Chapter 7

Single-Molecule Magnetic Tweezers Studies of Type IB Topoisomerases

Jan Lipfert, Daniel A. Koster, Igor D. Vilfan, Susanne Hage, and Nynke H. Dekker

Abstract

The past few years have seen the application of single-molecule force spectroscopy techniques to the study of topoisomerases. Magnetic tweezers are particularly suited to the study of topoisomerases due to their unique ability to exert precise and straightforward control of the supercoiled state of DNA. Here, we illustrate in a stepwise fashion how the dynamic properties of type IB topoisomerases can be monitored using this technique.

Key words: Single-molecule techniques, magnetic tweezers, topoisomerases, TopIB, spectroscopy.

1. Introduction

1.1. Topoisomerase Activity

Topoisomerases are enzymes that control the topological state of DNA, in particular supercoiling, by temporarily breaking one or two strands of the DNA double helix (1–3). They are generally classified as type I and type II topoisomerases, depending on the number of strands cut in their catalytic mechanism. Type II topoisomerases induce temporary double-strand breaks and have a strand passage mechanism that requires ATP hydrolysis. In contrast, type I topoisomerases act on a single strand of DNA and remove one or more supercoils by temporarily creating a single-strand break and rotating the DNA around the remaining intact strand. Topoisomerase IB (TopIB), a eukaryotic enzyme, releases the torsion built up in a supercoiled DNA in a manner that is very different from type II topoisomerases and does not require ATP for the reaction. The enzyme releases the torsion from the DNA by

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surrounding the double-stranded DNA (dsDNA) like a clamp and temporarily cleaving one of the two strands. The accumulated torsional forces in the DNA are then spun out by swiveling about the intact strand. After a number of turns, the topoisomerase IB again firmly grabs the spinning DNA and neatly ligates the broken strands back together again (**Fig. 7.1**).

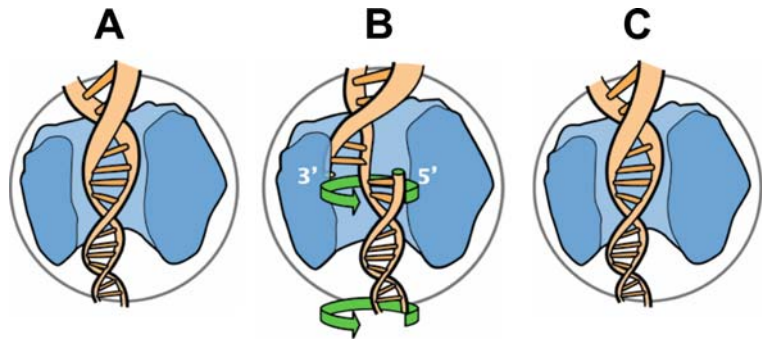

Fig. 7.1. **Schematic of the supercoil removal mechanism of topoisomerase IB**. The enzyme binds (non-covalently) to supercoiled double-stranded DNA (**A**). Topoisomerase IB then binds covalently to one strand of the DNA via its active tyrosine, creating a nick in the DNA strand. Rotation around the remaining DNA strand that acts as a swivel releases supercoils (**B**). Finally, the topoisomerase–DNA bond is broken and the DNA nick is resealed (**C**).

1.2. Magnetic Tweezers and DNA Manipulation

Magnetic tweezers (MT) are a single-molecule technique that permits the application of both stretching forces and torques to single DNA molecules (4). They are particularly well suited to the study of topoisomerases, as they enable straightforward and precise control of the supercoiling of DNA molecules (5–8). In the magnetic tweezers, a DNA molecule is tethered in a flow cell between a glass surface and a paramagnetic bead by means of non-covalent bonds which can resist forces on the order of ~100 pN (**Fig. 7.2**). Above the flow cell, a pair of permanent magnets or an electromagnet is suspended on a motorized stage (**Fig. 7.2**), exposing the flow cell to a magnetic field B (9). The magnetic field exerts an upward stretching force F on the bead given by

$$\vec{F} = \frac{1}{2}\vec{\nabla}\left(\vec{\mathbf{m}} \cdot \vec{\mathbf{B}}\right) \qquad [1]$$

where $\vec{\mathbf{m}}$ is the induced magnetization of the bead in the external magnetic field $\vec{\mathbf{B}}$. The magnitude of the stretching force can be controlled by controlling the distance of the magnets from the flow cell. Additionally, the magnets can be rotated, which in turn controls the rotation of the DNA-tethered beads. Rotating the bead attached to a torsionally constrained DNA molecule changes its linking number Lk. Starting from a torsionally relaxed molecule, the change in linking number is initially absorbed by elastic

