Published in final edited form as:

Methods Mol Biol. 2018 ; 1703: 139–152. doi:10.1007/978-1-4939-7459-7_10.

Single-Molecule Magnetic Tweezer Analysis of Topoisomerases

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Abstract

Magnetic tweezers (MT) provide a powerful single-molecule approach to study the mechanism of topoisomerases, giving the experimenter the ability to change and read out DNA topology in real time. By using diverse DNA substrates, one can study different aspects of topoisomerase function and arrive at a better mechanistic understanding of these fascinating enzymes. Here we describe methods for the creation of three different DNA substrates used in MT experiments with topoisomerases: double-stranded DNA (dsDNA) tethers, “braided” (intertwined or catenated) DNA tether pairs, and dsDNA tethers with single-stranded DNA (ssDNA) regions. Additionally, we discuss how to build flow cells for bright-field MT microscopy, as well as how to noncovalently attach anti-digoxigenin to the coverslip surface for tethering digoxigenin-labeled DNAs. Finally, we describe procedures for the identification of a suitable DNA substrate for MT study and data collection.

Keywords

Single-molecule; Magnetic tweezers; Functionalized DNA; Flow cell; Noncovalent anti-body attachment; Bright-field microscopy; Topoisomerases

1 Introduction

Single-molecule force experiments conducted with magnetic tweezers (MT) have provided insights into how topoisomerases alter DNA topology [1]. In MT experiments, DNA molecules are tethered at one end to a surface and at the other end to a paramagnetic bead, which can be manipulated using a magnetic field. MTs are ideally suited for studying topoisomerases since it is relatively easy to rotate the magnetic bead attached to the DNA and introduce supercoiling of individual DNA molecules or catenations (“braids”) of pairs of DNAs, mimicking supercoiled and catenated DNA configurations found in the cell. Single molecule MT experiments have provided a better understanding of the enzyme-bridged strand passage mechanism for type IA and IIA topoisomerases and the constrained swivel mechanism for type IB and IC [2–8]. They have also allowed comparisons between topoisomerases in the same sub-type and different homologs. Furthermore, single molecule MT experiments have uncovered aspects of topoisomerase mechanism, such as rates along multistep pathways that could not be discerned from bulk experiments [9, 10].

Some of the most time-consuming aspects of magnetic tweezer experiments are the steps leading up to data collection: making DNA substrates, generating DNA tethers, and identifying a suitable tether for data collection. Part of what makes these tasks challenging is the variety of methods available to achieve them. For MT experiments a DNA substrate is needed that has been functionalized on both ends. In most experiments, at one end the DNA has a digoxigenin (dig) functionalization (dig handle) for attachment to an anti-dig coated coverslip. At the other end, a biotin (bio) functionalization (bio handle) is used for attachment to streptavidin coated paramagnetic beads.

Once the DNA is attached on one end to a glass slide and on the other to a paramagnetic bead, thus creating a DNA tethered bead, a magnetic force is applied. The magnetic field causes the DNA substrate to stretch, whereas rotation of the magnets rotates the bead and concomitantly the DNA. Monitoring the height of the bead allows determination of the length of the DNA tether. By rotating the magnetic bead, excess supercoils or braids can be introduced into the DNA, which in turn affects the height of the DNA substrate. The activity of topoisomerases on the DNA can then be monitored by tracking in real time the height of the bead, which is directly related to the number of turns introduced.

Here we discuss general methods used in our laboratory for magnetic tweezer experiments, including the creation of three types of DNA substrates, flow cells for bright-field MT microscopy, noncovalent attachment of anti-digoxigenin (anti-dig) to the cover glass (which can be used for both bright and dark-field microscopy), and generation and characterization of DNA tethers for data collection. We do not discuss the design, construction, and operation of MT instruments, which have been described else-where, for example in [11, 12].

