GRABBING THE CAT BY THE TAIL: MANIPULATING MOLECULES ONE BY ONE

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Methods for manipulating single molecules are yielding new information about both the forces that hold biomolecules together and the mechanics of molecular motors. We describe here the physical principles behind these methods, and discuss their capabilities and current limitations.

OPTICAL TWEEZERS
Focused photon fields.

PIEZO-ELECTRIC
Describes a device that expands or contracts as a voltage is applied to an internal crystal.

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"I know a man who grabbed a cat by the tail and learned 40 per cent more about cats than the man who didn't."

Mark Twain

Until quite recently, scientists could only investigate chemical processes on a bulk level. The forces and stresses that molecules exert on each other or develop in the course of reactions were not directly measurable. During the past few years, this situation has changed rapidly thanks to the development of methods for manipulating single molecules[1–4]. Methods such as OPTICAL TWEEZERS and scanning force microscopy (SFM)[5] are making it possible to follow, in real-time and at a single-molecule level, the movements, forces and strains that develop during the course of a reaction. These methods can be used to measure directly the forces that hold together molecular structures. They can also be used to exert external forces to modify the extent and even alter the fate of reactions in the hope of discovering rules that govern the inter-conversion of mechanical and chemical energy in these processes. This area of research can rightly be called 'mechanochemistry', and includes biochemical processes as diverse as protein folding[6], DNA elasticity[7–9], the protein-induced bending of DNA[10], the stress-induced catalysis of enzymes[11], the behaviour of molecular motors[12–15], and even the ubiquitous processes of protein–protein recognition[16].

Here we focus on the current capabilities and limitations of single-molecule manipulation methods, and provide guidelines for choosing the most appropriate method for a given problem.

Choosing the appropriate method

All single-molecule manipulation methods require two basic elements: a probe, which is usually of microscopic dimensions, that can generate or detect forces and displacements; and a way to spatially locate the molecules. As summarized in TABLE 1, the relevant force ranges, minimum displacements, probe stiffness, applications and practical advantages of each technique vary significantly.

Mechanical transducers

Mechanical force transducers apply or sense forces through the displacement of a bendable beam. The most common examples are SFM cantilevers[5] (FIG. 1) and microneedles[12] (FIG. 2). The spatial control of transducers can be accomplished efficiently by PIEZO-ELECTRIC positioners (FIG. 1a). Mechanical transducers have been used to investigate systems ranging from protein unfolding[6] and cell motility[17] to forces generated by motor proteins[12]. Mechanical transducers possess a linear response over a broad range of displacement and forces. Two important factors determine how mechanical transducers interact with single molecules: their size and stiffness. The effect of these parameters is described below and in BOX 1.

SFM cantilevers. Microfabricated cantilevers are available in a wide variety of sizes, shapes and materials. SFM devices are also commercially available, and some are specifically designed for manipulating single molecules[18] (FIG. 1b). The advantages of SFM are its high spatial range and sensitivity, its throughput (the ability to study many single molecules on a surface) and versatility.

HYDRODYNAMIC FIELD
A force field resulting from the momentum imparted by molecules in a flowing aqueous solution.

PHOTON FIELD
A force field resulting from the momentum imparted by photons in a beam of light.

For example, SFM can be used both as an imaging instrument and as a manipulation device, as first shown by Müller *et al.*[19] and further exploited by Oesterhelt and colleagues[20] (see below).

SFM cantilevers have stiffness (κ) ranging from 10^{-3} to 100 N m^{-1}. Stiffer cantilevers have lower sensitivities, as force is always detected by measuring a displacement that is inversely proportional to the stiffness. They are nonetheless useful when a given process (conformational changes, for example) requires the application of high forces. Although stiffer cantilevers experience correspondingly large force fluctuations owing to thermal motion (for a typical cantilever with $\kappa = 0.06$ N m^{-1}, the root-mean-square force fluctuation is ~16 pN; BOX 1), the signal-to-noise ratio of the measurement is independent of the stiffness of the cantilever[2,21]. In addition, drift over time caused by the thermal expansion and nonlinear voltage response of the piezo-electric crystals can further compromise the control of force on the sample. This is not a serious limitation, however, as ways to compensate for drift have been developed[22].

