A general method for manipulating DNA sequences from any organism with optical tweezers

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ABSTRACT

Mechanical manipulation of single DNA molecules can provide novel information about DNA properties and protein–DNA interactions. Here we describe and characterize a useful method for manipulating desired DNA sequences from any organism with optical tweezers. Molecules are produced from either genomic or cloned DNA by PCR using labeled primers and are tethered between two optically trapped microspheres. We demonstrate that human, insect, plant, bacterial and viral sequences ranging from ~10 to 40 kilobasepairs can be manipulated. Force-extension measurements show that these constructs exhibit uniform elastic properties in accord with the expected contour lengths for the targeted sequences. Detailed protocols for preparing and manipulating these molecules are presented, and tethering efficiency is characterized as a function of DNA concentration, ionic strength and pH. Attachment strength is characterized by measuring the unbinding time as a function of applied force. An alternative stronger attachment method using an amino–carboxyl linkage, which allows for reliable DNA overstretching, is also described.

INTRODUCTION

Protein–DNA interactions play a critical role in the molecular biology of all organisms. For example, the ~3.3 billion base pairs human genome is estimated to code for at least several thousand DNA-binding proteins, including transcription factors, nucleases, repair proteins, topoisomerases, structural proteins, and DNA and RNA polymerases.

A wide variety of methods exist for studying protein–DNA interactions, including DNase footprinting, sucrose gradient sedimentation, gel mobility shifts, fluorescence spectroscopy, imaging by electron microscopy and X-ray crystallography.

Over the last decade another approach involving mechanical manipulation of single DNA molecules has been developed. Manipulation of DNA by optical tweezers was pioneered by Chu and co-workers, and extended by Bustamante and co-workers (1–4). This method has since been applied to study many fundamental biochemical processes, including transcription, replication, chromatin unraveling, viral DNA packaging and helicase translocation (5–12).

Because protein–DNA interactions are vital to all organisms and these interactions are often sequence dependent, it is desirable to have a general method for manipulating DNA sequences from any organism. Here we describe and characterize such a general method in which selected DNA sequences from a variety of organisms are tethered between two microspheres. Besides providing useful protocols and a characterization designed to optimize the efficiency of the method, this work also serves to test the notion that the global elastic properties of these long, AT-GC balanced DNA molecules are largely independent of sequence (as opposed to the local properties on a <100 bp scale, where sequence-dependent bending and twisting occurs) (15–19).

METHODS

Genomic DNA preparation

Escherichia coli DNA was obtained by growing a 2 ml culture of DH5α competent cells (Invitrogen Corp.) in Luria–Bertani (LB) broth (Fisher Scientific) overnight at 37°C on an orbital shaker. The cells were pelleted at ~3000 g in a microcentrifuge tube and the pellet was lightly dabbed with a sterile cotton swab. DNA was then extracted and purified using the QIAamp DNA Blood Mini Kit (Qiagen) following the “buccal swab spin protocol” as per the manufacturer’s instructions with the following modifications: 10 mg/ml protease K (Roche Biochemicals) was substituted for the supplied protease, a total of 1% SDS was included during the lysis step, and the lysis was incubated overnight. These modifications were found to result in a higher yield of higher purity DNA.

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Table 1. List of DNA sequence targets that were tested along with selected properties and PCR primers used

