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Tying a molecular knot with optical tweezers

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Filamentous structures are abundant in cells. Relatively rigid ones, such as microtubules and actin filaments, serve as intracellular scaffolds supporting movement and force, and hence their mechanical properties are crucial to cellular functions. DNA storage and DNA-protein interactions depend critically on the flexibility of DNA, particularly because proteins often bend DNA sharply[1]. So far, mechanical characterisation of these filaments has been made without precise control of the filament shape, e.g., through the observation of thermal motion[2-5] or from the response to an external force such as flow[6-8] or pull[9-12]. Controlled buckling of a microtubule has been reported[13], but the analysis of the buckled shape was rather involved. Here we show a novel method of continuously controlling the radius of curvature of a molecular string: tying a knot with optical tweezers. Actin filaments broke at a knot when the knot diameter became <0.4 μm. The pulling force required was ~1 pN, $<10^{-2}$ times the tensile strength of a straight filament. The flexural rigidity of actin remained unchanged down to this diameter. A single DNA molecule was also knotted, opening the possibility of studying curvature-dependent interactions with associated proteins. Knotted DNA was stronger than actin, and hence DNA may serve as a manipulation tool.

To manipulate an actin filament, two polystyrene beads coated with myosin were held with dual-beam optical tweezers[14]. A fluorescent actin filament was moved toward the beads, by moving the sample stage, and one end was attached to a bead, and then the other end to the other bead. Subsequently, one end was manipulated with the optical tweezers, as shown in Fig. 1, to form a knot. To facilitate the manipulation, the medium contained 50%(w/w) sucrose which suppressed the brownian motion. When the end was to cross the filament (Fig. 1, images 3, 5 and 7), the end beads were brought either up or down by changing the microscope focus while the remainder of the filament stayed at the same height in the medium. The process of knotting took <1 min. Note that the transparent grip by the optical tweezers allowed continuous manipulation during knotting, without a shift of the grip as would be required in knotting with fingers or mechanical tweezers.

Once knotted, the filament was pulled stepwise to reduce the diameter of the knot and, thus, to establish the relation between the tension and knot diameter. In Fig. 2a, the upper bead was moved upward, while the lower bead was held weakly in a stationary trap. The tension on the filament was estimated from the displacement of the lower bead from the centre of the optical trap (Fig. 2c). Under a tension appreciably above the noise level, the knot diameter was too small to be resolved in the microscope image. Therefore, the diameter was estimated by comparing the intensity of the knotted region with that of a straight region(s) (Fig. 2a). Alternatively, a knot diameter was measured in the image when the diameter was 0.6-0.8 μm. Starting from this reference point, further increase in the distance between the end beads was equated to the decrease in the circumference of the knot (after correction for the apparent increase in the filament length at high tensions due to suppression of the brownian fluctuation, of which the amount was estimated in unknotted filaments) . The two methods gave consistent results, although the latter method was subject to a larger error at the reference point.

In Fig. 2d, the tension (T) is plotted against the knot diameter (D) estimated from the fluorescence intensity. Data from 26 knotted filaments are combined. Although the scatter is large, the data are consistent with the smooth solid line representing the relation $T = 2\kappa/D^2$ expected for a homogeneous rod with a flexural rigidity κ. [The elastic energy U of a ring of diameter D formed from a straight cylinder is given[15] by $U = 2\pi EI/D$, where E is the Young's modulus and I the moment of inertia of the rod ($EI = \kappa$). Pulling a knotted cylinder by $d\ell$ reduces the knot diameter by $dD = d\ell/\pi$, and thus the tension required is $T = -dU/d\ell = 2\kappa/D^2$.] The flexural rigidity calculated from this equation was $(5.5\pm0.2)\times10^{-26}$ N·m^2 (mean±standard error) over the plotted diameter range. This value is close to the rigidity reported for free actin filaments undergoing brownian motion[3-5] of $(5.8\text{-}7.4)\times10^{-26}$ N·m^2, which corresponds to a persistence length ($= \kappa/k_BT_A$, where k_B is the Boltzmann constant and T_A the absolute temperature) of 14-18 μm (an early estimate[16] was ~6 μm.) Thus, an actin filament bent to a radius of only 0.2 μm is as flexible as a free filament for which the radius of curvature induced by brownian motion is, on the average, tens of micrometres. An indirect estimate of κ for bent actin also supports this notion[11].