A promising development in SFM methodology is the fabrication of smaller, but still soft, cantilevers[23]. Their small physical dimensions allow them to have higher sensitivity and faster response times[24]. Being soft, these small cantilevers allow high spatial resolution without a subsequent increase in force fluctuations. This higher spatial resolution stems from the distribution of thermal fluctuations over a broader frequency range, thus decreasing the noise at biologically relevant frequencies (BOX 1).

The scanning force microscope has been used successfully to study the mechanism of unfolding in proteins. Fernandez and co-workers[6] unfolded a protein made of repeating immunoglobulin-type domains by pulling it with an SFM cantilever. To obtain refolding rates, they allowed the protein to refold for a varying amount of time before it was re-extended. These authors also determined how the unfolding force varies with pulling speed. Extrapolation of these data to zero force yielded a pulling speed of 0.013 nm s^{-1}, which, when divided by the extension required to unfold one domain (28.4 nm), matches the unfolding rate determined by chemical denaturation experiments (4.9×10^{-4} s^{-1}). So, mechanical unfolding experiments measure the same interactions as their chemical analogues, and have the potential to follow secondary-structure-unfolding events.

Microneedles. Because of their dimensions (typically 50–500 μm long and 0.1–1 μm thick), glass microneedles are usually softer than cantilevers (TABLE 1). This property gives them an advantage over SFM cantilevers for probing delicate biological systems. Microneedles are not commercially available, however, and the devices to detect their displacement are less standardized than in SFM. Two general approaches for displacement detection have been reported: imaging the microneedle itself [12,25–28]; and using a chemically etched optical fibre that projects light from its tip onto a photodiode[29–31] as a microneedle. This latter method has been used to measure the stretching of twisted DNA[31] and to study the binding of RecA to stretched DNA[30].

Microneedles have also been used successfully to quantify the force that myosin exerts on F-actin[12,25,27]. In the first of these experiments, fluorescently labelled F-actin filaments were attached to a microneedle coated with monomeric myosin (FIG. 2). This filament was brought into contact with a myosin-coated surface. The subsequent bending of the microneedle corresponded to the combined force (9.6 pN) exerted by no more than 53 interacting myosin heads (at least 0.2 pN per myosin head). This average force is comparable to that exerted in a muscle during contraction[32] (~1 pN). More recent studies, using complex experimental geometries (FIG. 3a), have determined the step size of myosin on actin to be 5.3 nm (REF. 25), and the force generated during the actual power stroke to be ~3–5 pN (REFS 21,27).

External field manipulators

External fields provide another approach to the manipulation of single molecules. Examples are HYDRODYNAMIC, magnetic and PHOTON fields. Unlike mechanical transducers, external fields act on molecules from a distance. These fields can be used to exert forces on

Table 1 | Overview of single-molecule manipulation methods

Methods	$F_{min-max}$ (N)§	X_{min} (m)§	Stiffness (N m^{-1})	Applications	Practical advantages
Cantilevers*	10^{-11}–10^{-7}	10^{-10}	0.001–100	Protein/polysaccharides[6,64] Bond strength[65,66]	High spatial resolution Commercially available
Microneedles*	10^{-12}–10^{-10}	10^{-9}	10^{-6}–1	Myosin motor force[12] DNA/titin strength[26,28]	Good operator control Soft spring constant
Flow field‡	10^{-13}–10^{-9}	10^{-8}	n.a.	DNA dynamics[38] RNA polymerase[36]	Rapid buffer exchange Simplicity of design
Magnetic field‡	10^{-14}–10^{-11}	10^{-8}	n.a.	DNA entropic elasticity[8] Topoisomerase activity[41]	Specificity to magnets Ability to induce torque
Photon field‡	10^{-13}–10^{-10}	10^{-9}	10^{-10}–10^{-3}	Protein motors[13,14] Protein unfolding[52]	Specific manipulation High force resolution

*Mechanical transducers: probes are bendable beams; spatial location is by beam deflection. ‡External field manipulators: probes are microscopic beads; spatial location is by bead displacement. §These numbers represent only empirical, not absolute limits. ($F_{min-max}$, force range; X_{min}, minimum displacement.)