2 Materials

2.1 Creation of the DNA Substrate

1. Plasmid DNA of the desired tether length with appropriate restriction enzyme sites (a large range of sizes can be used, with shorter DNA (~6 kilobases (kb) or shorter) providing less noisy data than longer DNA molecules (~10 kb), but fewer supercoils can be introduced into shorter DNA tethers). We have based a number of studies on the plasmid pFOS1 (~10 kb) and on derivatives of the pMAL-pIII plasmid (~6.7 kb), mutated to contain the desired cut sites [9, 10].
2. Restriction enzymes appropriate for creating the desired tether length (in our experiments, NotI, XmaI, SacI, ApaI), T4 DNA ligase, T4 Polynucleotide Kinase (PNK) (New England Biolabs (NEB)).
3. Digoxigenin-11-dUTP alkali stable (Dig-dUTP) (11093088910, Roche) and Biotin-16-dUTP (Bio-dUTP) (11093070910, Roche).
4. For single attachment DNA—DNA oligonucleotides with a single 5' biotin or digoxigenin functionalization designed to amplify the desired size of DNA via PCR (Integrated DNA Technologies (IDT)). Singly attached DNA is used when only one strand of the duplex needs to be attached, leaving the second strand free to rotate [10, 13]. This type of attachment is used in braiding experiments where

it is desirable to let the duplex rotate without supercoiling. The drawback of singly attached DNAs is that they are fragile and the DNA can detach easily under force. For example to create a 6 kb tether from pFOS1 use these two primers: Dig handle: 5'-(Dig) GCTCGTCGTTTGGTATGGCTT-CATTC-3' Bio handle: 5'-(Bio) GATGAAGGTAACTGCCCACCGATC-3'

5. For multiple attachment DNA—DNA oligonucleotides for amplification of ~250 base pairs (bp) functionalized handles with cleavage sites (Bio handle—NotI cleavage site; Dig handle—XmaI or ApaI cleavage site) and amplification of a spacer (ApaI and SacI cleavage sites) (IDT) (see Fig. 1). DNA with multiple attachment sites in both strands is more resistant to high forces and ideal for supercoiling experiments.
6. Standard reagents and equipment for PCR, dNTPs, Taq polymerase, PfuTurbo polymerase.
7. PCR purification and gel extraction kits (Qiagen).
8. Annealing buffer: 10 mM Tris pH 8.0, 50 mM NaCl, and 1 mM EDTA.

2.2 Fabrication of Flow Cells for Bright-Field Magnetic Tweezers

1. Streptavidin-coated paramagnetic beads (many different sizes and brands are available, we use Dynabeads MyOne Streptavidin T1, 65601, Invitrogen or Streptavidin Magnetic Beads, S1420S, NEB).
2. Vortexer.
3. Bovine serum albumin (BSA) (many different purities available, we use A7030_10G, Sigma-Aldrich).
4. Phosphate buffered saline (PBS) (17–516Q, Lonza) and ethanol for dilutions.
5. Micro Mill (MF70, Proxxon).
6. Cylinder Diamond Drill Bit—3/4 mm.
7. 25×75 mm microscope glass slides.
8. 24×50 mm microscope glass coverslips (12–544E, Thermo Fisher Scientific).
9. Double sided tape and epoxy for sealing the flow cells.
10. Sheep anti-Digoxigenin (anti-dig) Fab fragments (11214667001, Roche).

2.3 Identifying DNA Substrate

1. Tube rotator.
2. Magnetic tweezers instrument.
3. Single molecule buffer. The buffer should be selected based on the topoisomerase to be studied. For example, our buffer for bacterial topoisomerase I is: 50 mM Tris-HCl pH 8.0, 120 mM NaCl, 1 mM MgCl₂, 0.2 mg/mL BSA. The BSA concentration can be increased if more passivation is needed.

3 Methods

3.1 Creation of the DNA Substrate

3.1.1 dsDNA Tether with Single Attachment (for Creation of Braided Double Tethers)—When creating braided DNA molecules, two DNA molecules are attached to the same magnetic bead, so that when the magnet is turned, the DNA molecules twist around each other, braiding the molecules together and thus mimicking two catenated DNA molecules. To create braids it is necessary to either nick the DNA or to create DNA substrates that have only one attachment to the coverslip and bead, respectively. In this way the tethers cannot be supercoiled, but can still form braids. Here we describe how to make single attachment tethers. Another alternative is to follow the protocol for multiple attachment tethers and introduce a nick into the DNA using a nickase [10].