When the knot diameter became ~0.36 µm, or when the tension in the filament became ~0.9 pN, the filament broke at the knot (Figs. 2a and 3). The fracture occurred, in most filaments, within 10 s after the tension was raised to ~1 pN. The time elapsed before the fracture tended to be shorter for a higher tension, as in the case of unbinding of actin from myosin[17] or α-actinin[18], but the scatter in the present data precluded precise analysis of the time dependence. Without knotting, we were unable to break an actin filament: the filament was detached from a myosin-coated bead at a tension between 3-14 pN, consistent with the unbinding force between myosin and actin[17] of ~10 pN. The tensile strength of straight actin filaments is[12] ~600 pN. An actin filament thus resists straight pull but easily yields to bending. The critical tension of ~1 pN for knotted actin did not change appreciably in three sets of conditions that potentially increase the stability of an actin filament: an increase in the KCl concentration to 100 mmoles per litre of the solution containing 50% sucrose, the addition of $MgCl_2$ at 5 mmoles per litre, or the incubation with ~60 fold molar excess of phalloidin for 8 h. Also, changing the duration of fluorescence excitation, which might cause photo damage, between 2-10 min did not affect the results. Thus, the small fracture strength of knotted and bent filaments is unlikely to be an experimental artefact. Twisting, too, greatly reduces the fracture strength of actin filaments[12]: 10-µm filaments twisted by only one turn in either direction gave a tensile strength of ~200 pN, threefold reduction from the strength of straight actin.

At the knot diameter of 0.36 µm, as shown in Fig. 3c, the amount of shear per actin monomer is ~0.15 nm at the filament radius of 2.5 nm, half the maximal radius of an actin filament[19]. This relatively small shear, about half the interatomic distances, appears to be sufficient to break the bonds between actin monomers on the time scale of seconds. If an actin filament is modelled as a homogeneous rod with a flexural rigidity of 5.5×10^{-26} N·m^2, a lateral pull of only 2.8 pN at the centre between two supports separated by 0.35 µm (Fig. 3d) would produce a curvature with a radius of 0.18 µm. Even a single myosin molecule can produce this much of force[20]. Actin filaments in a cell may be easily broken, if forces act perpendicularly on them, e.g., through α-actinin[18]. [Note that the rigid supports in Fig. 3d were introduced for the

purpose of calculation. The supports exert a force of 1.6 pN in the direction perpendicular to the filament. The combination of these two forces with the pull of 2.8 pN at the top is sufficient to produce the radius of curvature of 0.18 μm.]

A single molecule of DNA has been knotted similarly (Fig. 4); even multiple knots could be introduced in a single DNA molecule (not shown). To facilitate knotting, unstained actin filaments were added in the medium at 10 μmol/kg in addition to 46%(w/w) sucrose (unstained DNA may also serve this purpose, because inadvertent knotting was observed in a thick solution of DNA[6]). Thus, the knot encircled many actin filaments, and the filaments were bundled when the DNA was pulled taut. This is evident in Fig. 4, where the knotted DNA was first pulled upward (image 15) and released (image 16), then pulled downward (image 17) and released (image 18). The knot position remained unchanged during the total of three such cycles (the additional cycles are not shown), indicating that bundled filaments in the knot prevented the knot from moving. We have been unable to break knotted or unknotted DNA with the optical tweezers. One of the beads escaped from the optical trap, as shown in Fig. 4 (images 4-5 and 21-24), indicating that the fracture strength was greater than 15 pN. Unknotted DNA has been reported to transform into an overstretched state[9,10] at 65-70 pN and breaks[7] at 476 pN.

We have shown that both the relatively rigid actin filaments and flexible DNA can be knotted by attaching two beads at the ends. At present, knotting takes tens of seconds and hence requires a medium with a high effective viscosity which suppresses the brownian motion. However, rapid knotting should be possible by electronically programming the movement of the optical tweezers (and the microscope stage). If the viscosity is made low enough, one can infuse a desired medium after knotting. At least two applications are conceivable, other than the mechanical measurements described here. One is to study the curvature dependence of the interaction of intracellular filaments with associated proteins or ligands. For example, RNA polymerase has been shown to be released from DNA when the DNA is pulled straight[21]. Measurements of association constants at precisely controlled curvatures are desirable, also for the dynamics of cytoskeletal network where many protein molecules interact with cytoskeletal

filaments. The other possibility is to use a knot as a manipulation tool, as in the bundling of actin filaments by DNA. One might be able to constrict a cell into halves by tying it in a knot, for mimicking cytokinesis or for artificial compartmentalisation, or one could constrict a cell protrusion such as a dendrite. The force from optical tweezers may not be strong enough for such operations, but one could make three knots in a single DNA molecule and, using the two knots at the ends, fasten the DNA string to two microneedles (coated with suitable glue) attached to manipulators.

