**P Pili Elongate Seven Times under Mechanical Tension**

One simple and straightforward use of laser tweezers in microbiology is to analyze mechanical and structural properties of proteins and protein assemblies—in particular, the unfolding and refolding of polymeric filaments. By attaching one end of the polymer to a surface and the other end to a bead trapped by laser tweezers, the polymer can be stretched while the effects of that stretching can be measured (Fig. 1). The resulting elongation-versus-force curves give information about the elasticity, forces, and energy required to unfold the quartenary structure and to break the protein filament.

Recently, Ove Axner at Umea University in Umea, Sweden, and collaborators applied this method to investigate unfolding forces in P-pili...
without use of denaturing agents such as urea. *E. coli* that colonize the urinary tract are exposed to mechanical host defenses, including the shear force that urine flow generates. These bacteria attach to host surfaces through P-pili, which bind to glycolipids on epithelial cell surfaces. Although urine removes other bacteria that bind to hosts through various other fimbrial adhesins, P-pili enable *E. coli* cells to resist this cleansing action. P-pili, which are 68 Å in diameter and about 1 μm long, are composed of about 1,000 copies of the principal structural protein, PapA. PapA subunits are coupled to their nearest neighbors by β-strand complementation and are arranged in a right-handed helical conformation (Fig. 2).

Pili are formed by the tight winding of a much thinner structure, according to Esther Bullitt and Lee Makowski of Boston University School of Medicine in Boston, Mass. Based on electron microscopic data, they suggest that a structural transition allows the pilus to unravel without depolymerizing, producing a thin, extended structure five times the length of the original.

Recent experiments by Axner and his collaborators confirm that mechanical forces induce the helical quaternary pilus structure to unfold. They mounted bacteria onto activated beads attached to a coverslip. A second bead was trapped in the laser tweezers and moved near trapped individual bacterial cells (Fig. 1). Once the pilus attached to the bead, force was applied on the pilus by continuously enhancing the distance between the bacterium and the center of the laser tweezers. The force on the bead—and, thus, the tension acting on the pilus—increased with increasing displacement of the bead from the center of the laser tweezers.

The force-versus-elongation curves show a characteristic profile: At a force of 27 pN, the helical structure abruptly unfolds, which is consistent with the electron microscopic data, presumably following sequential breakage of interactions between adjacent PapA subunits. At about 100 pN, the overall elongation was 7 times its unstretched length. The elongation process was entirely reversible; when tension was released, the pilus shortened to its initial length.

These findings suggest that bacterial P-pili may have intrinsic elongation properties that allow them to support tensions over a broad range of lengths. Furthermore, since unfolding of the quartenary structure is completely reversible, folding may enable bacteria to reestablish close contact to host cells once shear flow stops.

**Type IV Pili Are Dynamic Polymers That Generate High Molecular Force**

Optical tweezers are also useful for analyzing kinetics and force generation of single molecular motors or motor complexes. In living bacteria the technique is restricted to analyzing molecular motors that are located in or connected with the cell envelope.

Type IV pili are polymeric filaments that extend several micrometers from the cell envelope. They are involved in binding not only to mammalian cells but also to abiotic surfaces; further, they are important for surface motility, biofilm formation, and horizontal gene transfer. Pili are assembled when pilin subunits polymerize, and they come apart when those subunits depolymerize. PilF, a distinct member of AAA ATPases, supports pilus polymerization, and another member of the AAA ATPase family, PilT, is required for twitching motility (Fig. 3A).

Functional PilT significantly modifies the response of the cytoskeleton of host mammalian cells, say Magdalene So, Alexey Merz, Heather Howie, and coworkers at Oregon Health Science University in Portland. Does PilT act as a molecular motor that powers type IV pilus retraction? And does the mechanical force generated by pilus retraction influence the dynamics of the host cytoskeleton during adhesion?
Merz, who is now at the University of Washington in Seattle, Michael Sheetz of Columbia University in New York, N.Y., and their collaborators used laser tweezers to show that type IV pili generate mechanical force when they retract into the bacterial cell body. Moreover, in mutant strains that do not express the PilT retraction protein, pilus retraction is inhibited, suggesting that PilT acts as the molecular motor that moves the pilus tip either directly or indirectly by enhancing pilus depolymerization.

By lowering the concentration of pili to a single pilus per cell, we determined that a single retracting pilus generates a molecular force that exceeds 100 pN. By comparison, a single myosin II molecule from muscle cells generates a force of about 5 pN.

With wild-type Neisseria gonorrhoeae, pilus retraction is highly processive, and does not stop, pause, or reverse. However, when the concentration of the pilus retraction protein PilT is reduced, we see force-induced elongation of the pilus. Pilus-induced tension may be important for the cytoskeletal response of host cells to adhesion of Neisseria. Vice versa, the level of PilT, and thus pilus dynamics, is regulated during infection. Therefore, PilT levels might help to control interactions between bacteria and host cells by controlling these tensions.

This series of experiments shows that type IV pili are coupled to an extremely powerful molecular motor, probably PilT, that works processively. However, the reversibility of the machine can be perturbed by varying the concentration of the putative molecular motors. The nanoscopic motor may use a mechanism similar to that of a macroscopic linear motor (Fig. 3C). Thus, the molecular motor may be connected to the polymer to power its translocation. However, there is no experimental evidence for a toothed-wheel or toothed-belt mechanism for this molecular motor.