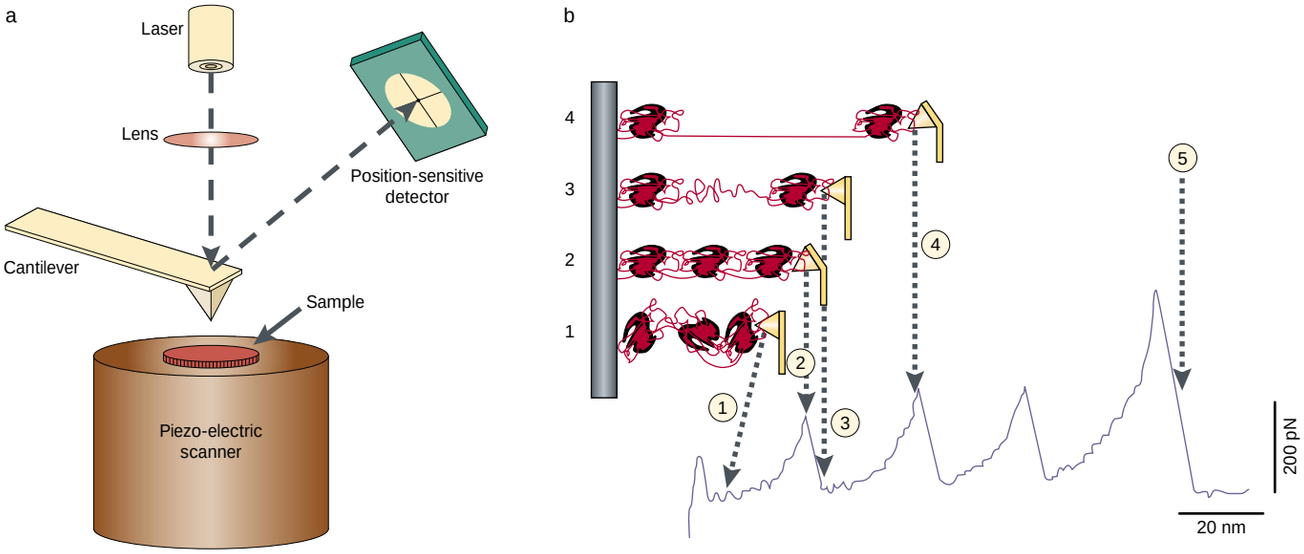


Figure 1 | **Applications of the scanning force microscope (SFM). a** | The principal SFM components. Laser light is focused onto the back of a cantilever that ends with a nanometre-scale tip. The reflection and corresponding position of the tip is detected by a position-sensitive photodiode. A piezo-electric scanner moves the sample in all directions, enabling the tip to scan topography or to extend molecules attached to the surface. **b** | Diagrams and force curves showing the mechanical unfolding of repeating immunoglobulin-like domains[6,64]. As the distance between the surface and tip increases (from state 1 to state 2), the molecule extends and generates a restoring force that bends the cantilever. When a domain unfolds (state 3), the free length of the protein increases, relaxing the force on the cantilever. Further extension again results in a restoring force (state 4). The last peak represents the final extension of the unfolded molecule before detachment from the SFM tip (state 5).

molecules by acting either on the molecules themselves, or through 'handles' such as glass beads, polystyrene beads or metallic particles attached to the molecules. The various external fields give different degrees of control over the magnitude and stability of the applied forces.

Flow fields. Flow fields exert forces on objects through the transfer of momentum from the fluid to the object (FIG. 3b). In LAMINAR FLOW, the drag force between a moving liquid with viscosity η and velocity v and a stationary bead handle of radius r can be calculated using STOKES'S LAW. For spheres of any size in water, Stokes's law remains valid for forces up to ~10 nN, beyond which turbulence becomes a factor. Forces up to ~10 nN can therefore be applied reliably.