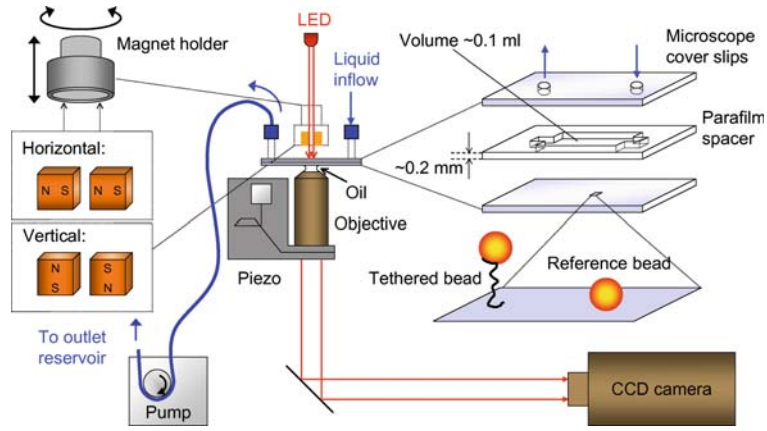

Fig. 7.2. **Schematic of the magnetic tweezers setup**. The basic components are shown schematically: the inverted microscope, the CCD camera, the flow cell system with fluid inlet and outlet connected to a pump, and the LED illumination. Shown in more detail are the flow cell with a tethered and a reference bead and pairs of magnets in horizontal and vertical geometry. Both horizontal and vertical magnet pairs provide field gradients appropriate for magnetic tweezers, however, with different characteristics. Lipfert et al. have carried out an extensive study of the different magnet geometries for magnetic tweezers (Jan Lipfert, Xiaomin Hao, and Nynke H. Dekker (2009) Quantitative modeling and optimization of magnetic tweezers. *Biophys J* 96, 5040–5049).

twist deformations and increases *Tw* of the molecule, while the Writhe *Wr* remains unchanged. In this regime, the torque Γ increases linearly with the number of turns *N*:

$$\Gamma = \frac{C}{L_c}(2\pi N) \qquad [2]$$

where *C* is the torsional modulus, $C \approx 90\ k_B T$ for DNA (10–12), and L_c is the length of the DNA. If one continues to rotate the magnets, the molecule undergoes a buckling transition where the additional mechanical energy is no longer stored as an elastic twisting deformation but rather in a loop, which leads to an observable decrease in the end-to-end extension of the DNA molecule (**Fig. 7.3**). Further rotations past the buckling transition do not increase *Tw*, and the torque remains constant. Instead *Wr* increases as plectonemic supercoils are formed, further decreasing the end-to-end distance of the molecule in a linear fashion (see the linear slopes shown as solid lines in **Fig. 7.3**).

Strick et al. have proposed a simple and approximate model for this plectonemic regime (13, 14), in which the postbuckling torque is equal to

$$\Gamma_B = \sqrt{2L_p k_B T F} \qquad [3]$$

where *F* is the force applied to the DNA molecule and $L_p \approx 50$ nm is the persistence length of DNA. The decrease in the length of the tethered molecule per turn in this approximate model is given by

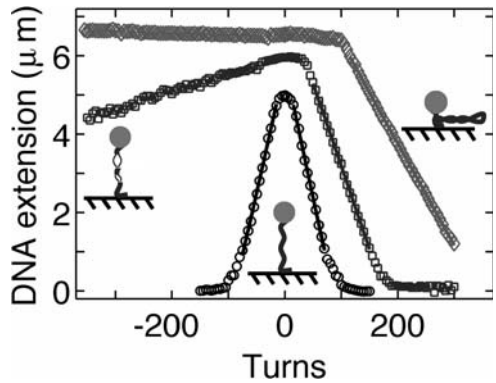

Fig. 7.3. **DNA extension as a function of linking number measured using the magnetic tweezers**. The data shown are for stretching forces of 0.25 (*black circles*), 1 (*dark gray squares*), and 5 pN (*light gray diamonds*). Initially, at zero turns, the DNA is torsionally relaxed. Introduction of positive turns eventually results in (positive) supercoiling of the DNA for all three measured forces. Negative turns result in (negative) supercoiling at low forces (*black symbols* and *line*), but at higher forces, the DNA melts locally under negative torque (*gray symbols*).

$$\Delta z = \pi \sqrt{\frac{2 L_p k_B T}{F}} \qquad [4]$$

It is the linear response of the DNA end-to-end extension to the number of induced supercoils that has to date proven so very useful to the study of topoisomerases, including TopIB. Since the supercoils are removed from the DNA as soon as the topoisomerase cuts through one of the two DNA strands, an increase in the length of the DNA is observed. Relating changes in length to changes in the degree of supercoiling, Koster et al. were able to determine the exact number of turns removed by the topoisomerase between "cutting" and "sealing" (**Fig. 7.4**) (6). In addition, by varying the applied force (and hence the torque in the plectonemic regime), the authors could monitor the torque dependence of the average number of turns removed. Finally, by monitoring the rate at which supercoils were removed by TopIB, the existence of friction of the rotating DNA in a cavity of the enzyme could be demonstrated. This methodology was recently also extended to study the influence of TopIB inhibitors on the dynamics of the enzyme (8).

