Near-field-magnetic-tweezer manipulation of single DNA molecules

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We have developed an instrument for micromanipulation of single DNA molecules end labeled with 3-μm-diameter paramagnetic particles. A small, permanent magnet that can be moved as close as 10 μm to the particle being manipulated can generate forces in excess of 200 pN, significantly larger than obtained in other recent "magnetic-tweezer" studies. Our instrument generates these forces in the focal plane of a microscope objective, allowing straightforward real-time observation of molecule extension with a position resolution of approximately 30 nm. We show how our magnetic manipulation system can be combined with manipulation and force measurement using glass micropipettes to allow rapid switching between measurements in fixed-force and fixed-extension ensembles. We demonstrate the use of our system to study formation of DNA loops by an enzyme which strongly binds two copies of a specific 6-base-pair sequence.

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Micromanipulation of single DNA molecules allows precise study of the proteins which process DNA, in ways which are impossible using traditional methods of solution-phase biochemistry [1]. It is now possible to directly observe and physically characterize fundamental biological processes such as the reading of DNA sequence [2], DNA replication [3], and the activity of enzymes which split the DNA double helix into single strands [4]. Development of the techniques for manipulation of single DNAs is of wide interest to biological physicists and molecular biologists.

In general, these types of experiments are done using few-micron-diameter particles which are attached to the ends of the DNAs being manipulated; methods to control these particles include optical trapping (OT), glass microfibers (GM), magnetic tweezers (MT), and atomic-force microscopy (AFM) [1]. OT allows measurement of piconewton forces and nanometer displacements on such particles [1], but has the drawbacks that application of constant, well-calibrated forces is technically challenging, and the intense laser spot can photodamage the molecules being studied. GM offer similar force and position resolution, but with the drawback that the fiber must be attached to the molecule being studied. AFM cannot measure forces <10 pN [1], and also is fundamentally a fixed-position approach [1]. OT and AFM setups also have the disadvantage of being quite expensive.

Conventional MT using permanent magnets about 1 mm away from the particles being manipulated allow precisely calibrated constant forces to be applied with 10–femtonewton resolution [1], but cannot apply large forces; for the 2.8-μm-diameter particles widely used [5] the limit is usually about 20 pN. Also, MT does not allow fixing of particle position [1]. In addition, traditional MT pulls the DNA perpendicular to the viewing (objective focus) plane, making measurement of DNA extension dependent either on dynamic refocusing or on calibrations based on out-of-focus bead images [5]. Three nonvertical versions of MT were reported [6–8] where DNA is pulled at an angle to the focus plane. Also, MT which generates forces in the focal plane has been reported [9], but not demonstrated to be usable for single DNA experiments. However, truly transverse MT used to pull DNA in the focal plane has not been developed.

In this article we describe a hybrid method for manipulation of single DNA molecules, using a truly transverse magnetic-tweezer system where the magnet is in solution and thus can be very close to the end-attached particles being manipulated, allowing large and constant forces to be generated if needed. At any time we are able to use glass micropipettes to move the particles to essentially fixed positions in space, and to measure tensions via pipette bending. Our approach allows us to do either fixed-position measurements as in GM, or fixed-force MT measurements. In addition, our approach allows observation of the entire molecule contour in the same focal plane, allowing precise and fast absolute measurement of end-to-end extension with <30-nm precision.

Experiments were done in 500-$\mu\ell$ volume wells, made by gluing a plastic ring on a #1 cover glass. We left the wells open at the top, allowing micropipettes to be inserted from above (Fig. 1). The well was mounted on an inverted microscope (Olympus IX-70); $10\times$ and $40\times$ noncontact objectives were used to bright-field-image pipettes and particles. Experiments use up to three glass micropipettes, plus a roughly 200-μm-diameter permanent magnet particle mounted at the end of a tapered glass rod.

One "bead-catching" micropipette and the magnet particle are mounted on motorized three-axis micromanipulators (MP-285, Sutter Instruments, Novato, CA). A "loading" pipette is mounted in a manual three-axis manipulator (Taurus, World Precision Instruments, Sarasota, FL), and a fourth "force-measuring" micropipette is mounted on the microscope in a fixed position in the sample cell. The bead-catching pipette has an inside diameter of about 2 μm; the loading pipette has an inside diameter of about >20 μm. The force-measuring pipette has a tip inside diameter of about 2 μm, but is pulled so as to have a very long taper so that its bending force constant is about 200 pN/μm. The pipettes are prepared from 1-mm-diameter glass capillaries (TW100F-6, World Precision Instruments) using a heater-puller (model P-97, Sutter Instruments).