The advantages of using flow fields for single-molecule experiments include the fact that the liquids surrounding the tethered macromolecule can be easily replaced. This feature is important in many single-molecule studies of enzymes, which require varying buffer conditions. Moreover, the flow can be used to introduce new beads or biomolecules.

To calculate the drag force, the size of the bead handles and the actual flow velocity must be known. Furthermore, an accurate drag-force calculation requires the bead handle to be stationary in the flow, such as in the case of a bead tethered to a surface by a piece of DNA (FIG. 3b). In addition, as single-molecule experiments are almost always carried out inside a microchamber, force determination should also take into account the modification of Stokes's law, owing to the coupling, through water, between the bead and the boundaries of the microchamber[8,33,34]. Finally, it should be kept in mind that, because the drag coefficient of an object scales largely with its longest dimension[35], often the friction coefficient of a long polymer such as DNA for example is comparable to that of the bead handle and cannot be neglected.

The flow-field manipulation technique was demonstrated in the earliest single-molecule study of DNA elasticity[8]. In that study, biotinylated DNA was attached by one end to a streptavidin-coated glass surface and its other end was attached to a magnetic bead (FIG. 3b). With this set-up, different tensions were applied to single

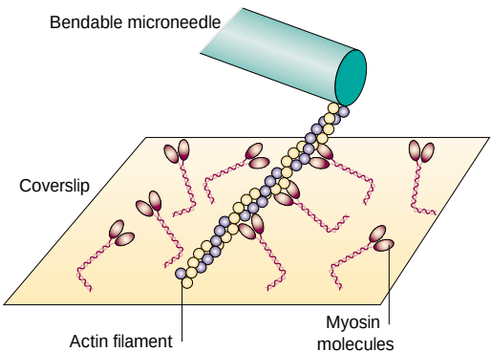


Figure 2 | **Using a microneedle to measure the force of myosin acting on actin.** A bendable microneedle coated in myosin heads (not shown) catches an actin filament. This filament is brought into contact with a glass coverslip coated in myosin molecules. In the presence of ATP, the myosin drags the actin filament across the coverslip and generates a force on the microneedle, which is observable by video-fluorescence microscopy.

LAMINAR FLOW
A flow of molecules in which neighbouring molecules have linearly dependent velocities, that is, not a turbulent flow.

STOKES'S LAW
$F_{drag} = 6\pi r\eta v$

RADIATION PRESSURE
The pressure on an object that arises from photon collisions rather than from bombarding molecules.

DNA molecules by changing the flow rate in one direction relative to an orthogonal magnetic force. These results have led to a precise description of DNA elasticity[7,9]. A recent study of transcriptional pausing and arrest of RNA polymerase (BOX 2) used computer-controlled fluid flow, which applied force to sub-piconewton resolution on active single RNA polymerases[36]. The flow-field technique has also been used by Chu and co-workers[37–39] to characterize the rheological properties of individual DNA molecules.

Magnetic fields. Magnetic fields can be used to manipulate and apply forces to biomolecules that are tethered to magnetic particles, as most biomolecules have zero magnetic susceptibility. Very stable and small forces can then be created with magnetic fields from either permanent magnets or electromagnets.

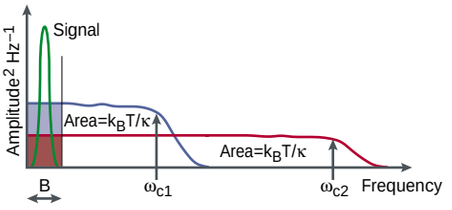
The forces generated with permanent magnets on small magnetic beads ($r < 3$ µm) are usually below 10 pN (REFS 8,40). A drawback of magnetic fields is that the magnetic forces often have to be measured indirectly. For example, the forces acting on magnetic beads can be calibrated by determining the velocity attained by the beads in liquid in a given field and by using Stokes's law[8]. However, an elegant and direct measurement of magnetic force has been implemented by Strick *et al.*[40,41] by using the equipartition theorem (BOX 1).