1. After deciding the length of DNA tether desired, design two oligonucleotides to amplify by PCR a portion of a plasmid to create the desired length DNA (3 kb to >10 kb; we typically use 6 kb, which results in a ~2 μ m DNA tether). Purchase one of the oligonucleotides with a 5' digoxigenin group incorporated, for example from IDT, which will be used for attaching to the anti-dig coated coverslip surface. To the second oligonucleotide add a 5' biotin group, which will attach to the streptavidin coated magnetic bead. For a 6-kb tether made from plasmid pFOS1 use the primers listed in Subheading 2.1, **item 4**.
2. The oligonucleotides serve as primers for use in a standard PCR procedure for PfuTurbo polymerase to create a linear DNA fragment. For the 6 kb DNA from pFOS1 use an annealing temperature of 55 °C and an extension time of 6 min.
3. Purify the resulting DNA using standard commercially available PCR purification kits. Store concentrated stock at –20 °C. Dilute a working stock to ~0.5 ng/ μ L just before using (see Note 1).

3.1.2 dsDNA Tether with Multiple Attachments (for Single Unnicked DNA Tethers)—In order to supercoil a single tether, multiple attachments are required between the dig and bio functionalized DNA and the coverslip and bead, respectively. If there is only a single attachment on either end, the tether can rotate around the attachment, which prevents supercoiling. The presence of nicks in the DNA backbone also results in an inability to supercoil. To achieve multiple attachments approximately 25 bio or dig functionalized dUTPs are randomly incorporated into the DNA on either end of the substrate to increase the likelihood of two or more attachments, including one on each strand of the DNA.

1. Decide on the DNA length desired and chose (or create) a plasmid close to the desired size, so that when digested with two restriction enzymes the resulting product can be purified (the piece cut should be <50 bp, so it can be readily removed using standard PCR purification kits). The choice of enzymes for digestion is also important, since ideally the cut sites would not have any T's in them, as in later steps this DNA will be ligated to handles where the T's are replaced by U's for functionalization (the presence of U's might inhibit later

digestion). Typically we use NotI and XmaI with a derivative of pMAL-pIII plasmid that has been mutated to have the appropriate cut sites (*see* Fig. 1a). After digestion purify the product using a PCR purification kit, leaving only a long linearized DNA.

2. Using an oligonucleotide which introduces a single restriction site (either a NotI or XmaI site) amplify a shorter DNA region, we typically use 250 bases long, which will serve as a functionalized handle. We use NotI for the Bio handle and XmaI for the Dig handle. Taq polymerase is used for creating the handles by PCR, since Taq polymerase can incorporate functionalized dUTPs. The ratio of Bio-dUTP or Dig-dUTP to dTTP determines the amount of random incorporation of the functional groups in the handle. For shorter handles (~250 bp) we use a high percentage (50%), whereas for longer handles (1000 bp) we use a lower one of 10%. For the 250 bp handle PCR reactions, we use 50% functionalized dUTP or a 1:1 ratio of dUTP to dTTP. Specifically, we use 1 μ L of functionalized dUTP (1 mM) per 50 μ L reaction, and 1 μ L of a 50 $\times$ stock of dNTPs containing 2 mM dATP, 2 mM dGTP, 2 mM dCTP, and 1 mM dTTP (*see* Note 2).
3. After making the handles by PCR, purify the products using a PCR purification kit. Follow this by digestion with either NotI (for Bio handle) or XmaI (for Dig handle) and PCR purify again.
4. In one tube, mix 75 ng each of the Bio and Dig functionalized handles with 250 ng of the long, linearized double stranded DNA and ligate overnight at 16 $^{\circ}$ C (*see* Fig. 1a). Stop the reaction by denaturing the ligase at 65 $^{\circ}$ C for 15 min and store the reaction at 4 $^{\circ}$ C. The ligated DNA can be used for up to 3 days, although the ability to form tethers diminishes with time. On the first day of use, we typically find that ~1 in 4 single tethers are intact, i.e., the DNA made multiple attachments and was not nicked (*see* Note 3).