With this setup we can inject bead+DNA structures into

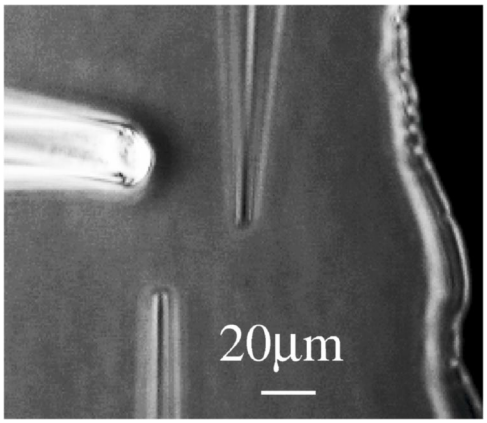

FIG. 1. Three glass micropipettes and a submillimeter-size magnet are involved in the experiment: (1) The upper pipette is the movable catching pipette (2-μm opening diameter) used to catch a bead pair connected by a single DNA. (2) The lower pipette is the force-measuring pipette ($\sim$2-μm opening size) mounted on the microscope stage. It has a long taper so as to have a force constant $\sim$200 pN/μm deflection from its equilibrium position. Calibration is done by the technique in Ref. [13]. (3) The left pipette is the loading pipette ($\sim$20-μm opening size) used to inject the prepared DNA sample into the open cell made by plastic ring. Once a single-DNA pair is found, the catching pipette delivers its nonmagnetic end to the force measuring pipette. Fluctuations of the magnetic end can be used to calibrate force; alternately, it can be moved by the catching pipette to do a fixed-extension experiment. (4) The object to the right is the front of the submillimeter-size magnet glued to a glass pipette. It can be moved to any position in the cell.

our sample cell, and grab them with the catching pipette using fluid suction. Then, we can use the pipettes to stretch DNAs strung between a pair of beads, measuring forces using deflection of the force-measuring pipette, rather like GF or AFM. Alternately, we can use the small magnet to apply a constant force to the paramagnetic particle. This setup does not require high-performance optics; all measurements of this paper can be done using a simple 40×0.5-NA noncontact objective with bright-field illumination. The objective has a working distance of more than 300 μm, allowing the small magnetic particle to be moved into (or below) the objective focal plane; this allows the force applied to a paramagnetic particle to be directed to the focal plane. Our experiments are therefore typically done about 200 μm above the glass.

We first determined that large forces could be generated by our near-field magnetic tweezer, using micropipette deflection. We used the loading pipette to inject 2.8-μm-diameter paramagnetic particles (M-280, Dynal Biotech, Oslo, Norway); we then grabbed them with the catching pipette using suction. One particle was then transferred to the force-measuring pipette, where it was again held using suction. We then moved the small, permanent magnet to a series of positions from 15 to 1000 μm away from the trapped bead. Using spatial correlation analysis of video images, we measured pipette shifts for different magnet positions (Fig. 2, inset: upper image shows pipette position at highest force; lower image shows pipette position at zero force). Each measurement was done using a 20-s time series;

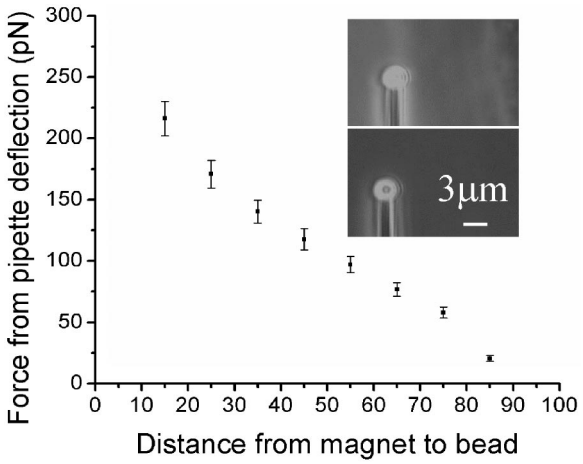

FIG. 2. The deflection of a force-measuring pipette holding a magnetic bead is shown as a function of distance between the bead and the front of the magnet. The force-measuring pipette was calibrated using the technique described in Ref. [13] to have a force constant of 137 pN/μm. The deflection was then converted to the force applied on the magnetic bead. The magnet used in this experiment can provide more than 200 pN on the magnetic bead at a distance about 15 μm away.

error bars reflect the calibration error. Following this experiment, we calibrated the bending moment of the pipette to be 137 ± 9 pN/μm using the procedure of Ref. [13]. Figure 2 shows that forces of up to 200 pN can be readily generated. This is comparable to forces generated on micron-size beads using an electromagnet, but without any severe constraints on sample size, imposed by the requirement that magnet poles be close together [9]. We have found that micropipettes can be used as force cantilevers to measure forces as small as 5 pN, even in our relatively noisy open-cell setup.