Magnetic force has been used to apply torsional stress to individual DNA molecules. Strick *et al.*[40,41] used the fact that magnetic beads have a preferred magnetization axis that makes them orientate with an external field and rotate when the field rotates. In this way, individual DNA molecules, torsionally constrained between a coated glass surface and a magnetic bead (FIG. 3c), were under- or overwound to determine the force–extension behaviour of supercoiled DNA. More recently, this same set-up was used to investigate the relaxation of a supercoiled DNA by single topoisomerase II molecules (BOX 2).

Photon fields. Optical tweezers developed rapidly after their power was shown by Ashkin and colleagues[42] in the 1980s. Optical tweezers rely on forces imparted to matter by scattering, emission and absorption of light. The RADIATION PRESSURE, which stems from the momentum change as light refracts off an object[43] (FIG. 4a), allows objects (a bead, for example) to be held in a focused laser beam, with which it is possible to generate a spring-like force. As with mechanical transducers, the stiffness of the optical trap is an important parameter for force and position resolution (BOX 1). In general, the spring stiffness is much smaller than that of a cantilever (TABLE 1). The force exerted on a refractive object depends on the power of the laser, the dimensions of the object and the difference in the refractive index between the object and its surrounding medium[44,45].

To apply forces in the piconewton range with tens of milliwatt power, beads are required that are at least one wavelength in diameter because for smaller beads the trapping force scales as the third power of the bead radius[2]. Such beads can then be attached to macromolecules[46,47]. There are several unique advantages of optical tweezers over other external field techniques. First, the radiation pressure will only trap a bead near the focus of the laser beam, so the photon field does not simultaneously affect other beads. Second, the momentum transfer between the trapped object and the laser beam can easily be calibrated against displacement and force, thus providing a method of direct, high-resolution force and

Box 1 | Balancing signal, thermal noise and time resolution



Every object in solution is bombarded constantly by surrounding molecules. As a result, a spring-like device such as a cantilever, a microneedle or a bead in an optical trap experiences a mean-square displacement noise, $\langle\Delta x^2\rangle$, proportional to the temperature, T, and inversely related to spring stiffness, κ ($\langle\Delta x^2\rangle = k_BT/\kappa$, where k_B is the Boltzmann constant). This is the so-called equipartition theorem. If the device is linear, its corresponding mean-square force noise is $\langle\Delta F^2\rangle = \kappa k_BT$. Stiff mechanical transducers such as cantilevers ($\kappa = 0.06$ N m^{-1}) experience larger force fluctuations but smaller displacement fluctuations than do soft transducers such as a bead in an optical trap ($\kappa = 10^{-4}$ N m^{-1}).

As it happens, fluctuations are not spread out uniformly over all frequencies. The spectrum of fluctuations of an object is determined by the proportionality that exists between its ability to absorb thermal energy and its ability to dissipate it by friction. This result is embodied in the 'fluctuation-dissipation theorem':

$$\left\langle \Delta x^2(\omega) \right\rangle_{eq} = \frac{2k_BT}{\gamma(\omega_c^2 + \omega^2)} \qquad (1)$$

In EQN 1, $\langle\Delta x^2(\omega)\rangle_{eq}$ is the mean-square amplitude of fluctuations per unit frequency of the device at frequency ω, and γ is the friction coefficient of the device. The 'corner frequency', $\omega_c = \kappa/\gamma$, is the frequency above which the system cannot respond to an external stimulus. The corner frequency sets a limit to the rate at which processes can be observed and measured experimentally. A 1-µm diameter bead in a typical optical trap has a corner frequency of 1,000 Hz, whereas a commercial cantilever, 100 µm long and 10 µm wide, has a corner frequency of 6,000 Hz and thus data can be gathered six times faster. The figure shows the effect of increased corner frequency on fluctuation distribution. For the same stiffness κ and bandwidth B, the signal-to-noise ratio of the measurement will be higher for the transducer having the larger corner frequency ω_{c2} (that is, the transducer with the smaller dimensions) than for ω_{c1}.