Methods

Materials. Rabbit skeletal actin and myosin were prepared[22] and stained[23] with phalloidin-tetramethylrhodamine B isothiocyanate conjugate (Fluka). Amino-polystyrene beads (0.9 μm diameter, Polysciences) were covalently bound with myosin[11]. To tie an actin knot, a solution containing, per kg, 500 g of sucrose, 7 mmol imidazole (pH 7.4), 7 mmol KCl, 3 mmol 2-mercaptoethanol, 5 g glucose, 0.3 g glucose oxidase, 0.07 g catalase, 7 mg myosin-coated beads, and 0.15 nmol labelled F-actin was infused in an observation chamber[23] pretreated with 10 mg/ml BSA. λ-phage DNA was biotinylated at both ends[21] and attached to streptavidin-coated beads[21] (0.95 μm) in a solution containing, per kg, 460 g sucrose, 6 mmol HEPES (pH 7.4), 17 mmol KCl, 0.6 mmol EDTA, 7 mmol 2-mercaptoethanol, 2 g glucose, 0.1 g glucose oxidase, 0.02 g catalase, 7 mg streptavidin-coated beads, 0.1 g α-casein, 13 μmol F-actin, 7 pmol DNA, and 70 nmol POPO-3 (Molecular Probes).

Microscopy. Actin filaments and DNA were knotted on an inverted fluorescence microscope (Diaphot TMD, Nikon) equipped with dual optical tweezers and two video cameras for simultaneous observation of phase-contrast images of beads and fluorescence images of actin filaments or DNA[14]. The trap stiffness of the tweezers was measured after each experiment by trapping a free bead at a reduced laser intensity and observing the width of the brownian motion within the trap[14]; correction for the finite temporal resolution of the video camera was not necessary because the bead motion was slow in the viscous media. The position of a bead was measured as the centroid of the phase-contrast image of the bead. Temperature, 23±3 °C.

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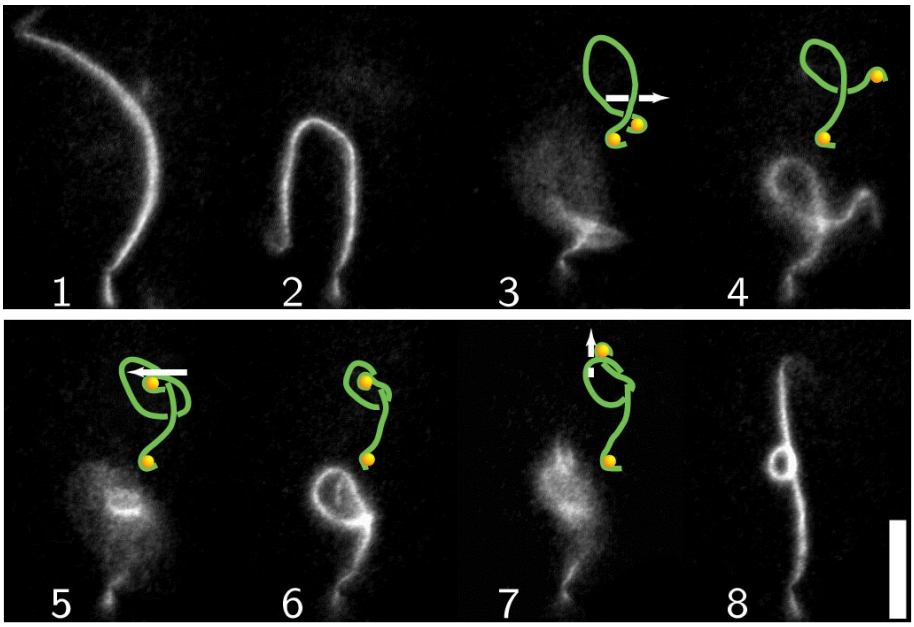

Figure 1 Tying a knot in an actin filament. Explanatory drawings are added in images 3-7. In images 3 and 7, the microscope focus was moved to below the filament to bring down the end beads which were trapped at the focus level; the focus was above the filament in image 5. Bar, 10 µm.