1.3. The Magnetic Tweezers Microscope

1. Typically, the microscope is assembled in an inverted configuration for straightforward sample handling.
2. The focal length of the tube lens must be taken into account when computing the magnification of the experimental configuration. A calibrated grid (stage micrometer/G390010000, Linos, Germany) is useful for verifying that the magnification achieves the expected value.

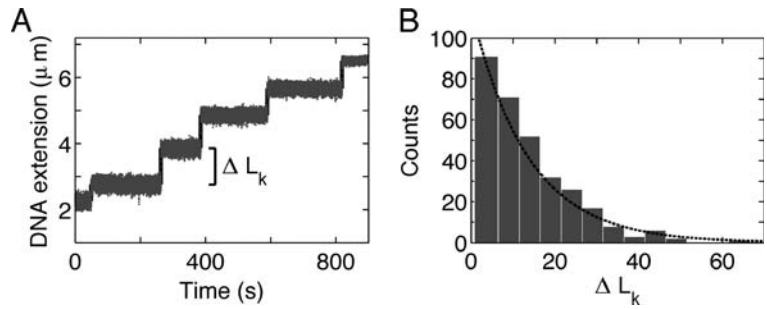

Fig. 7.4. **Single-molecule topoisomerase IB supercoil removal observed in the magnetic tweezers**. (**A**) Time trace of the DNA extension at a stretching force of 1 pN in the presence of wild-type human topoisomerase IB. The DNA is initially (time = 0) prepared in a positively supercoiled state by rotating the magnets +120 turns. Each time a topoisomerase molecule removes supercoils, a step in the extension is observed. Using the relationship between supercoils and the DNA extension (from the slope of the rotation curves, *see* **Fig. 7.3**), the steps in extension can be converted to a change in linking number ΔL_k. (**B**) A histogram of supercoil removal step sizes for human topoisomerase IB at a stretching force of 1 pN. The histogram is accumulated by recording many traces like the one shown in *panel A*. The data shown here comprise a total of $N = 309$ events and are well described by an exponential distribution (*dashed line*) of the form $P(\Delta L_k) = \exp(-\Delta L_k/\langle \Delta L_k \rangle)$ with $\langle \Delta L_k \rangle = 13.6 \pm 1.5$.

3. To reduce drift, it is recommended that the microscope be built as compactly as possible.

Magnetic tweezers setups are now also commercially available (www.picotwist.com). Our setup is similar to the one used by Croquette and coworkers (4) and described in further detail elsewhere (6) (I. D. Vilfan, J. Lipfert, D. A. Koster, S.G. Lemay and N. H. Dekker (2009) Magnetic tweezers for single-molecule experiments. P. Hinterdorfer and A. van Oijen (eds.), *Handbook of Single-Molecule Biophysics*, Springer).

1.4. The Magnetic Tweezers Software

1. In the magnetic tweezers, the force F applied to the DNA molecule is given by the following expression:

$$F = \frac{k_B T l}{\langle \delta x^2 \rangle} \qquad [5]$$

where k_B is Boltzmann's constant and T is the absolute temperature. Thus, to determine F, one needs to measure the extension of the molecule l and the variance of the bead excursions $\langle \delta x^2 \rangle$. These parameters are determined from the acquired data by image analysis of the diffraction pattern recorded by the CCD.

2. To monitor l, we use a method first designed by Gosse and Croquette, in which the objective's focal plane (OFP) is accurately shifted in the vertical direction while imaging the

diffraction rings of the bead attached to the nucleic acid (15). In this manner, a calibration profile is generated correlating the diffraction pattern of the bead to the distance between the bead and the OFP (I. D. Vilfan, J. Lipfert, D. A. Koster, and N. H. Dekker, Springer Handbook of Single-Molecule Biophysics, 2009).When this calibration profile is interpolated, the vertical displacements of the bead can be measured with an accuracy of up to ~10 nm (15). If one then measures a similar calibration profile for a reference bead fixed to the surface in the proximity of the tethered bead, the distance between the surface and OFP can be determined. Finally, the difference between the two measured distances yields l.

3. An additional advantage of tracking both a reference bead and the DNA-tethered bead is that this differential measurement minimizes the effect of drift between the objective and the sample holder (i.e., flow cell).
4. Processing of the bead's in-plane fluctuations allows one to compute $\langle \delta x^2 \rangle$. Changes in the x position of the bead are computed via cross-correlation analysis of the intensity profiles displayed by a bead at subsequent time intervals (16, 17). As in the case of tracking the vertical position, in-plane tracking can be carried out at subpixel resolution to an accuracy of a few nanometers (15).
5. In practice it can be advantageous, in particular for large forces ($\geq$10 pN), to determine the pulling force not directly from equation [5] but from Fourier analysis of the beads' fluctuations (I. D. Vilfan, J. Lipfert, D. A. Koster, and N. H. Dekker, Springer Handbook of Single-Molecule Biophysics, 2009).