Single λ-DNA molecules were prepared for use in our apparatus by covalently attaching short, chemically labeled single-stranded oligomers (3′biotin-cccgccgctgga and 3′digoxygenin-tccagcggcggg) to their ends as done by Smith *et al.* [6]. The resulting molecules carry a biotin at one end and a digoxygenin at the other, allowing them to be bound at one end to a 2.8-μm-diameter paramagnetic streptavidin-covered particle (M-280, Dynal Biotech), and at the other to a 3-μm-diameter nonmagnetic polystyrene bead (Polybead-Amino 3.0-μm microspheres, Polysciences Inc., Warrington, PA) coated with antidigoxygenin (Roche Diagnostics Corp., Indianapolis, IN). After 8 h incubation of DNA and beads in phosphate-buffered saline (PBS, 140-mM NaCl, *p*H 7.4, BioWhittaker, Cambrex Bio Science Walkersville Inc., Walkersville, MD), we inject bead-DNA-bead structures into our sample cell using the loading pipette. We grab the nonmagnetic particle of a bead pair on the catching pipette, and transfer it to the force-measuring pipette.

At this point, the nonmagnetic particle is held by suction at the end of the force-measuring pipette, while the paramagnetic (PM) particle is in solution, tethered by the DNA (Fig. 3, inset). Thus, one observes thermal fluctuations of the paramagnetic bead. As the permanent magnet is moved closer to the PM bead, the molecule extends to near its 16.5-μm contour length (assuming the force remains well below the

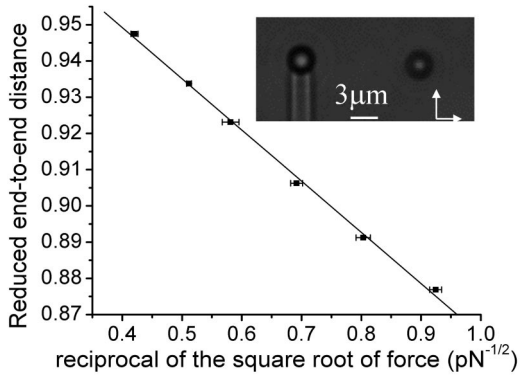

FIG. 3. Using the transverse fluctuations of the paramagnetic bead, the force applied to a single DNA can be measured. A λ-DNA bead pair was caught in PBS and its end-to-end distance, z, was studied, as a function of the applied force determined by the transverse fluctuation of the magnetic bead. Six lengths were measured at six different forces between 1 and 6 pN. We plot z/L vs $\sqrt{1/f}$ here. The slope of the linear fit is -0.14 $\sqrt{\text{pN}}$, corresponding to $A=52$ nm for $T=300$ K, via use of the worm-like chain model (see the text).

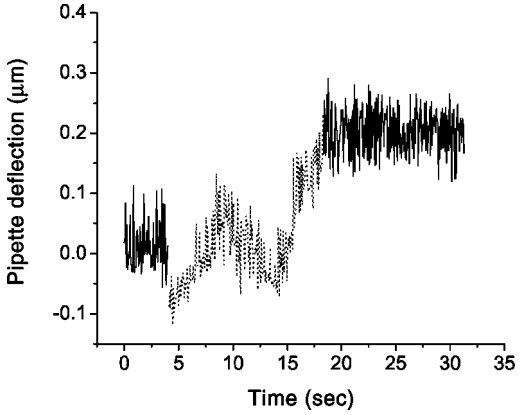

FIG. 4. Calibration of force-measuring pipette using magnetic tweezer and single DNA. For time <5 s, magnetic force was <1 pN, and deflection of pipette reflects essentially its zero-force position. During times between 5 and 18 s, magnet is moved; variations in pipette deflection reflect hydrodynamic noise. For time >18 s, magnet is at a position close enough to generate a measurable pipette deflection of 0.2 μm; by simultaneously measuring paramagnetic bead fluctuations, we are able to determine that the force being applied is 33 pN, which allows the pipette bending moment to be determined (see the text for details.)