3.1.3 dsDNA Tether with a ssDNA Bulge with Multiple Attachments (for Type IA Topoisomerases Experiments)—

Type IA topoisomerases require a single stranded binding region for activity. Negatively supercoiled DNA has melted regions that provide a substrate for the type IA topoisomerases *in vivo*. When using a MT, there is less noise in the data at higher forces, however, at forces >0.4 pN the DNA melts when negative supercoils are introduced resulting in no change in the height of the DNA [14]. To overcome this issue, it is typical to introduce a ssDNA region to the dsDNA tether, so that when the DNA is positively supercoiled there is still an available substrate for the type IA topoisomerase to bind to [2]. This allows data to be collected at higher forces (>0.4 pN).

1. Follow Subheading 3.1.2, **steps 1–3** for creating multiple attachment dsDNA tethers, but replacing XmaI in the Dig Handle with ApaI, as in this case the ligation will be to a spacer instead of the long linearized DNA (*see* Fig. 1b). This allows the use of the same linearized DNA used in Subheading 3.1.2.
2. Use a standard procedure to amplify by PCR a spacer DNA region to position the ssDNA bulge at the desired distance from the Dig handle. The spacer would have

ApaI and SacI restriction sites on either end. After digestion with only SacI, purify the spacer using a PCR purification kit. Typically, we start with 100 µg of spacer, since multiple purification steps cause significant loss of product.

3. Purchase oligonucleotides with at least 20 bp of overlapping complementary regions on either side of the desired bulge, which is located in the middle of one of the oligos. For a 27 nucleotide (nt) bulge we use the sequence 5'-AATCGGCATTGCGCAAACCAAGACAG-3' and for a 12 nt bulge 5'-AACGTGCCAAGA-3' [9]. The oligonucleotides should have overhanging sticky ends that allow for ligation to the digested spacer (SacI) and the long linearized DNA (XmaI) respectively. The SacI overhang for the annealed oligonucleotides needs to be 5' phosphorylated; we purchase the oligonucleotides prephosphorylated. Anneal the oligonucleotides by heating in a heat block to 95 °C for 3 min and allow cooling to room temperature (RT) in the heat block in annealing buffer (*see* Note 4).
4. Conduct a test to find the best ligation conditions. It is necessary to find appropriate conditions to minimize self-ligation of the annealed oligonucleotides or the SacI digested spacer. Typically we supply excess of the annealed oligonucleotides compared to the spacer. It is important to identify the desired ligation product in a gel before scaling up to a larger reaction. Once the best conditions have been identified, ligate the spacer DNA to the annealed oligonucleotide containing the bulge and gel-purify (*see* Note 5).
5. Digest the other side of the spacer (corresponding to the Dig handle) with ApaI and ligate the Spacer/Bulge to the digested Dig handle. Gel-purify the product as in Subheading 3.1.3, **step 4**.
6. In a 40 µL reaction, phosphorylate 75 ng of the Dig handle/Spacer/Bulge with T4 PNK following the manufacturer's protocol (using T4 DNA ligase buffer to facilitate the next step). This will phosphorylate the overhang on the bulge oligonucleotides corresponding to XmaI.
7. After denaturing the T4 PNK, take the 40 µL reaction and add 75 ng of digested Bio Handle and 250 ng of linearized DNA, ligating overnight at 16 °C (*see* Fig. 1b). Denature the ligase at 65 °C for 15 min and store the DNA at 4 °C. DNA can be used for up to 3 days, although the ability to form tethers diminishes with time. On the first day of use, we typically find ~1 in 30 single tethers can be supercoiled (not nicked).

3.2 Creation of Flow Cells for Bright-Field Magnetic Tweezers

Magnetic tweezer instruments can either illuminate the flow cell through the objective or by passing light through the flow cell to the objective, for dark-field or bright-field imaging respectively. In both cases the anti-dig coated coverslip must be the one closer to the objective. We use an inverted bright-field microscope, which requires the coverslip to be on the bottom side of the flow cell and closer to the objective [15]; a different flow cell construction is used when the objective is above the flow cell. The flow cell described here was adapted from ones used for fluorescence microscopy [16].