| Organism          | Size (bp) | Genes   | Source               | Primers, forward and reverse ('5'→'3')                                                                 | %GC  |
|-------------------|-----------|---------|----------------------|--------------------------------------------------------------------------------------------------------|------|
| λ. Phage (virus)  | 10 051    | p9-p20  | NEB                  | CTGATGAGGTTCGTGTCCTGAAAATCGGGTAATCC, ATACGCTCGATATCCGAAAGAAGTAAGTCTGAGGATGAGGCGAGGATCTGAGG | 57.6 |
| Drosophila (insect) | 14 001   | actI, CG2118 | Embryos                 | GCCGTAACAATGAGAAGCGTGGTGGAGGTG, ATCTGGGCGGCTGGAAGGTAGTGCAGTGG | 45.6 |
| Human             | 15 138    | tpa     | Cheek cells           | CTTTCACTGTGCTGTAACCTCTCTTCTGTTGTTCC, ACTGTGCTCTCTGACCCATGGCAGAGAGGGCGTTTCT | 49.2 |
| Human             | 15 138    | tpa     | BAC clone             | CTTTCACTGTGCTGTAACCTCTCTTCTGTTGTTCC, ACTGTGCTCTCTGACCCATGGCAGAGAGGGCGTTTCT | 49.2 |
| Arabidopsis (plant) | 20 527    | At1g15170, 15230 | Young leaves          | GCTCTGAAGATATAGGGACCTGATGATCC, AAAAGATGCCACGCTCCTACCCACAAAG, GGAGATTAGAGAAAGAAGATGCCGAGGAG | 49.6 |
| E.coli (bacteria) | 25 340    | topA-sapA | Liquid culture        | CTGATGAGGTTCGTGTCCTGAAAATCGGGTAATCC, ATACGCTCGATATCCGAAAGAAGTAAGTCTGAGGATGAGGCGAGGATCTGAGG | 48.9 |
| λ. Phage (virus)  | 40 368    | p9-p78  | NEB                  | GCCGTAACAATGAGAAGCGTGGTGGAGGTG, ATCTGGGCGGCTGGAAGGTAGTGCAGTGG | 57.6 |

Drosophila DNA was obtained by growing embryos as described previously (20). Embryos were frozen in liquid nitrogen and stored at −80°C. Approximately 100 µl of embryos were thawed and dabbed with a sterile cotton swab. The DNA was then extracted and purified using the QIAamp DNA Blood Mini Kit (Qiagen) following the ‘buccal swab spin protocol’, substituting 10 mg/ml proteinase K for the supplied protease (which resulted in a higher yield and purity).

Human DNA was obtained by firmly scraping the inside of one of our cheeks ~10 times with a sterile cotton swab. The DNA was then extracted and purified using the QIAamp DNA Blood Mini Kit (Qiagen) following the ‘buccal swab spin protocol’ without modification.

Arabidopsis DNA was obtained from ~100 mg (wet weight) of young leaves. The leaves were frozen in liquid nitrogen and disrupted by using a small pestle to grind them into a powder in a microcentrifuge tube. The DNA was then extracted and purified using the DNeasy Plant Mini Kit (Qiagen) as per the manufacturer’s instructions, using water in the final elution step. We found that DNA eluted in the supplied elution buffer failed to work in our PCRs.

λ DNA purchased from New England Biolabs was used directly.

Extracted genomic DNA samples were characterized by UV spectroscopy (absorbance at 260 nm) and gel electrophoresis. These measurements indicated that DNA concentration following purification ranged from 5 to 50 ng/µl, and that fragment lengths were predominantly ~15–25 kb in length.

BAC DNA preparation

The UCSC genome browser and sequence alignment software was used to identify an appropriate BAC clone bracketing genomic sequences of interest (21). Here we chose the clone CTD-2240D16 (from Caltech Library D) containing sequences from chromosome 14 that code for human tissue plasminogen activator. A culture of E.coli carrying this clone was obtained from Invitrogen. A pipette tip was dipped in this lysis solution and added to the lysate, and the elution buffer was preheated to 65°C. We found that these modifications improved the recovery of long BAC DNA constructs.

Synthesis and labeling by PCR

The UCSC genome browser was used to identify human and Drosophila DNA sequences of interest, and the NCBI databases were used to identify E.coli, λ, and Arabidopsis sequences. PCR primers were selected using GeneRunner software (except for those targeting lambda and human sequences, which were recommended by Eppendorf) (Table 1). All primers were chosen to have a melting temperature of ~62–68°C to limit necessary modifications of reaction conditions. Forward primers were labeled at the 5' end with biotin-TEG and reverse primers were labeled at the 5' end by digoxigenin (DIG) (via an amino-C6), such that a DNA molecule could be tethered as shown in Figure 1. All primers were purchased from Operon Biotechnologies. All PCRs were carried out using the Triplemaster PCR system (Eppendorf), which combines Taq DNA polymerase, a proof reading enzyme and a processivity enhancing buffer additive.