Measurements are often performed in a narrow band (bandwidth B) around the frequency of the signal (see figure). Assume that a molecule attached to a transducer can generate a force F. Then, the signal-to-noise ratio (SNR) of the measurement, for $B << \omega_c$, is given by EQN 2:

$$S/N = F/\sqrt{2\gamma k_BTB} \qquad (2)$$

In general, therefore, the SNR can be increased by decreasing the bandwidth of the measurement (that is, by averaging the signal over longer times). Decreasing the bandwidth reduces the time resolution of the measurements, however, so this approach is limited by the frequency of the biological event of interest. Note that this ratio is independent of stiffness[2,21]. Thus, a soft transducer is not a more sensitive detector than a stiff one: as κ decreases, the noise increases exactly as fast as the sensitivity.

The physical meaning of the SNR expression is the following: the total area under the curves in the figure is a constant equal to k_BT/κ. As the noise spreads over a larger frequency range for a transducer with a higher corner frequency, the fraction of total noise observed in a given bandwidth (shaded areas) can be reduced by increasing $\omega_c = \kappa/\gamma$, that is, by decreasing γ (see figure). Thus, the SNR and the time resolution of force measurements can only be improved by reducing the dimensions of the transducer[18,62].

NANOTECHNOLOGY
Any technological development that exceeds standard lower size limits of modern microfabrication techniques (hundreds of nanometres or less).

position measurement (<1 pN and <10 nm, respectively). Last, the applicable force range for photon fields (10^{-13}–10^{-10} N) is highly relevant for biological systems.

A major disadvantage of optical tweezers is laser damage to active biological systems. The negative effect of laser exposure on the working lifetime of biological complexes has been shown in RNA polymerase experiments. Exposure to laser light of a wavelength most compatible with biomolecules (λ = 835 nm) still decreased the active lifetime of this enzyme from more than 300 s, when not exposed to laser light, to 89 s at 90 mW of laser power[48]. Also, the data throughput is relatively low because, unlike in flow fields, only one molecule is handled at a time.

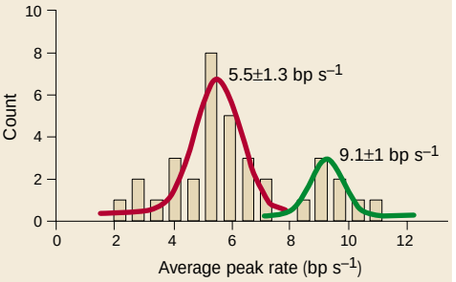
Manipulation of biological systems with optical tweezers began with relatively large objects, such as bacteria, yeast and mammalian cells[49,50]. But optical trapping, combined with microsphere handles linked to molecules of interest, has quickly become a principal tool for manipulating and measuring the force-producing properties of various molecular motors, including kinesin moving along microtubules[13], actomyosin complexes[28], RNA polymerase[36,48] (BOX 2) and DNA polymerase[11] (FIG. 4b). Moreover, optical trapping has also been used both to measure the elastic properties of DNA[47] and to characterize the mechanical unfolding of proteins[52,53].

What next?

The future of biomolecular manipulation depends on three factors: the integration and further development of single-molecule techniques; progress in the field of NANOTECHNOLOGY; and the use of high-throughput systems such as MICROFLUIDICS. These factors will facilitate the application of single-molecule methods to more complex problems, in particular to *in vivo* systems.

Already the power of integrating SFM imaging and pulling has been demonstrated in a study of bacteriorhodopsin[20]. Oesterhelt *et al.*[20] first imaged a crystalline region of membrane-embedded bacteriorhodopsin, then pulled on the last two helices, F and G, of the seven bacteriorhodopsin transmembrane helices. As these two helices unfolded and left the membrane, the polypeptide chain extended and began pulling on the next helix pair. In this manner, the whole

Box 2 | What can single-molecule manipulation tell us about biology?