1. Clean the commercial streptavidin coated paramagnetic beads by taking 20 μL from the bottle (vortexed prior to evenly suspend the beads) and dilute with 200 μL of PBS. Vortex for at least 1 min. Pellet the beads with a magnet and remove the PBS. Repeat three times. Resuspend the beads in 120 μL of 0.4 mg/mL BSA for a final concentration of ~ 0.6 mg/mL and store at 4 $^{\circ}\text{C}$ (*see* Note 6).
2. Deposit reference beads on the coverslip by mixing 5 μL of cleaned beads with 45 μL of 100% ethanol. Spread the mixture on the coverslip and place the coverslip on a heat plate set at 80 $^{\circ}\text{C}$ until the liquid has evaporated (~ 15 s) [17].
3. Drill two holes diagonally from each other (*see* Fig. 2) in a glass slide using a micro mill equipped with a 3/4 mm diamond drill bit. Using double-sided tape create a channel between the inlet and outlet as shown in Fig. 2. Place the coverslip on top (with the bead-coated side facing the glass slide) and gently, but firmly press until the coverslip and tape are completely affixed. Carefully cut-off the excess tape. Mix a small amount of 5-min epoxy glue and apply it to the gaps in the tape at the ends of the coverslip. Allow it to sit for 30 s and wipe clean, so no excess epoxy remains. The epoxy will have seeped under the coverslip and sealed both ends of the flow cell (*see* Fig. 2). If the epoxy glue does not form a good seal, more epoxy glue can be added for an additional 15 s and then wiped away. To create a liquid reservoir, cut the end off of a pipet tip and place it around the inlet. Apply epoxy glue to create a watertight seal for the reservoir. Allow to dry [16].
4. Resuspend anti-dig at 1 mg/mL with PBS, aliquot, and freeze at -20 $^{\circ}\text{C}$ for long term storage. Take a working stock out and store at 4 $^{\circ}\text{C}$. For noncovalent attachment, add 10 μL of 1 mg/mL anti-dig to 40 μL of PBS, mix well by pipetting, and use the pipet to introduce liquid into the flow cell through the inlet until it emerges from the outlet. Pipet slowly to allow the liquid to spread out in the flow cell. After the flow cell is filled, liquid can enter the flow cell by capillarity, but flow can be aided by wicking with a tissue on the output end of the flow cell. 50 μL should completely fill the channel and leave a small amount in the reservoir to prevent drying [18]. Place the slide into a sealed plastic box on a raised platform, with PBS in the bottom of the box to maintain humidity, and incubate overnight at 4 $^{\circ}\text{C}$ (*see* Note 7).
5. For dark field microscopy, noncovalent attachment can also be used (in these flow cells the coverslip is on the top side of the cell). On the day of the experiment mix 5 μL of cleaned beads with 43 μL of 100% ethanol and 2 μL of 1 mg/mL anti-dig. Spread the mixture on a coverslip and lay on a heat plate set at 80 $^{\circ}\text{C}$ until evaporated (~ 15 s) (*see* Note 8).
6. Before using a flow cell, flow in at least four flow cell volumes (FCV) of 0.4 mg/mL BSA (~ 200 μL) and incubate for at least 5 min to passivate the flow cell. Add the BSA to the reservoir and pull through the flow cell using a tissue. Longer incubation with BSA can reduce the ability to form tethers and for this reason it is important to adjust the time of incubation.

3.3 Creating the Tether and Identifying the DNA Substrate

In order to successfully conduct an experiment the generation of tethered DNA molecules attached to both the coverslip and the bead is essential. Since many tethers may not be torsionally constrained (having either nicks or too few attachments), it is important to create many tethered molecules and to examine them for their suitability for the experiments.