PCRs (50 µl) were carried out using reagent concentrations recommended by Eppendorf. No supplemental Mg²⁺ was added to the reaction buffer. Reactions were run in a 24-well Hybaid PCR Sprint thermocycler using 200 µl thin-walled PCR tubes (Fisher Scientific). Because some reactions required tuning to obtain optimum results, the thermocycling parameters and quantity of template DNA used in each incubation was 5 min, 195 µl isopropanol was added to the lysate, and the elution buffer was preheated to 65°C. We found that these modifications improved the recovery of long BAC DNA constructs.
reaction are listed in Table 2. Identical conditions were used with the amino-labeled primers as with the DIG labeled ones. We note that in some cases the method of purification of the genomic DNA strongly influenced the results. For example, an attempt to prepare the Arabidopsis construct by phenol-chloroform extraction and isopropanol precipitation of genomic DNA, resulting in ~50–150 kb fragments, was not successful. Instead, DNA tethers were successfully produced when using the Plant Mini Kit (Qiagen, Inc.), which produced fragments predominantly ~20–25 kb.

**Optical tweezers**

Two different optical tweezers instruments were used for measurements. In the first, one microsphere was held in an optical trap while the other was held by suction on the end of a micropipette, as described previously (10). In this apparatus the DNA was stretched between the microspheres by moving the micropipette with a piezoelectric nanopositioning stage. In the second instrument, the two microspheres were held in two separate optical traps and the DNA was stretched by displacing one trap with an acousto-optic deflector. This configuration is similar to that used in studies of single actin–myosin interactions (22).

**Microsphere preparation**

Two hundred microliters of 0.5% (w/v) 2.2 μm diameter streptavidin coated microspheres (Spherotech, SVP-20-5) were washed twice to remove any free streptavidin by pelleting at 10 000 g in a microcentrifuge tube and resuspending them twice in 200 μl phosphate-buffered saline (PBS), pH 7.4, 1 M NaCl with 0.1 mg/ml BSA. To prepare anti-DIG coated microspheres, 200 μl of 0.5% (w/v) 2.8 μm diameter Protein G coated polystyrene microspheres (PGP-20-5; Spherotech) were washed twice by pelleting at 10 000 g in a microcentrifuge tube and resuspending them twice in 200 μl PBS buffer. After the second wash, the microspheres were resuspended in 20 μl PBS and 5 μl 200 μg/ml of anti-DIG (Roche Molecular Biochemicals) was added. The microspheres were incubated on a rotisserie (Barnstead Labquake) at room temperature for 30 min and then washed three times in 200 μl PBS and resuspended in 20 μl PBS. These microspheres (3–6 μl) were loaded into a 1 ml tuberculin syringe (Becton Dickinson, Co.) for injection into the microfluidic chamber.

**DNA tethering procedure**

Labeled DNA was first attached by one end to the streptavidin beads in a bulk reaction as follows: 3 μl of appropriately diluted DNA (ranging from ~2.5 to 500 ng/μl, such that DNA: microsphere stoichiometry varied from ~1:1 to ~200:1, as discussed in Results) was mixed with 27 μl of microspheres and incubated for 30–60 min at room temperature on a slowly rotating rotisserie (Barnstead Labquake). These microspheres (5–10 μl) were diluted in 0.5 ml of PBS and loaded into a syringe for injection into the microfluidic chamber.

DNA tethers were formed in situ (inside the flow chamber), as follows: first, a single anti-DIG microsphere was trapped in the first optical trap (in the single beam system this microsphere was then transferred onto the tip of the micropipette by applying suction). Then, a streptavidin microsphere (carrying DNA) was trapped in the second optical trap and brought nearly in contact with the anti-DIG coated microsphere for ~10 s in an attempt to form a tether. Usually the same anti-DIG microsphere was used in up to five trials before discarding it.