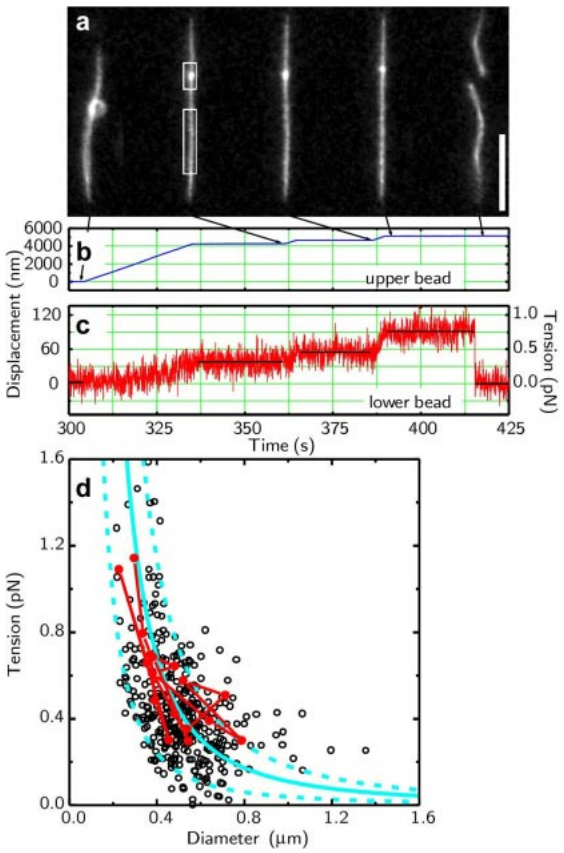

Figure 2 Measurement of the relation between the tension (T) and knot diameter (D). **a**, Fluorescence images of the filament. D was estimated from the back-ground corrected intensities per pixel, I_{knot} and $I_{straight}$, averaged over respective rectangles: $D = (I_{knot} - I_{straight})\ell_{knot}/\pi I_{straight}$, where ℓ_{knot} is the length of the rectangle enclosing the knot portion. Bar, 10 μm. **b,** Movement of the upper bead trapped at the stiffness of 0.086 pN/nm. **c,** Displacement of the lower bead in a trap at 0.0086 pN/nm. T equals the displacement times the stiffness. The filament broke at the knot at 415 s. The abscissa is the time elapsed after knotting, during which several pull-release cycles were made. **d,** Tension-diameter relationship for 26 actin filaments. Data for one filament are connected with red line, in the order of observations, to demonstrate that the knot diameter changed reversibly in response to the tension change. Cyan lines represent $T = 2\kappa/D^2$ for $\kappa = (5.5\pm3.6)\times10^{-26}$ N·m^2; solid line, the average; dashed lines, standard deviations for the 356 data points. Estimated uncertainty for each point is ±0.2 pN in T (mainly due to drift in the trap position) and ±0.2 μm in D (from the differences in D values obtained by the two methods explained in text).