1. Mix the DNA with cleaned beads by creating two identical tubes, each containing 2 μL of $\sim 0.5 \text{ ng}/\mu\text{L}$ of the DNA substrate and 2 μL of the cleaned beads ($\sim 0.6 \text{ mg}/\text{mL}$). Do not mix by pipetting the DNA and beads as pipetting can introduce nicks into the DNA. Place the tubes on a tube rotator for 5 min. Dilute the beads/DNA with PBS to a volume of $\sim 55 \mu\text{L}$. Pipet the mix into the flow cell reservoir and pull through by placing a tissue at the outlet. Add an additional 55 μL of PBS to the reservoir to prevent drying, but do not flow. Incubate the beads/DNA mix in the flow cell for 10 min (*see Note 9*).
2. Place the flow cell in the magnetic tweezer set up and apply magnetic force to the flow cell by lowering the magnets. Beads that are not well attached will float up to the top of the flow cell; turning the magnet can help to detach poorly tethered beads (*see Note 10*). If after looking at the slide more tethers are desired, repeat BSA passivation and redo Subheading 3.3, **step 1**. As many as three repetitions are sometimes necessary to generate a good covering of tethers, particularly if the DNA is not fresh.
3. To find a suitable DNA tether for the experiments, a reference bead and tethered DNA bead must be in close proximity, so that both can be tracked simultaneously. The reference bead and the tethered bead can be distinguished as they will be at different heights. For longer tethers, the two beads will be in very different focal planes and are easy to distinguish. Very short tethers are more difficult to distinguish and sometimes it is necessary to observe the movement of the beads to assess whether they are attached or tethered. In addition, turning the magnet a few turns at low force will result in a change of height of the tethered bead due to supercoiling. Intact tethered beads will not only be of the correct length, they will also change height when the magnet is turned [19]. A good reference bead will not move at all when the magnet is turned (these are the beads deposited with the EtOH). The proximity of the tether and reference bead will be determined by the size of the imaging field [12].
4. After locating a DNA tether suitable for the experiment, a look-up table is created by changing the height of the microscope objective by known amounts and taking a radial profile of the reference and tethered bead separately [12]. In this way, it is possible to measure the length of the tethered DNA with respect to the reference bead. Once the look-up table is available, it is possible to calibrate the force by measuring the variance of the bead movements and relating it to the position of the magnets. Measuring the force at several magnet positions allows a calibration so a desired force can be set (*see Note 11*). After these two calibrations are done, a plot of the height of the DNA against the number of turns introduced can be generated for different forces. For a single DNA tether start

with a curve at ~0.4 pN, since at this force the height will change for both positively and negatively supercoiled tethers, allowing for manual recentering of the tether if necessary (*see* Note 12). After the force has been calibrated and the tethered DNA has been centered, it is necessary to collect an extension vs. turns curve for the force(s) planned for the experiment. These plots have a characteristic shape (“hat curves”) and significant deviation from the expected shape indicates that the DNA substrate is unsuitable for the experiments [14] (*see* Fig. 3).

5. The linear portion of the hat curve can be used to translate extension into excess linking number or number of braids introduced for data analysis [12]. For experiments, single molecule buffer is introduced by capillary flow, since it is very gentle and reduces the likelihood of breaking the tether. Liquid pooled at the outlet can be absorbed by a tissue. The protein of interest is also diluted in single molecule buffer and introduced to the flow cell (2 nM is a good starting protein concentration in our experience).

4 Notes

1. In some cases, the DNA does not readily create tethers between the coverslip and bead (few tethers are seen when examined with the microscope), in these cases diluting a new aliquot of DNA can be helpful. Increasing working stock to 1 ng/μL can also increase the number of double tethers.
2. The ratio of functionalized dUTPs is important, since handles without sufficient functional groups can form fewer attachments. If this happens, when the magnet is rotated the DNA appears to be nicked (nonsupercoilable), as it can rotate around the single attachment.
3. Instead of ligation the day before the experiment, the DNA can also be ligated in bulk, gel purified, and stored at –20 °C, like the single attachment tether. However, significant amounts of the DNA can be lost in the gel purification step and the likelihood of DNA nicking is increased, as opposed to using the DNA directly from the ligation reaction.
4. The full oligonucleotides [9] used for the creating the 27 nt ssDNA bulge are:
Oligo containing overhangs for both SacI and XmaI ligation (opposite the bulged oligo): 5′ - CCGGCCGCATTA
GCAGCGTACGCTCAGCTTGGCGATCACGTAGTGGGCG
AAATCTGTCAGCT–3′ Oligo containing the 27 nt bulge (5′ phosphorylated for ligation): 5′ - /5Phos/CCCTGACAG
ATTCGCCCCACTACGTGATCGAATCGGCATTGCGCAAACCAAGACAGC
CCAAGCTGAGCGTACGCTGCTTTAATGCGG–3′
5. For smaller spacers, a 2% agarose gel can help separate the ligation products, making it easier to excise the correct fragment.