For the trials done with varying salt (NaCl), the binding of the DNA to the streptavidin microspheres was done in the same manner described above, except these microspheres were then diluted in 0.5 ml of 20 mM Tris–HCl, pH 7.8, with 0–2 M NaCl, instead of in PBS buffer. For the trials with varying pH, the following 10 mM buffers were used: acetate (pH 4), citrate (pH 5.6), phosphate (pH 7), Tris–HCl (pH 8.5), carbonate (pH 9.9) and phosphate (pH 11.8). An appropriate amount of NaCl was added to each so as to keep the total ionic strength at 150 mM (23). All measurements of tethering efficiency were done using the single beam optical tweezers system.

**Covalent DNA attachment**

A covalent DNA attachment strategy was also used as an alternative to DIG-anti-DIG. In this method, 10 or 25 kb amino-labeled molecules were crosslinked to 2.8 μm diameter carboxyl functionalized polystyrene microspheres (CP-25-10; Spherotech) in a bulk reaction. The PCR primer for the 25 kb construct was synthesized with an 5′ amino C6 modifier (Operon) while the 10 kb primer was synthesized with a 5′ amino C12 modifier and two internal amino C6 dT modifiers (at positions 12 and 24) (IDT). Following PCR, the amino-labeled DNA was purified by dialysis against 20 mM HEPES buffer (pH 7.5) on a floating filter pad (Millipore VSWP02500) followed by phenol-chloroform extraction, isopropanol precipitation, ethanol wash and resuspension in 20 mM HEPES, pH 7.5. These steps allow for removal of PCR proteins, primers and Tris (which, owing to its containing primary amines, may interfere with crosslinking).

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**Table 2. PCR parameters used for each construct**

| PCR parameters          | λ 10 kb | Dro sophila | Human | Arabidopsis | E.coli | λ 40 kb |
|-------------------------|---------|-------------|-------|-------------|--------|---------|
| Quantity of template DNA (ng) | 20      | 250         | 170   | 120         | 300    | 20      |
| Initial Denaturation    | 93°/3.00| 93°/3.00    | 93°/3.00| 93°/3.00   | 93°/3.00| 93°/3.00|
| # of cycles constant    | 10      | 10          | 10    | 10          | 10     | 10      |
| Denaturation            | 93°/0.15| 93°/0.15    | 93°/0.15| 93°/0.15   | 93°/0.15| 93°/0.15|
| Anneal/Extension        | 62°/0.30/68°/8.00 | 68°/16:00 | 68°/16:00| 62°/0.30/68°/17:00 | 68°/27:00 | 68°/21:00|
| Numbers of cycles ramping | 20      | 17          | 17    | 27          | 12     | 8       |
| Denaturation            | 93°/0.15| 93°/0.15    | 93°/0.15| 93°/0.15   | 93°/0.15| 93°/0.15|
| Anneal/extension        | 62°/0.30/68°/8.00 | 68°/11:00 | 68°/11:00| 62°/0.30/68°/17:00 | 68°/21:00 | 68°/27:00|
| Increase per cycle      | +0:20   | +0:20       | +0:20 | +0:20       | +0:20  | +0:20   |
We found that either one- or two-step crosslinking methods worked well. In both protocols, 5 μl of 5% (w/v) carboxyl microspheres were washed twice in 20 μl of 100 mM MES buffer, pH 6.0, and resuspended in 10 μl of MES. In the one-step method, ~20 ng of DNA is added to the washed beads and 1 μl of 40 μg/μl 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (Pierce Biotechnology) freshly dissolved in water was added. The reaction was incubated at room temperature for 15 min and then an additional 1 μl of 40 μg/μl of freshly dissolved EDC was added. This addition was repeated after 15 min and the sample was then allowed to react for another hour. Tris–HCl, pH 7.6, was then added to 100 mM to quench the reaction. Prior to injecting these microspheres into the flow chamber BSA was added to 0.1 mg/ml to block non-specific adhesion of DNA to the microspheres.