$\approx$60-pN forces which disrupt the B-DNA structure [14]), and the thermal fluctuations become greatly reduced. In this regime, fluctuations may be used to measure the force on the PM bead and therefore the tension in the attached DNA [5]. The fluctuations transverse to the force direction are related to the applied force by

$$f = \frac{k_B T \langle z \rangle}{\langle (\delta x)^2 \rangle}, \tag{1}$$

where $\langle z \rangle$ is the average end-to-end extension of the molecule. In our setup, $\langle z \rangle$ and $\langle (\delta x)^2 \rangle$ are simultaneously measured using straightforward real-time x-z bead tracking. Figure 3 shows results for a series of such measurements. We have plotted the reciprocal of the square root of the measured force versus the reduced extension, which falls on a straight line. This is expected given the semiflexible-chain elastic response of a single DNA, calculated in an expansion in inverse powers of force [10,11]

$$\frac{\langle z \rangle}{L} = \left(1 - \sqrt{\frac{k_B T}{4Af}} + \cdots \right), \tag{2}$$

where A is the DNA persistence length. The slope of the straight-line fit of Fig. 3 determines the persistence length $A=53\pm2$ nm, in agreement with other measurements made under similar aqueous buffer conditions [12,6,11].

Figure 3 indicates that the magnetic-tweezer system is capable of resolving forces down to 1 pN. In our current setup forces down to 0.5 pN are readily measured: the main limiting factor is mechanical noise due to our open cell. We also note that this experiment shows that our tweezer system can generate forces in the focal plane. This follows from the simultaneous focus of the two beads (e.g., see Fig. 3); if the magnetic gradient direction (and thus the force applied to the paramagnetic bead) were out of plane, we would observe the beads to be in focus at different vertical positions. Finally, we note that it may be useful to apply out-of-focus plane forces in some experiments; our system is able to pull a paramagnetic particle away from, or even towards, the glass cover slip, simply by changing the vertical position of the permanent magnet particle.

The force-measuring pipette may be calibrated following the experiment using the procedure of Ref. [13]. We have also used the MT to check the calibration. If a single DNA is held as described above, forces in the 10- to 30-pN range allow simultaneous measurement of pipette bending and paramagnetic bead fluctuations. Figure 4 shows how this can be done. The initial position of the magnet was more than 300 μm away from the free paramagnetic bead, tethered through a single λ-DNA to the nonmagnetic bead held by the force-measuring pipette. The force at this magnet position was less than 1 pN.

We then moved the magnet to a much closer final position; the dotted line in the middle of Fig. 4 shows the hydrodynamic noise generated by this motion. Then, deflection of the force-measuring pipette from its initial position (Fig. 4, left solid curve) to the final position (right solid curve) was measured to be 0.20 ± 0.02 μm. At the final magnet position, the length of the DNA was measured to be $\langle z \rangle = 16.17\pm0.03$ μm, and the variance of the transverse fluctuation of the magnetic bead was measured to be $\langle (\delta x)^2 \rangle = 0.0020\pm0.0001$ μm^2. The force was determined to be $f = [k_B T \langle z \rangle]/\langle (\delta x)^2 \rangle = 33\pm2$ pN. Using the force measurement, we therefore conclude that the force-measuring pipette has a bending constant of 165 ± 25 pN/μm. Calibration of the force-measuring pipette gave a result of 140 ± 10 pN/μm, using the method of Ref. [13]. This double calibration thus gives consistent measurements of pipette stiffness.

In our current setup, our ability to measure forces and bead/pipette positions is limited primarily by mechanical

noise. Currently we cannot reliably measure forces below about 0.2 pN, nor can we resolve bead displacements below about 30 nm. Both of these measurement limits are connected with the open-well design of our current setup; we have roughly 1 cm^2 of open air-water interface through which our pipettes are inserted. By using a sample cell which is closed except for small $\approx mm^2$ ports through which pipettes enter, we should be able to reduce mechanical noise drastically, and be able to measure $\approx$0.01-pN forces and $\approx$10-nm bead displacements, as in conventional MT [5] setups.