2. Materials

Our measurements employ a home-built microscope equipped with a pair of permanent magnets whose position and rotation can be computer controlled. This MT setup is described in -**Section 2.1** (*see* **Note 1**).

2.1. Magnetic Tweezers Microscope

1. Microscope objective: An Olympus ACH 100X 1.25 numerical aperture objective (Olympus, Japan) is recommended for measurements of a single pair of beads, 60X for multibead tracking (18).

2. Nanometer-accurate objective positioner: A PI PIFOC P-721.CDQ piezo-driven microscope objective nanofocusing/scanning device (Physik Instrumente, Germany).
3. High-speed CCD camera: A Pulnix TM-6710CL CCD camera (to track the bead positions at 60 or 120 Hz).
4. Frame grabber: A NI PCIe-1429 frame grabber (National Instruments, USA) to read out the CCD camera images.
5. Permanent magnets with high magnetization: Cubic 5 × 5 × 5 mm neodymium–iron–boron (NdFeB) magnets (W-05-N50-G, Supermagnete, Germany).
6. Motorized stage (to control the magnets' position): A PI M-126.PD motorized stage (Physik Instrumente, Germany).
7. Motor to control the magnets' rotation: A PI C-150 (Physik Instrumente, Germany).
8. Syringe pump (Cole-Parmer, IL, USA) for buffer exchange in the flow cell.
9. Computer and software: A Dell Precision T5400 work station (Dell, USA) and software custom written in Labview 8.2 (National Instruments, USA) are used to control all of the components and for data acquisition.

2.2. Magnetic Tweezers Software

The software to control the magnets, read out the CCD camera, and track the DNA-tethered bead and reference bead positions in x, y, and z in real time is written in Labiew 8.2 (National Instruments, USA).

2.3. Flow Cells for Magnetic Tweezers

1. Coverslips: Glass coverslips 24 × 60 mm, 130 μm thickness (Merzel Gläser, Germany).
2. Polystyrene solution: 100,000 MW polystyrene powder (Sigma-Aldrich, USA) dissolved in toluene (1% polystyrene by weight).
3. Anti-digoxigenin antibodies: Fab fragments of anti-digoxigenin antibodies (Roche Diagnostics, The Netherlands) dissolved at 100 μg/ml in PBS.

2.4. Buffer Solutions

1. PBS: Dissolve phosphate-buffered saline tablets (Sigma-Aldrich, USA) in deionized MilliQ water to obtain a 1X solution. Filter through a 0.22-μm pore size Millex GV syringe driven filter unit (Millipore Corporation, USA) to remove impurities and to filter sterilize the solution. The solution can be stored at 4°C and should be replaced after 6–8 weeks.

2. Topoisomerase reaction buffer: 10 mM Tris-HCl, pH 8.0, 50mM KCl, 1 or 10 mM $MgCl_2$, 1mM DTT (dithiotheitol), 200 μg/ml BSA (bovine serum albumin), and 0.1% Tween-20 (molecular biology grade). Make up the solution in deionized MilliQ water and filter through a 0.22-μm pore size Millex GV syringe driven filter unit (Millipore Corporation, USA) to remove impurities and to filter sterilize the solution. The solution can be stored at 4°C and should be replaced after 6–8 weeks.
3. BSA or poly-L-glutamic acid solution: 10 mg/ml BSA or poly-L-glutamic acid (15,000–30,000 molecular weight, Sigma-Aldrich, USA).

2.5. DNA Constructs for Magnetic Tweezers

1. Supercos1 plasmid DNA (Stratagene, The Netherlands). The plasmid contains two nicking sites *Bbv*CI.
2. pbluescrIISK plasmid DNA (Stratagene, The Netherlands).
3. λ-phage DNA (Promega, The Netherlands).
4. LB (per l): 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl. Adjust the pH to 7.0 and autoclave to sterilize.
5. Midi kit: Qiafilter plasmid midi kit (Qiagen, Germany).
6. Restriction enzymes and buffers (New England Biolabs, MA, USA): *Mlu*I, *Xho*I, *Not*I.
7. Gel extraction kit: Nucleospin extract II kit (Macherey-Nagel, Germany).
8. PCR primers (for sequences *see* **Section 3.2**).
9. bio-dUTP: Bio-16-dUTP (biotin-16-2′-deoxyuridine-5′-triphosphate) (Roche, The Netherlands).
10. dig-dUTP: Dig-11-dUTP (digoxigenin-11-2′-deoxyuridine-5′-triphosphate) (Roche, The Netherlands).
11. T4 DNA ligase and buffer (New England Biolabs, MA, USA).
12. Go taq polymerase kit (Promega, WI, USA).
13. Phase lock tubes (Eppendorf, Germany).
14. Phenol:chloroform:isoamylalcohol: (49.5:49.5:1) (J.T. Baker, NJ, USA).
15. TE: 10 mM Tris–HCl, pH 8, 1 mM EDTA.