In the two-step method, the microspheres were first activated by adding 1 μl of 40 μg/μl EDC and 100 μg/μl N-hydroxysulfosuccinimide (Sulfo-NHS; Pierce, #24510) freshly dissolved in water. The reaction was incubated at room temperature for 15 min and then an additional 1 μl of freshly dissolved EDC and Sulfo-NHS was added. This addition and incubation was repeated and the microspheres were then washed twice in 20 μl of 50 mM HEPES, pH 7.5, and resuspended in 10 μl HEPES. DNA (60 ng) was immediately added and allowed to react for 2 h at room temperature. Tris buffer and BSA were added to these microspheres, as in the one-step method.

The procedure for tethering the molecules in the optical tweezers was the same as for the anti-DIG labeled molecules, but with the biotin–streptavidin linkage being formed in the flow chamber. BSA (0.05 mg/ml) was also included in the buffer in the flow chamber.

RESULTS AND DISCUSSION
Manipulation of desired sequences
To demonstrate that we could manipulate specific DNA sequences from a variety of organisms, we targeted seven arbitrary sequences in the human, Drosophila, Arabidopsis, E.coli and bacteriophage λ genomes (Table 1). Genomic DNA was purified from each organism and PCR amplification and labeling were carried out as described in Methods. Each of these DNA constructs was successfully manipulated by optical tweezers by following the tethering protocols described above. High precision extension measurements confirmed that the tethered molecules had lengths consistent with the targeted sequences, as discussed below. Based on these results, which use DNA from a broad variety of sources, we expect that this method can easily be used to manipulate desired sequences from virtually any organism. Although some limitations have been reported for long-range PCR (24,25), continuing advances in the method have greatly expanded the range of lengths and sequences that can be amplified (26). Segments up to ~50 kb and sequences containing up to 75% GC content have been amplified using the TripleMaster PCR system (26–29). Long-range PCR has also been used recently to scan across entire bacterial genomes, suggesting that a majority of DNA sequences can be successfully amplified (30). Here we successfully prepared seven arbitrarily chosen sequences ranging in length from ~10 to ~40 kb and having ~41–58% GC content.

We also showed that cloned DNA could be used to generate desired sequences. BAC clones (in E.coli) spanning the human genome, as well as the genomes of many other organisms, have been produced during genome sequencing projects. For example, BAC clones of human DNA have been produced at Caltech and Children’s Hospital of Oakland Research Institute (http://www.tree.caltech.edu/ and http://bacpac.chori.org/). To demonstrate that these could be used, we targeted the same human sequence that we targeted when using genomic DNA, i.e. that containing the gene sequence for tissue plasminogen activator. This construct was successfully tethered and exhibited the same elastic behavior as the construct generated using genomic DNA, as described in more detail below. We note that these DNA tethering methods may also have other applications, such as in DNA microarray technology or molecular electronics (13,14).

Prior to use in the optical tweezers PCR products were analyzed by agarose gel electrophoresis (Figure 2). Cycling conditions were tuned to obtain a strong signal in a single band, although weaker secondary bands were often observed before optimization of the cycling parameters. In some cases it proved difficult to obtain a completely pure product even after these adjustments (e.g. the λ 40.4 kb sample). Presumably the amplification of undesired products could be further minimized through further optimization of the primers and reaction conditions. Fortunately, secondary products represented a small fraction of the total and were always of significantly shorter length than the desired products. In practice, tethering of undesired products in the optical tweezers was rare (<10%, in the worst case) and could easily be distinguished as having significantly shorter lengths.