Two recent studies have demonstrated the ability of single-molecule techniques to elucidate new aspects of enzyme kinetics. First, Davenport *et al.*[36], by using a single RNA polymerase (RNAP) molecule moving along a DNA strand attached to a bead in a flow field, determined that RNAP can operate in at least two modes, one slow and one fast. The figure illustrates the averaged peak rates of single RNAP molecules, showing that they can be in a slow or a fast transcription state.

Molecules in the slower mode paused more readily than did the faster molecules, and a high correlation between the pausing and the complete stopping of individual molecules was observed. Together, these results imply that a temporary pause in transcription is the first kinetic step towards a complete halt in activity. Furthermore, they indicate that identical enzymes can exist in different microstates with distinct functions, opening the possibility of yet another level of control over gene expression in the cell.

Second, Strick *et al.*[41] examined the activity of type II topoisomerase (topo II) on DNA attached to magnetic beads and supercoiled by the application of a rotating magnetic field (FIG. 3c). Topo II was observed to unwind DNA, which was detected as discrete 90-nm jumps in the DNA length. This observation supports the claim that topo II catalyses the relaxation of two supercoils per molecule of ATP hydrolysed[63]. However, mechanically extending the DNA strand did not accelerate topo II catalysis. In fact, at saturating ATP concentrations, topo II activity decreased by a factor of three as the force was raised from 0.3 pN to 5 pN, suggesting that the rate-limiting step of the reaction is directly affected by the applied force.

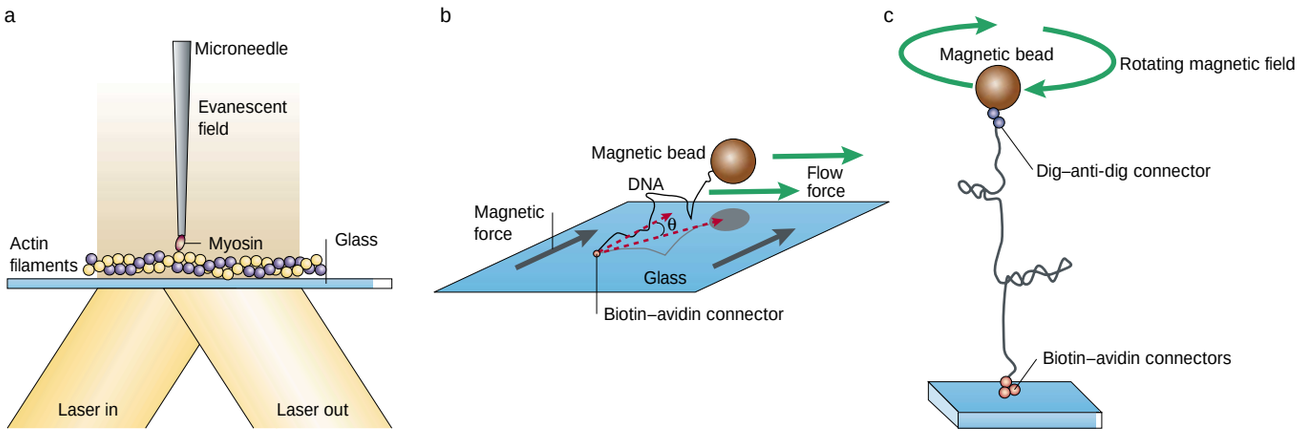


Figure 3 | **Geometries of typical single-molecule experiments. a** | A single myosin head attached to a microneedle moves along an actin filament. The motion of the myosin is observed with a laser by total internal reflection fluorescence microscopy, while the forces are detected by observing the displacement of the microneedle[25]. **b** | DNA tethering a magnetic bead to a point on the glass slide[8]. A magnetic force, which can be determined by means of Stokes's law (see text), is applied perpendicularly to a flow force. The latter, and therefore the total resultant force acting on the molecule, can be determined from the known magnetic force and the angle between the DNA and the magnetic field, θ. The combined magnetic and flow fields can be used to stretch the DNA more than that achievable with the magnetic field alone. **c** | A rotating magnetic field is used to under- or overwind double-stranded DNA tethered between the glass slide and a magnetic bead[40,41]. The resulting supercoils (plectonemes) can be studied by measuring the displacement of the bead perpendicular to the glass slide as a function of the magnetic force.