6. Volumes can be adjusted based on the concentration of the original beads. Over time the beads become more concentrated and should be resuspended in larger volumes.
7. The amount of anti-dig used can be adjusted depending on the application. To create more double tethers, use more anti-dig. If too many double tethers are seen, use less anti-dig.
8. This procedure can also be used with the bright-field flow cell, but is slowed by the drying time for the epoxy. Increasing the amount of anti-dig used leads to more aggregation of the beads on the coverslip.
9. The incubation time outside and inside the flow cell can be adjusted depending on the substrate. Longer incubation of the DNA and beads before adding to the flow cell can increase the amount of multiply tethered beads, while longer incubation in the flow cell can increase the number of tethered beads observed. When trying to form double tethers, double both incubation times (as well as increasing the anti-dig).
10. These beads can later cause problems in bright-field imaging, since the free beads stay at the upper surface between the slide and the light source. This problem can be alleviated by flowing in PBS or buffer with the magnet lifted, to push the free beads out of the flow cell.
11. For double tethers it is important to do the force calibration after ensuring that the DNA molecules are not braided, which is apparent by looking at a height vs. turns plot. Any braids in the DNA will affect the force calibrations.
12. We have found that tethers can gain twists during the preparation process and are not always centered at zero. For tethers with bulges, there will be a slight asymmetry in the curve, which makes it more difficult to assess the centering of the molecule. For double tethers there will be a high point at the center of the curve [20], which results from a large drop in height after the introduction of the first braid.

Acknowledgments

We thank members of the Mondragón and Marko laboratories for discussions and assistance. Research was supported by the NIH (R01 GM051350 to A.M., and R01 GM105847 and U54 CA193419 (CR-PS-OC) to J.F.M.) and the NSF (MCB-1022117 and DMR-1206868 to J.F.M.). K.H.G. was supported by a Dr. John N. Nicholson Fellowship and an NRSA predoctoral training grant (T32 GM008382).

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Fig. 1.

DNA molecule construction. **(a)** A multiple attachment dsDNA tether can be used to study a variety of topoisomerases using either *negative* or *positive* supercoils. After PCR amplification and digestion of the Bio and Dig functionalized handles, they are ligated to a linearized DNA. **(b)** For studying type IA topoisomerases, introducing a ssDNA bulge provides a binding site even when the DNA is positively supercoiled. After PCR amplification and digestion with SacI, the spacer can be ligated to the annealed ssDNA bulge and gel purified. Next the spacer/ssDNA bulge construct is digested with ApaI and ligated to the Dig handle. The Dig handle/spacer/ssDNA bulge is then phosphorylated at the XmaI site and ligated to the linearized DNA and the Bio handle

Fig. 2.

Flow cell construction. For bright field microscopy, this flow cell uses capillary flow to gently change the buffer, without affecting the tethered DNA. The flow channel is created between two holes drilled in a glass slide using double sided tape to delineate the width of the channel. A glass coverslip coated with beads is gently applied to the double sided tape and pressed firmly down. Epoxy glue is then used to seal the edges of the flow cell. A reservoir is constructed using the end of a pipet tip and sealed to the glass slide with epoxy glue to prevent leakage [16]

Fig. 3.

Characterization curves. **(a)** Typical hat curves showing the change in DNA extension when turns are introduced into a dsDNA tether. At a force of 0.4 pN, it is possible to introduce both negative and positive supercoils. As the force increases, the DNA melts when negative supercoils are introduced, no longer changing its length. The curve was measured using a 9.5 kb dsDNA tether which has multiple bio and dig attachments. The linear region of the hat curve, after plectonemes have begun to appear, can be used to translate DNA extension data into excess linking number. **(b)** When a ssDNA region is introduced into a DNA tether, the hat curve becomes slightly asymmetric [9]. However, it still has the same overall characteristics as a dsDNA tether; at low forces introduction of negative supercoils changes the DNA extension, whereas at high forces the DNA melts. The hat curve was measured using a 9.5 kb DNA tether with a 27 nt ssDNA bulge. **(c)** When two single-strand attached dsDNA tethers are anchored to the same bead, it is possible to “braid” (intertwine) the two DNA molecules, which mimics catenated DNA molecules [10]. The introduction of the first turn causes a drastic change in length due to the introduction of the first braid. The hat curve was measured using a 6 kb single attachment dsDNA tether at 2 pN