Tethering efficiency
As DNA molecules are tethered by bringing pairs of microspheres into contact one at a time, it is important to optimize the tethering conditions: too little DNA results in many microspheres failing to tether, while too much DNA results in tethering of multiple molecules on single microspheres. As
the number density of the spheres is very low (~0.5 × 10^9 per ml), most samples of DNA must be heavily diluted to have a high likelihood of obtaining single tethers. Here, DNA is first incubated with streptavidin microspheres in bulk for 30–60 min to tether the biotin-labeled ends. In theory, since biotin binds streptavidin with very high affinity one could simply set the microsphere:DNA stoichiometry to 1:1 and wait for the binding reaction to proceed to completion, whereupon the distribution of DNAs per bead would be expected to follow a Poisson distribution with ~37% of microspheres having exactly one DNA tethered. Our experience indicates that after ~5–10 h of incubation fewer tethers are detected than predicted by these considerations, suggesting that the binding had not reached completion or that some molecules may have been improperly labeled, have degraded or adhered in a manner that prohibits binding to the second microsphere. Thus, in practice we find it convenient to use a 3-fold excess of DNA and an incubation time of ~30–60 min. Further binding is essentially stopped by the ~100-fold dilution of the sample prior to injection into the flow chamber. Systematic measurements show that tethering efficiency for the 10.1 kb λ DNA construct varies from 0 to ~100% multiple tethers as the DNA: microsphere ratio is increased from 1:1 to 200:1 (Figure 3). In this particular titration, the 3:1 ratio, yielding ~30% single tethers and <5% multiple hookups, proved to be a convenient reaction condition.

In some experiments one may test by elastic measurements, or by twisting with a rotary pipette (31), whether a single DNA is tethered in the optical tweezers. For these experiments it is sometimes convenient to use a higher DNA concentration during sample preparation and reject multiple tethers during data taking. However, when studying protein–DNA complexes, the elasticity may be altered and it may not be possible to discern single tethers a priori. In this case, it is better to accept a lower tethering efficiency in order to minimize the chance of multiple DNA tethers, which may invalidate certain datasets. We often like to use conditions where about 1 in 10 trials yields a hookup. Our data on tethering efficiency (Figure 3) are useful as a guide on what reaction conditions to use. However, we find that there is often significant variation depending on the particular DNA and microsphere samples being used. We often find it necessary to titrate the amount of DNA up or down by a factor of 3 to find optimal tethering conditions.

Depending on the biochemical process being studied, one may wish to tether DNA molecules under a variety of solution conditions. Here we have characterized the dependence of tethering efficiency on salt (NaCl) and pH. In these experiments, the first attachment (biotin–streptavidin) was formed during a 30–60 min. incubation in standard buffer conditions, as described above, while attempts to form the DIG–anti-DIG linkage were made under conditions of varying salt and pH. As shown in Figure 4, tethering worked quite well from 0 to 2 M NaCl, with the highest efficiency at 150 mM NaCl. This efficiency may be attributed to electrostatic screening, which presumably allows the DNA and microspheres, which are both negatively charged, to approach more closely. We note that problems with tethering often occur when using divalent cations, such as Mg^{2+}, above ~5–10 mM. These conditions tend to cause non-specific adhesion of the microspheres to each other and/or to the DNA, and in this case it is helpful to use a blocking agent such as BSA. We usually include 0.1 mg/ml BSA during tethering incubations. While BSA mitigates non-specific adhesion, we found that increasing

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Efficiency of DNA tethering following 30 min incubation with streptavidin microspheres versus stoichiometry. Black, light gray and dark gray bars indicate percentages of trials that yielded zero, one or multiple DNA tethers, respectively.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Efficiency of DNA tethering in the microfluidic chamber. (A) Dependence on NaCl concentration. (B) Dependence on pH. Black, light gray and dark gray bars indicate percentages of trials that yielded zero, one or multiple DNA tethers, respectively.
the concentration of BSA to above 1 mg/ml can also reduce DNA tethering efficiency to inconvenient levels.

DNA tethering worked at pH values ranging from 5.6 to 9.9, with optimal results at pH 7–8.5 (Figure 4). Non-specific adhesion of the microspheres to each other and/or to the DNA was often observed at pH 5.6 and 9.9. Tethering did not work at all at pH 4 or 11.8, presumably because of denaturation of the DNA and/or proteins under these extreme solution conditions. At pH 4 there was also significant precipitation of BSA, which tends to clog the flow chamber, adhere to the beads and interfere with optical trapping. A small amount of precipitation was also observed at pH 5.6, although DNA manipulation was still quite workable at this pH.