2.6. Superparamagnetic Beads

We employ streptavidin-coated Dynal MyOne beads (Invitrogen, USA) for most measurements. MyOne beads have a nominal diameter of 1.05 μm and exhibit good homogeneity in size and magnetic content, such that the variation in stretching forces from bead to bead is less than 5–10%. For measurements with higher stretching forces (see below), we also use streptavidin-

coated ≈ 1 μm diameter MagSense beads (MagSense, IN, USA), which have a higher content of magnetic material, or streptavidin-coated Dynal M280 beads (Invitrogen, USA), which have a diameter of 2.8 μm.

2.7. Topoisomerase Stocks

Topoisomerases can be purchased commercially or purified following overexpression in-house (6, 8). We routinely use human TopIB purchased from Topogen (TopoGEN, FL, USA). It is also available from Inspiralis (Inspiralis, UK).

3. Methods

3.1. Assembly of Flow Cells for Magnetic Tweezers

Flow cells are made from microscope cover slides on the top and bottom with a parafilm spacer in the middle. The bottom slides are coated with a layer of polystyrene to enhance the absorption of anti-digoxigenin antibodies that are used to specifically bind functionalized DNA molecules to the surface (*see* **Note 2**).

1. Drill holes in the microscope cover slips that are going to be the top slides for the flow cells. The holes are required for the fluid inlets and outlets to the cell (*see* **Note 3**).
2. Clean the top slides by sonication for 10 min in ethanol or isopropyl alcohol. Blow dry with compressed air or nitrogen stream.
3. Rinse the bottom slides with deionized MilliQ water. Blow dry with compressed air or nitrogen stream.
4. Coat the bottom slides with polystyrene: Fill the bottom of a small glass beaker with polystyrene solution. Rinse a thick 70 × 24 microscope cover slide with MilliQ water, blow dry with compressed air or nitrogen, and place in the beaker. Coat the bottom slides by dipping them into the beaker with tweezers and using the surface tension between the glass slides to evenly cover one surface with the polystyrene solution. Place the slides with the coated surface facing up into a glass Petri dish.
5. Heat the coated bottom slides in an oven at 100°C for 60 min.
6. Cut out spacers from a double layer of parafilm that form the reaction chamber of the flow cell, with channels that will connect to the holes of the top slide. This is easily done using a scalpel and a metal mould (*see* **Note 4**).

7. Assemble the flow cells: Place the parafilm spacers on the polystyrene-coated side of the bottom slides and close the top with the cleaned top slides. Using tweezers, place the assembled cell on a heater plate set to 80–100°C for ≈5 min (*see* **Note 5**). Allow the cells to cool off for a few minutes at room temperature.
8. Fill the flow cells with anti-digoxigenin solution using a standard pipette.
9. Cover the inlet and outlet holes with small pieces of parafilm.
10. Store the readymade flow cells in plastic Petri dishes, with a small piece of paper towel or Kimwipe soaked with MilliQ water to keep the cells from drying out. Seal the Petri dishes with parafilm.
11. Incubate for at least 12 h at 4°C (*see* **Note 6**).
12. Prior to mounting the cell, clean the microscope objective and apply a drop of objective oil, if an oil immersion objective is used.
13. Mount the flow cell on the magnetic tweezers microscope and connect the syringe pump to the outlet channel. Check the flow cell for leaks.
14. Passivate the flow cell surface by adsorbing inert proteins: Load into the flow cell BSA or poly-L-glutamic acid solution and incubate for 15–30 min to passivate the part of the surface not occupied by the nucleic acid attachment points (6, 19–22) (*see* **Note 7**).

3.2. Preparation of the Magnetic Tweezers DNA Constructs

The DNA construct described here is ≈21 kb in length, which corresponds to a contour length of ≈7 μm. Longer or shorter molecules can be used, depending on the requirements of particular measurements. To reduce the noise on traces and observe single steps in great detail, shorter constructs will improve the signal-to-noise ratio on the time traces (23). In contrast, for the determination of the step size distribution (see below), it is advantageous to use fairly long constructs so that a larger number of supercoils can be initially introduced. A long construct with a large number of initial supercoils reduces the bias of the distributions stemming from the finite number of supercoils (24). The ≈21 kb construct described here presents a compromise between the two requirements. The protocol can be modified to generate longer or shorter DNA constructs by using the same labeled ends and by changing the DNA construct that is used for the middle portion of the tether (Supercos1-lambda1,2 in this protocol) by a longer or shorter DNA construct.