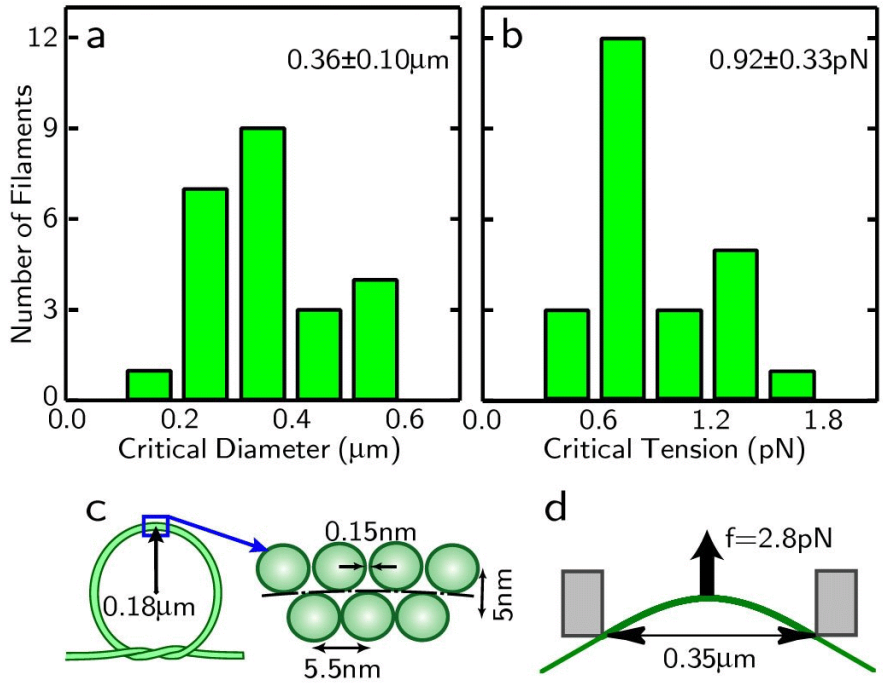

Figure 3 Critical knot diameters (**a**) and tensions (**b**) at which actin filaments broke at the knot. The average±standard deviation are shown (n=24). **c,** A highly schematic diagram of an actin filament bent to a radius of curvature, r, of 0.18 μm. Ellipsoids represent actin monomers. **d,** Force f required to bend a rod supported at two points[15]. For the support separation L_0 of 0.35 μm, f = 2.8 pN produces r = 0.18 μm at the pulled point. [After correcting typographic errors in ref. 15, we obtained a relation $f = (EI/r^2)\tan\theta_0$, where θ_0 is a parameter (the angle between ***f*** and the filament at the support edge) determined by $L_0/r = \int_{\theta_0}^{\pi/2} d\theta\{2(\cos\theta_0/\cos\theta)^{1/2}\cos(\theta-\theta_0)\}$.]

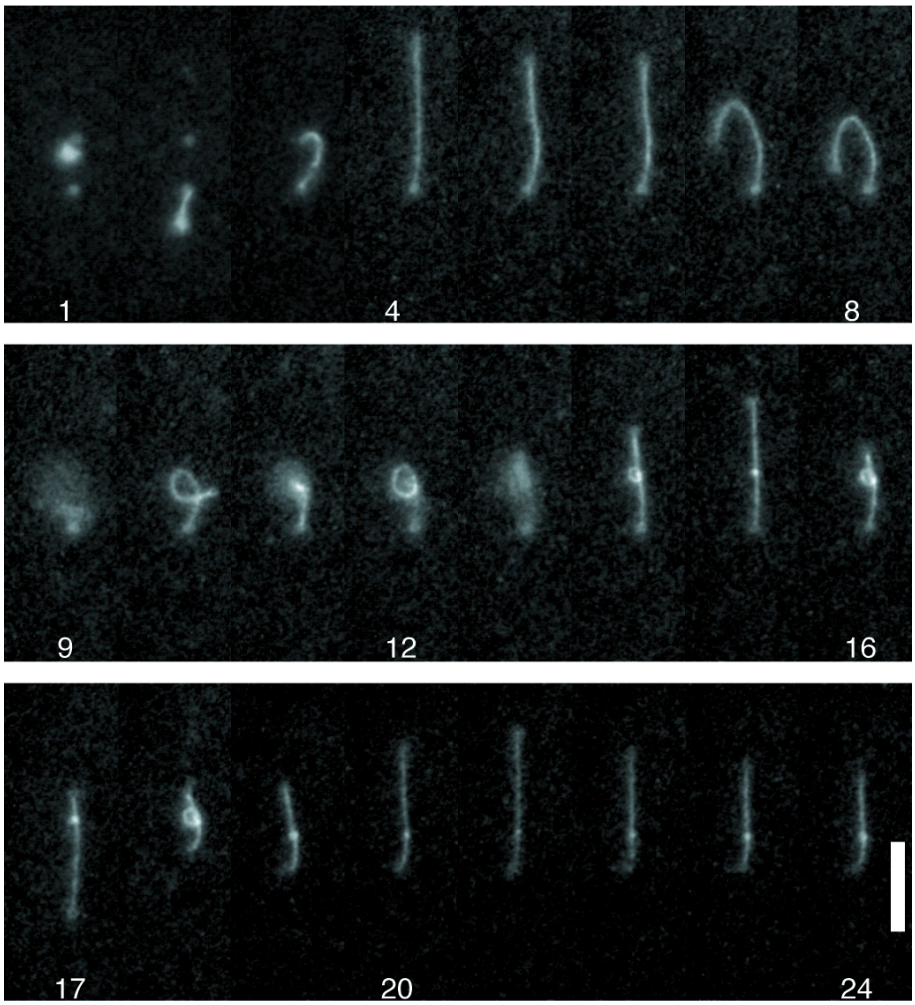

Figure 4 Knotting a single DNA molecule. Two beads were held with optical tweezers, and a DNA molecule stained with POPO-3 was attached to the lower bead by moving the microscope stage (images 1-2). The stage was moved upward to pull the DNA, and the other end was attached to the upper bead (image 3). The upper bead was then moved with the optical tweezers to straighten the DNA (4) and to confirm firm attachment; the upper bead escaped from the trap when the upper trap went too far (4-5). After re-trapping, a knot was made as in Fig. 1a (6-14). The knot diameter was made smaller and larger by moving the upper bead up (15) and down (16), or moving the lower bead down (17) and up (18). After two more cycles (not shown), the upper bead was brought up until it escaped from the trap without rupture in the DNA (21-24). The knot diameter in image 21 is estimated <0.2 μm from its intensity. Bar, 10 μm.