Force-extension measurements

To test the repeatability of optical tweezers measurements on the prepared DNA constructs, force-extension measurements were performed on small ensembles of molecules. Force data were recorded at 5 kHz and averaged for 0.25 s at each extension to reduce noise due to Brownian motion of the trapped bead. This force measurement was done at 20 discrete values of the extension to obtain each individual dataset. As shown in Figure 5, the individual datasets for each construct were in close agreement and datasets for different constructs had the same basic shape. For reasons discussed below, all of these measurements were made using the dual optical tweezers instrument.

The largest ensemble of measurements was taken on the 25.3 kb E.coli DNA construct and comprised 57 datasets recorded on several different days over the course of a month. To quantify measurement reproducibility, a histogram of measured extensions at 25 pN is plotted in Figure 6. The SD is 22.2 nm (~65 bp) and the SE is 2.9 nm (~85 bp), which indicates a narrow distribution for physical measurement of a single molecule. For comparison, similar measurements done with the single beam optical tweezers system, in which one microsphere was manipulated using a micropipette, yielded a much larger SD of ~700 nm. These larger variations are due to the fact that DNA molecules may attach at any point on a microsphere and a microsphere held by the micropipette is not free to rotate. This effect leads to an uncertainty in the absolute molecular extension on the order of the radius of the microsphere (~1000 nm or ~3000 bp), which can present a problem in experiments where high precision is desired. In the dual beam optical tweezers both microspheres are free to rotate and thus align when the DNA is stretched so that this uncertainty is avoided.

In the dual tweezers system the small residual SD in the extension measurement can be completely attributed to inherent variation in the diameters of the microspheres. They have a reported SD in diameter of ~2% (~50 nm), as determined by transmission electron microscopy measurements (Spherotech). We attempted to correct individual datasets for this variation by recording the extension at each pair of microspheres contact, but no repeatable signature of contact could be identified to improve the accuracy further. Fortunately, this error is relatively small and unlikely to cause problems in most types of experiments, which in many cases involve measuring relative changes in extension. To the extent of our apparatus’ ability to discriminate length differences, these measurements show that the individual molecules are behaving identically. As these data were recorded over the course of a month, this finding also indicates that our dual beam optical tweezers instrument is highly stable, with a systematic drift of less than ~10 nm/week.

As a second test, we compared measurements on the 15.3 kb human DNA construct prepared in two different ways: from the genomic DNA from cheek cells versus from the BAC clone. The sets of force-extension curves for these two samples fall closely on top of each other as seen in Figure 5. The mean extension for the construct produced using genomic DNA was <x> = 5040 nm with a SD of σ = 29 nm, while for the construct produced from the BAC clone (measured ~1 month later) we obtained <x> = 5080, σ = 47 nm. This correspondence suggests that the two constructs are the same, at least to within tens of nanometers, and is consistent with our estimate of ~10 nm/week long-term instrumental drift.

Figure 5. Force-extension datasets recorded for six different DNA constructs. For each construct a small ensemble (indicated by the number, n) of independent measurements were made. The two datasets for the human DNA construct, from genomic and BAC DNA, are not separately visible due to their near perfect overlap. Note that although the Arabidopsis construct was successfully tethered, a full ensemble of data was not recorded.

Figure 6. Histogram of measured extensions at 25 pN for 57 different tethered molecules of the E.coli 25.3 kb sequence. These measurements were made over the course of about one month.
Next we sought to check that the molecules were behaving in a manner consistent with the targeted construct lengths. The elasticity of DNA molecules has been shown to agree with the behavior predicted by the worm-like chain (WLC) model (4,17,32–34). In this model, the fractional extension of a molecule (defined as the end-to-end distance divided by the contour length) is a universal function of the applied force. The extension of molecules of different contour lengths at a given force is therefore expected to be proportional to the contour length (35). To confirm that this was true for all of our DNA constructs we compared the ratios of average molecular extensions measured at 25 pN to the ratios of number of basepairs of the constructs. We chose to calculate ratios instead of absolute values because such ratios should depend only on the relative lengths of the constructs and should therefore be independent of instrument calibration. As shown in Figure 7, there is excellent agreement between the measured and predicted ratios. The average deviation from the expected ratio is only 0.3% and the maximum deviation was 2%.