3.2.1. Construction of the Supercos1-Lambda1,2 Vector

1. Digest the Supercos1 plasmid with *Mlu*I to delete the *Mlu*I fragment and then self-ligate the backbone of the plasmid. (This will remove one *Blv*CI nicking site.) Transform the ligation product into *Escherichia coli* and recover the plasmid DNA, designated Supercos1-*Mlu*I.
2. Amplify a 9.5-kb lambda fragment by PCR using the forward primer 5′ AAGGAAAAAA GCGGCCGCTACATCTCGAG ATGGTGCATCCCTCAAAACGAG and the reverse primer 5′ GGAAAGGGCCCGTAAAGTGATAATGATTATCATC, and λ-phage DNA as the template DNA.
3. Digest the Supercos1-*Mlu*I plasmid with *Not*I and ligate the PCR product from step 2 into the cut plasmid. After recovering the ligation product in *E. coli* and isolating the plasmid DNA from *E. coli* clones, select clones having the orientation where the *Not*I site is 10 kb away from the *Blv*CI site, designated Supercos1-lambda1-1.
4. Amplify a 5-kb lambda fragment by PCR using the forward primer 5′ TTGGCGCGC TTGATACATCAACTGCACCT-GATATTG and the reverse primer 5′ CCAGATCT ACGA CCTGCATAACCAGTAAG, and λ-phage DNA as the template DNA.
5. Digest the Supercos1-lambda1-1 plasmid from step 3 with *Bss*HII and *Bgl*II and ligate the PCR product from step 4 into the cut plasmid. Recover the ligation product into *E. coli*. This plasmid is designated Supercos1-lambda1,2.

3.2.2. Construction of the DNA Construct for Labeling

1. Inoculate the *E. coli* harboring the Supercos1-lambda1,2 plasmid into 100 ml of LB medium and grow overnight at 37°C.
2. Pellet the culture and isolate the plasmid DNA using the low copy protocol from the midiprep kit.
3. Digest approximately 18 μg of the Supercos1-lambda 1,2 plasmid with *Xho*I and *Not*I in NEB buffer 3, supplemented with BSA, according to the manufacturer's instructions. (The expected fragment lengths are 20,666 and 12 bp, which can be verified by gel electrophoresis.)
4. Purify the 20.6-kb fragment from the digest in step 3 using the gel extraction kit.

3.2.3. Preparation and Ligation of the Biotin and Digoxigenin Handles

1. Set up two PCR reactions to produce the biotin and digoxigenin fragments using the pbluescrIISK+ as the template DNA: In 50 μl volumes, make the PCR reaction solutions containing 0.2 μM forward primer 5′ GACCGAGATAGGGTTGAGTG, 0.2 μM reverse primer 5′ CAGGGTCGGAACAGGAGAGC, 0.2 mM dNTP, 100 ng of the pbluescrIISK+ plasmid DNA, 1X

PCR buffer, and 1 µl of Taq polymerase. To one of the reactions, add bio-dUTP (40 µM final concentration) and to the other reaction add dig-dUTP (40 µM final concentration).

2. Purify the PCR fragments using the gel extraction kit.
3. Determine the concentration of the DNA recovered from step 2 spectrophotometrically.
4. Digest approximately 8 µg of the biotin PCR fragment with *Xho*I according to the manufacturer's instructions. The expected digestion products are 534 and 665 bp.
5. Digest approximately 8 µg of the digoxigenin PCR fragment with *Not*I according to the manufacturer's instructions. The expected digestion products are 624 and 614 bp.
6. Purify the digested fragments from steps 4 and 5 with the gel extraction kit. These are designated the "handle constructs."
7. Determine the concentration of the handle constructs.
8. Ligate the handle constructs to the ends of the 20.6 kb Supercos1-lambda1,2 digested fragment from **Section 3.2.2**, step 5: Prepare the ligation mixture according to the manufacturer's instructions and using 7 µg of the 20.6 kb fragment with approximately a 20-fold molar excess of each of the handle constructs. Ligate overnight at 16°C.
9. Purify the ligation product by phenol:chloroform:isoamylalcohol extraction in phase-lock tubes and finally precipitate the DNA with ice-cold absolute ethanol.
10. Pellet the DNA from step 9 and resuspend the pellet in ~50 µl of TE to give a stock solution that is ~0.1 ng/µl. This is designated the "magnetic tweezers construct DNA" stock.

3.3. Preparation of Magnetic Beads and DNA for Magnetic Tweezers Measurements

3.3.1. Assembly of Tethered DNA Constructs in the Flow Cell

1. Sonicate the MyOne beads for 5 min.
2. Take an aliquot of 2 µl of the MyOne beads and dilute it with 10 µl of PBS. Wash twice with 10 µl of PBS using a magnetic pipette aid and resuspend in 10 µl of PBS.
3. Dilute 1 µl of the magnetic tweezers construct DNA stock from **Section 3.2**, step 10, into 50 µl of TE (*see* **Note 8**).
4. Add ca. 1–2 µl of the diluted DNA construct to the washed beads (*see* **Notes 9** and **10**).
5. Incubate the bead–DNA mixture for 15–30 min at room temperature.
6. Dilute the DNA–bead mixture into 100–300 µl PBS and load ca. 100 µl into the flow cell (*see* **Note 11**).