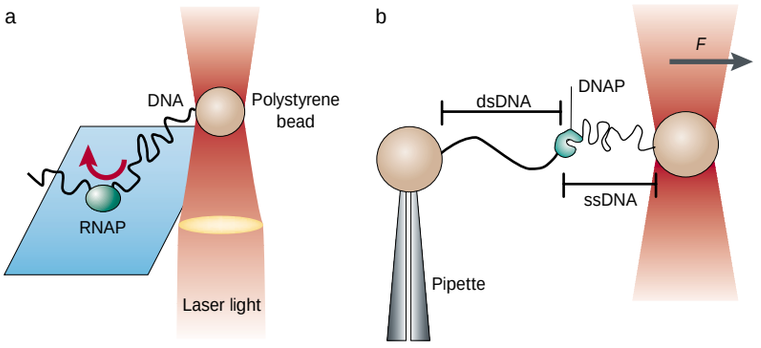


Figure 4 | **Geometries of typical optical-trap single-molecule experiments. a** | An optical trap measuring the force generated during transcription by a single RNA polymerase molecule (RNAP)[54]. During transcription, an RNAP bound nonspecifically to a glass slide must thread the template and do work against a load applied by the optical trap through a polystyrene bead attached at the end of the DNA molecule. **b** | A single-stranded DNA molecule, bound with a primer, connects a bead fixed at the end of a micropipette and a bead held in the optical trap. A feedback circuit is used to keep the DNA molecule at a fixed tension, *F*. As the DNAP converts single-stranded DNA into double-stranded DNA, keeping the tension constant requires the pipette to adjust its position relative to the optical trap by an amount proportional to the movement of the enzyme over the template[11].

protein was pulled out of the membrane, helix pair by helix pair, revealing details of the attractive forces between helices and the membrane. Finally, the same region was imaged to verify that only one bacteriorhodopsin molecule had been extracted from the membrane. Although this study illustrates the advantage of combining single-molecule techniques, the possibilities abound. For instance, combining single-molecule fluorescence with optical tweezers will make it possible to observe spectroscopic signals in response to mechanically induced changes.

Nanotechnology has yet to be effectively applied to single-molecule methods. Attaching nanotubes to SFM tips for improved imaging resolution has been demonstrated[54,55], but not fully exploited. Carbon nanotubes have also been used as mechanical tweezers capable of grabbing polystyrene particles[56]. In fact, these atomically thin tubes may prove to be an ideal building block for the next generation of single-molecule manipulation devices.

Ultimately, the goal of single-molecule manipulation is to access the machinery of a living cell. Although the task of characterizing molecular machines and organelles seems daunting, there has been exciting progress. Researchers have used microneedles to probe cells during cytokinesis[57,58], and have adapted force-mapping atomic-force microscopy to study the activity of actin under the membrane of living cells[17,59]. Magnetic beads may also prove useful, *in vivo*, as the magnetic field will not interfere with other cellular processes. Finally, motor proteins for cell motility are perfect targets for *in vivo* manipulation and single-molecule studies have already yielded information about their force, efficiency and regulation[60,61].

In the near future, scientists may come to see each cell as an individual with its own set of molecular machinery. By using methods for manipulating single molecules, biologists will be able to investigate the nature of molecular machines one by one, and infer from their behaviour those properties common to the population and those corresponding to specific substates. Indeed, what Mark Twain observed with cats may be equally true of biomolecules.

MICROFLUIDICS
Microscopic channels etched into a surface by modern microfabrication techniques for the purpose of transporting small amounts of solution from one place to another.

Links

FURTHER INFORMATION SFM overview | SFM in depth | Microneedle research page | Movies of flow fields stretching DNA | Theory of optical tweezers | Building optical tweezers | Background of optical tweezers | Microfluidics applications

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