An Active and Powerful Machine Drives DNA Uptake in Bacillus subtilis

When a bacterial cell is transformed genetically, DNA must move through the cell envelope. Double-stranded DNA with a contour length on the order of micrometers forms a coil with a diameter on the order of 1 μm. Translocation through a nanometer-sized pore is entropically not favorable because the cell wall reduces the...
number of allowed conformations for this molecule. For DNA to be transported efficiently, it is likely that a molecular machine pulls such DNA molecules through bacterial cell envelopes.

In stationary phase, *Bacillus subtilis* cells express several proteins that support efficient transport of DNA through the cell envelope (Fig. 3b). It is likely that they form a DNA transport machine to transport macromolecular DNA through the bacterial cell envelope, but little is known about the mechanism, regulation, and dynamics of this complex.

We designed experiments for measuring the rate of transporting single DNA molecules into bacterial cells. Bacterial cells were immobilized along a glass surface, and we used laser tweezers to bring DNA-coated beads close to single cells. Once the DNA bound to the bacillus, the bacterium began to take up the DNA, pulling the attached bead away from the center of the laser tweezers. Using a tracking algorithm, we determined the uptake velocity to be 80 bp/sec. This DNA transport was highly processive and irreversible. Even at high external forces of 40–50 pN, there was no significant reduction of velocity in DNA uptake, indicating that a powerful molecular machine supports DNA transport.

Where is this machine within the bacteria cell, and is its localization regulated? Various proteins responsible for DNA uptake form foci, mainly at the cell poles, according to Jeanette Hahn and David Dubnau of Public Health Research Institute in Newark, N.J. Using GFP-fusion proteins, they showed that competence proteins accumulate at the cell poles as transformability develops and then diffuse when cells no longer are competent for being transformed.

When we used the single-molecule assay, we found that DNA is taken up preferentially from the cell poles and that uptake colocalizes with those foci. The experiments suggest that the cell regulates the assembly and disassembly of its DNA transport machine at the cell poles. Moreover, this machine performs mechanical work against external forces, suggesting that DNA uptake in *B. subtilis* is actively driven rather than passive.

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**The Bacterial Flagellum Motor Rotates Stepwise**

Directly observing steps and measurement of step length in molecular motors reveals much about the fundamental mechanism of these protein-based machines. Although biologists who pioneered the use of laser tweezers measured the torque generated when *E. coli* cells rotate their flagella, they did not measure the elementary step size until recently.

Many species of swimming bacteria use similar molecular machines to rotate their flagella. Its rotor consists of a set of rings within the cytoplasmic membrane, each of which is up to 45 nm in diameter; the stator contains about 10 torque-generating units anchored in the cell wall at the perimeter of the rotor (Fig. 4A).

This machine generates torques of about 4,000 pN nm near stall. Because the radius of the rotor is about 20 nm, the machine generates a force of about 200 pN at its rim, making the bacterial flagellar machine even more powerful than the type IV pilus motor. Ions (H\(^+\), Na\(^+\)) drive the flagellar motor, which is powered by a transmembrane electrochemical gradient. According to one model, each passing proton (or...
proton pair) moves a torque generator one step (one binding site) along the periphery of the rotor. Any linkage between the torque generator and the rigid cell wall would become stretched, and releasing this tension would generate rotational movement. If correct, the flagellum works like a stepping motor in which torque is transmitted by toothed wheels (Fig. 4b).

Richard Berry and his coworkers at Oxford University in Oxford, United Kingdom, recently solved several technical problems, thus allowing them to observe individual steps while the bacterial flagellar motor rotates. First they immobilized an *E. coli* cell at a glass surface, then attached a 500-nm bead to one flagellum and used low-power laser tweezers to avoid interfering with its rotation. The interference between the illuminating laser light and the light scattered from the bead allowed them to detect the position of the bead with high resolution. They also used a chimeric flagellar motor that was driven by a Na\(^+\) instead of a H\(^+\) gradient, enabling them to limit extracellular Na\(^+\) and thereby slow flagellum rotation enough to detect 26 individual steps per revolution. This finding is consistent with 14° steps, which corresponds to the periodicity of the ring of the FliG rotor component, the proposed site of torque generation.

One of the next steps in understanding how bacteria use ion gradients to rotate their flagella will entail determining how ion flow through the cell envelope is coupled to rotational motion. Energy calculations indicate that single ions are not sufficient to generate a step of 14°, which is what the experiment seems to suggest. However, more than one ion may be involved in driving each elementary motor step.

**Additional Analytic Challenges for the Whole Cell, Single-Molecule Assay Appears Suited**

Optical force proves useful for manipulating single molecules and probing the forces that they, molecular complexes, and cells can generate. Initially, this approach was restricted to analyzing isolated proteins and their responses to external mechanical force in vitro.

However, although nanomanipulation cannot be used to investigate intracellular molecules, experiments with external molecules such as pili and flagella in intact cells are providing useful insights. For example, in contrast to results from in vitro experiments that characterized single molecular motors, maximum forces and processivity appear to be considerably higher in vivo without evidence of pausing or slippage. Perhaps the integrity of the molecular machines in intact cells accounts for this higher efficiency. One challenge facing investigators who use laser tweezers is to determine what perturbations affect the power and efficiency of these molecular machines in intact cells. Another challenge is to investigate the responses of molecular motors to changing physiological and environmental conditions.

**SUGGESTED READINGS**