To demonstrate how our setup can be useful in the physical study of DNA-protein interactions, we have carried out experiments on the type IIs restriction (cutting) enzyme BspMI (New England Biolabs, Beverly MA), which forms a complex that binds two copies of the DNA sequence 5′-ACCTGC. In the λ-DNA molecule this sequence occurs 41 times, and therefore up to 20 loops can be made. In the presence of Ca^{2+}, BspMI will bind two targets without cutting DNA [15,16]. In order to study the two-site binding of this protein, we first determined that a DNA in PBS with 100 uM of added $CaCl_2$ had the baseline force response of Fig. 3. This tells us that we have one DNA, and verifies that the Ca^{2+} ion does not by itself cause any DNA compaction. Next, with the magnetic tweezer we applied a force of 2 pN to extend the molecule. We then added BspMI to our sample well, to achieve a final concentration of 6 BspMI activity units/ml BspMI (note that 1 unit BspMI is approximately 20 ng of protein, estimated by the supplier, New England Biolabs Inc.). Over a period of 20 min we did not observe any change in DNA extension, while maintaining a constant force of 2 pN. At 1 pN we also observed no effect. At these forces, there is no possibility for DNA loops to form, since the DNA is fully extended (Fig. 3, inset).

Next, we used the catching pipette to move the magnetic particle to a fixed position about 8 μm away from the nonmagnetic bead, in order to allow the 16-μm molecule to encounter itself. After incubation for 10 min, we released the magnetic bead, thus placing the molecule under the 1-pN tension applied by the magnet nearby. The length of the DNA was shortened after the incubation, due to the presence of the BspMI enzyme. We then increased force to determine at what force the DNA loops would open. The critical force was determined to be 1.75±0.25 pN, above which the DNA's "lost length" can be completely recovered. The DNA gets its length back by distinct jumps, from opening of loops formed by the BspMI enzyme. To be sure the lost length of the DNA is due to the effect of the BspMI enzyme, we repeated the above procedure in the same buffer but without BspMI. The DNA always jumped back to its original length immediately when the magnetic bead was released.

Figure 5 shows such a loop-opening experiment done at ~4.5 pN. The length of the DNA was about 9 μm when the bead was released. When the measurement started it had a slightly larger length, ~10 μm (it took several seconds to start the measurement after the bead was released). All the loops on the DNA were opened in 300 s. Note that from T =215 s to T=225 s there are actually three jumps; the largest one is about 500 nm. The upper inset zooms in on the first 70 s to show small jumps, from which a ~150-nm jump, a ~50-nm jump, and a jump of about 30 nm can be identified. Fifteen distinct jumps of size ⩾30 nm were observed in this experiment.

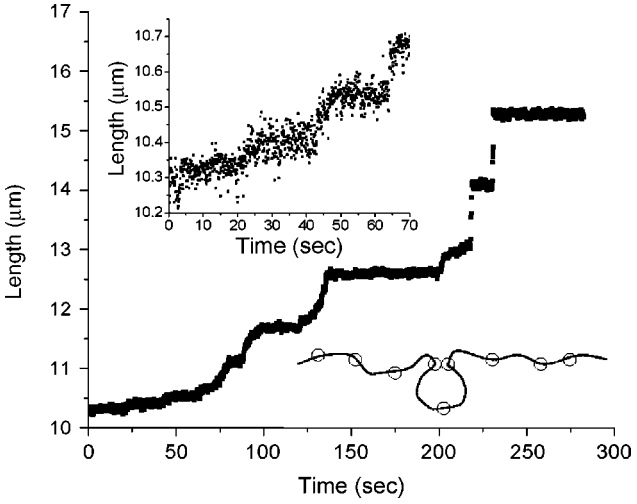

FIG. 5. Time series for opening loops along a λ-DNA, formed by the type IIs restriction (cutting) enzyme BspMI, at a force ~4.5 pN. We used the catching pipette to move the magnetic particle to a constant position 8 μm away from the nonmagnetic bead, allowing the 16-μm molecule to encounter itself in a solution of 6 units/ml BspMI in PBS with 100-μm CaC_2. After incubation for 10 min, we released the magnetic bead, thus placing the molecule under tension of 4.5 pN applied by the magnet. A series of jumps in DNA extension was observed, as the loops were opened. The largest jump was ~500 nm (note that between times of 215 and 225 s there are three jumps, the largest one about 500 nm). Upper inset shows the first 70 s to show small jumps of ~150, ~50, and 30 nm. A series of experiments of this type allowed us to determine a critical force of 1.75±0.25 pN for opening the BspMI loops.

This experiment indicates that the looping of DNA by BspMI can be controlled using force, and that the critical force needed to open the loops under our solution conditions is 1.75±0.25 pN. Our experiment is a direct observation of DNA looping by a type IIs restriction enzyme, and also a measurement of the strength of a DNA-protein-DNA synapse. This experiment demonstrates how our combined micropipette and near-field-magnetic tweezer can be a powerful tool for analysis of DNA-protein interactions.

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