7. Incubate the beads in the flow cell for 30–60 min (*see* **Note 12**).
8. Flush the flow cell with ca. 1 ml of PBS. Most of beads will be flushed out and a small fraction of beads will remain either stuck to the surface through unspecific interactions or tethered to the surface through the DNA. Ideally, the density of beads is such that there are one to a few stuck and tethered beads per field of view.
9. Move the magnets down to within 1–2 mm of the flow cell surface to apply a stretching force to the tethered beads (*see* **Note 13**).

3.3.2. Calibration of the Tethered DNA in the Magnetic Tweezers

1. Search for coilable molecules in the flow cell. By introducing 30–60 turns of the magnets at a force of ≈ 0.25 pN (*see* **Section 1.4** on how to determine the stretching forces), one can test whether a tethered molecule is coilable. By comparing the behavior under positive and negative turns at forces of ≈ 1 pN, it can be checked whether the molecules are attached by a single or multiple tether. Additionally, appropriate fixed beads stuck to the bottom surface need to be identified, which can serve as a reference bead. Instead of using unspecifically attached magnetic beads as reference markers, it can be beneficial to use non-magnetic (e.g., polystyrene) beads as reference beads.
2. Record look-up tables for the z-position tracking and verify that the tethered and reference beads can be tracked by the tracking software (*see* **Section 1**).
3. Flush in the topoisomerase reaction buffer, without the enzyme added.
4. Run a rotation curve (measurement of the DNA extension as a function of the number of turns) at a stretching force of ≈ 0.25 pN to determine the number of turns at which the extension is maximal and at which the DNA molecule is torsionally relaxed. Define this point as "zero turns." For this step, it is useful to fit the rotation curve locally with a parabola or Gaussian to determine the center position (*see* **Note 14**).
5. Record a force–extension curve: At a series of ~ 10 magnet positions, determine the average extension of the molecule from the z-trace. In addition, at each magnet position determine the stretching force from the fluctuations in the x or y position, as described in **Section 1.4**. Fit the resulting force-extension data by the worm-like chain equation using the polynomial approximation by Bouchiat et al. (25). The fitted values of the persistence length should be ≈ 50 nm (in practice, values in the range 45–55 nm are acceptable), and

the fitted contour length should be close (typically within 10%) to the value expected for the DNA construct that is used in the measurements, using the relationship $L_{DNA} = 0.34$ Å*bp.

6. Record a number of rotation curves of the DNA molecule at different forces, where the forces included should correspond to forces at which data of topoisomerase activity are desired (*see* **Fig. 7.3** for examples of rotation curves). For each of these curves, determine the slope of the rotation curves in μm/turn by fitting a straight line to the slope of the rotation curve in the plectonemic supercoiling region (**Fig. 7.3**, solid lines) (*see* **Note 14**).

3.4. Monitoring DNA–Topoisomerase IB Interactions

1. Flush in TopIB at a concentration of 0.5–20 nM in topoisomerase reaction buffer.
2. Adjust the magnets to exert the desired pulling force and rotate the DNA molecule into the plectonemic regime.
3. Track the bead's position to obtain a time trace with stepwise increases in the DNA extension (**Fig. 7.4A**; these length changes are below referred to as "steps"). Troubleshooting strategies in case too many or two few events are observed are outlined in **Notes 15–18**.

3.5. Analysis of Topoisomerase IB Time Traces

The time traces of topoisomerase activity (**Fig. 7.4A**) should be saved in an appropriate format, e.g., as ASCII text files. We further analyze the time traces offline to quantitatively dissect different aspects of TopIB activity. An analysis that has revealed important fundamental mechanistic aspects of topoisomerase enzymatic activity is to determine step size histograms, i.e. the distribution of the number of supercoils relaxed in individual steps (6, 7).

1. To analyze the step sizes, first identify all of the steps due to topoisomerase activity in the time traces of DNA extension (**Fig. 7.4A**). The steps can be identified using a sliding average window after low-pass filtering (6). Alternatively, use the algorithm developed by Kerssemakers et al. that detects steps in time traces without any a priori assumptions about the step size (26) (solid lines in **Fig. 7.4A**) (*see* **Note 19**).
2. Convert the changes in DNA extension determined from the steps in the time traces to changes in linking number per step, ΔL_k. The slope of the rotation curve, recorded at the appropriate force, in the linear plectonemic regime (**Fig. 7.3**, solid lines) gives the necessary conversion factor (in μm/turn).
3. The step size histograms for TopIB are well described by exponential distributions of the form

$$P\Delta(L_k) = exp\frac{-\Delta L_k}{\langle \Delta L_k \rangle} \quad [6]$$

where $\langle \Delta L_k \rangle$ is the mean number of supercoils removed per step. To obtain an unbiased estimate of the step size distribution and of $\langle \Delta L_k \rangle$, correct for the finite number of initial supercoils using the method of Koster et al. (24) (*see* **Note 20**).

4. By repeating the same analysis (steps 1–3) for time traces recorded at different stretching forces, establish the relationship between $\langle \Delta L_k \rangle$ and the stretching force. This provides further insights into the mechanism of topoisomerase activity (6, 7).