It is known that on short length scales (~10–100 bp) different DNA sequences may have dramatically different conformations and bending and torsional rigidities (15,16). For example, significant bending occurs when A-T tracts are repeated in phase with the helical pitch of the DNA (36). However, the optical tweezers measurements described here probe the global elasticity of long DNA molecules containing tens of thousands of basepairs. Two long, random DNA sequences with equal GC–AT content would not have different global elastic properties because local variations in compliance would be expected to average out over the lengths of the molecules. The constructs measured here are not purely random sequences, but they have fairly balanced GC versus AT content (ranging from ~41 to 58%). To within our measurement precision, we find that these different sequences exhibit the same elasticity. In future studies, following further improvements in instrument resolution, it may be of interest to compare elasticity measurements for sequences with highly skewed GC–AT content, or for sequences having long stretches of repeated DNA-binding motifs.

**Attachment strength**

When a tension greater than ~10–20 pN is applied to a single DNA tether we find that it usually unbinds from the microspheres in less than a minute. Molecules can sometimes be stretched to the overstretching transition point at ~65 pN, but at this force level the link usually breaks in less than a second. In cases where it is of interest to study protein–DNA interactions under high force, this unbinding may interfere with measurements. However, many experiments do not require application of such high forces for extended periods. As previous work has shown that DNA tethered via biotin–streptavidin can be stretched to forces above 65 pN for up to several minutes, we attribute the weakness of our linkage to rupture of the DIG–anti-DIG bond (4). While connecting each end of the DNA via biotin–streptavidin provides a strong linkage, it has the major disadvantage that both ends of the DNA are highly likely to bind to the same microsphere when tethering the DNA as we have described. This problem may be avoided by tethering the DNA in a flow, but this requires one to flow a solution of free DNA molecules into the chamber, which is often inconvenient. In any case, the DIG–anti-DIG link usually provides a sufficiently strong linkage for...
many protein–DNA interaction experiments to be carried out. A stronger method of attachment, in which an amino–carboxyl linkage is substituted for DIG–anti-DIG, is also described below.

We characterized the strength of the DIG–anti-DIG link by sharply ramping the force to a certain value and measuring the time it took for the tether to break. This measurement was repeated on an ensemble of 10.1 kb λ molecules at each force to determine the distribution of unbinding times (Figure 9). As the unbinding events are thermally activated events, the time intervals for unbinding are expected to follow an exponential distribution $P(t) \sim \exp(-t/\tau)$. Indeed, each of our distributions was well fit by this distribution, yielding a characteristic lifetime $\tau$ at each force. The fitted values of $\tau$ were 6.3 ± 2.0, 3.8 ± 0.7, 2.9 ± 0.3 and 2.0 ± 0.1 s at $F = 30$, 40, 50 and 60 pN, respectively.

**Stronger attachment via an amino–carboxyl linkage**

To obtain a stronger DNA tether we also conducted trials substituting an amino–carboxyl linkage (an amide bond) for the weaker DIG–anti-DIG bond (37). In this case, the DNA was first attached via an amino-labeled end to a carboxyl functionalized microsphere in a bulk reaction, and then tethered by the biotin end to a streptavidin coated microsphere in the optical tweezers. This methodology worked with similar efficiency as the DIG/biotin DNA tethering. Both single amino-labeled and triple amino-labeled molecules worked well. We found that such a linkage could sustain much higher forces for long lengths of time. Since a covalent amide bond is stronger than the biotin–streptavidin linkage, we believe that the strength of the tether is limited by the streptavidin–biotin link in this case. Molecules tethered in this manner could be reliably stretched multiple times past the overstretching transition at ~65 to ~85 pN, the maximum force that our optical tweezers could exert before the microsphere escaped from the trap. A molecule could also be held at a force of ~80 pN for up to several minutes without detaching, whereas a molecule tethered by DIG could rarely be stretched past the overstretch point without detaching.

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