Time traces of topoisomerase activity can be analyzed in a number of other ways, to quantitatively determine additional parameters and to reveal various further aspects of their mechanism. If time traces with sufficient temporal resolution were recorded, individual steps can be analyzed to determine the velocity of supercoil removal (6). This kind of analysis has provided particular valuable insights into the effects of TopIB inhibitors of the camptothecin class of chemotherapeutics on TopIB function (8, 27). In addition, time traces can be analyzed to determine the time between DNA cleavage and religation. This analysis, too, has been particularly useful to study the action of camptothecin-type TopIB inhibitors. The details of these analyses depend on the particular scientific question under study and are detailed in the literature.

4. Notes

1. Commercial MT systems that supply complete systems have recently become available (www.picotwist.com).
2. We typically prepare batches of five to ten flow cells at once. The cells can be stored at 4°C for several weeks without noticeable degradation. Each cell can last for several days of magnetic tweezers measurements.
3. We use a 190-070 Microetcher II sandblaster (Great Lake Orthodontics, NY, USA) to drill the holes.
4. A single layer of parafilm could be used if a particularly thin flow cell with a reduced volume is desired.
5. Pay attention that (1) the flow cell is well sealed, (2) the parafilm does not close off the holes that connect to the inlet and outlet, and (3) the glass slides are well aligned. To

ensure a good seal, it is recommended to stroke out bubbles in the parafilm using a large cotton swab. Poorly aligned glass slides can cause breaking and leaking of the cell upon mounting in the microscope.

6. The flow cells can be stored at 4°C for several weeks without noticeable degradation.
7. Alternatively, a monolayer composed of PEG and biotinylated PEG has been successfully used to reduce unspecific interactions between the inner surface and the components of the system (21). In addition, our laboratory has used nitrocellulose-based passivation of the flow cell surface (22), which is characterized by the ease of its preparation as well as high density of nucleic acid tethers.
8. The DNA suspended in TE buffer can be stored at 4°C for several weeks without significant degradation.
9. The amount of DNA added in this step can be adjusted: If the yield of tethered beads in the flow cell is very low, the amount of DNA added in this step should be increased. If many beads are tethered through multiple DNA molecules, the amount of DNA should be reduced.
10. When handling DNA constructs avoid unnecessary pipetting, as this can result in breaking or nicking of the DNA, in particular for long DNA constructs. Instead, mix solutions by shaking or gentle vortexing.
11. The dilution factor and amount loaded into the flow cell in this step can be adjusted, depending on the amount of beads present in the flow cell after the final incubation.
12. The surface of the flow cell should be covered with beads at this stage.
13. At pulling forces of $\geq$1 pN, tethered beads can easily be distinguished from beads stuck to the surface of the bottom slide by their different heights in the focus.
14. A Matlab (The Mathworks, USA) routine for this purpose is available from the authors upon request.
15. If there are no or too few stepping events during the tracking of the bead, we recommended to periodically (every 20–40 min) check for sticking of the bead and DNA to the surface. This can be accomplished by quickly changing the stretching force to a higher value ($\approx$ 5–10 pN). The bead should assume its new equilibrium position (at larger distances from the flow cell surface) almost instantaneously. If this is not the case, the flow cell surface should be treated with (additional) poly-L-glutamic acid (*see* **Section 3.1**) or a new flow cell should be used. If the rate of stepping events is very low ($<$1 step per $\sim$5 min) and if sticking has been excluded as a possible cause,

fresh topoisomerase reaction buffer containing TopIB solution should be flushed into the cell. If the event rate remains very low, a higher concentration of TopIB can be employed.

16. If there are too many stepping events: If the stepping events are observed in very short succession or if the molecule becomes uncoilable after introduction of TopIB, the data are uninterpretable as several TopIB molecules likely act on the DNA at the same time. Such "bursts" of activity are sometimes observed after introducing fresh TopIB-containing buffer. If the behavior persists after waiting for ~5 min, TopIB can be removed from the flow cell by flushing with a high-salt buffer (e.g., 1 M NaCl) and the TopIB concentration in the topoisomerase reaction buffer should be reduced.
17. The stepping behavior can be monitored in the plectonemic regime, i.e., for the range of extensions where the relationship between turns and DNA extension is approximately linear (**Fig. 7.3**). Once TopIB activity has removed all plectonemic supercoils, the DNA molecule needs to be recoiled by rotating the magnets. Recoiling of the magnets can be done manually, by following the online trace, or automatically by programming a threshold for rotation into the tracking routine.
18. Even though topoisomerase activity can sometimes be observed even after overnight incubation in a flow cell, the stepping activity typically decreases over time and it is necessary to flush in fresh TopIB buffer every ~60 min.
19. A Matlab implementation of the algorithm of Kerssemakers et al. is available from the authors upon request.
20. In general, for the accurate determination of step size histograms, it is advantageous to use fairly long DNA constructs, such that a large number of initial supercoils ($\geq$100) can be introduced.

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