Published in final edited form as:

Methods Mol Biol. 2018 ; 1665: 297–316. doi:10.1007/978-1-4939-7271-5_16.

Combined magnetic tweezers and micro-mirror total internal reflection fluorescence microscope for single molecule manipulation and visualization

Yeonee Seol and Keir C. Neuman

Laboratory of Single Molecule Biophysics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

Abstract

Magnetic tweezers are a versatile yet simple single-molecule manipulation technique that has been used to study a broad range of nucleic acids and nucleic acid based molecular motors. In this chapter, we combine micro-mirror based total internal reflection microscopy with a magnetic tweezers instrument, permitting simultaneous single-molecule visualization and mechanical manipulation. We provide a simple method to calibrate the evanescent wave penetration depth via supercoiling of DNA with a fluorescent nanodiamond labeled magnetic bead and a complementary method employing a surface immobilized fluorescent nanodiamond.

Keywords

Magnetic tweezers; TIRF; image tracking; single-molecule enzymology

1. Introduction

Magnetic tweezers (MT) is a highly versatile and technically simple to implement single-molecule manipulation technique that provides control over force and torque on micron-sized magnetic particles [1–4]. Magnetic tweezers have been used to study the mechanical properties of nucleic acids and nucleic acid-protein interactions [5–10]. Moreover, the technique is well-suited for the study of enzymes that affect DNA topology such as topoisomerases since the topology of rotationally constrained DNA molecules can be precisely manipulated and measured [11,12].

In a typical MT assay, a biomolecule such as DNA is attached to a micron-scale magnetic bead at one end and the surface of a microscope flow cell at the other end via a biotin-streptavidin linkage, an antigen antibody linkage, or a covalent chemical linkage [13]. An external magnetic field, typically, but not exclusively, imposed by a pair of permanent magnets, applies force and torque on the magnetic particle. The position of the magnetic particle is tracked in real-time by camera-based particle-tracking analysis with typical acquisition rates of 30 – 500 Hz for CCD cameras and up to 10 kHz for CMOS cameras [14–16,1,3]. A conventional magnetic tweezers instrument is capable of applying forces from a few femtonewtons (10^{-15} N) to hundreds of piconewtons (10^{-12} N) dependent on the size of the magnetic particle and the magnet configuration [17]. Force is calibrated as function of the height of the magnet relative to the surface of the flow cell by tacking the

Brownian motion of a magnetic particle tethered by a long DNA (>10 kb) and applying either equipartition theorem or power spectrum analysis [18–20]. Camera-based magnetic tweezers tracking routines generally obtain the three-dimensional position of a magnetic particle with an accuracy of ~1 nm (10^{-9} m) or better at a sample rate of ~100 Hz [14] and up to 10^{-10} m at lower effective sampling rates [14,15]. As is the case for all single molecule manipulation techniques, MT can only probe biological activities that lead to a change in the position of a magnetic particle associated with a change in DNA extension caused, for example, by a change in tension or torsion applied to the DNA or by protein enzymatic or binding activity that alters the extension or mechanical properties of the DNA. However, for enzymes that bind or move along DNA without altering the mechanical properties of the DNA, these activities cannot be detected. This limitation can result in an incomplete or ambiguous understanding of DNA-enzyme interactions. Questions concerning the number of enzymes associated with activity, their binding affinity, their direction of movement on the DNA, and the relationship between binding and mechanical activity, among many others are challenging, if not impossible, to resolve solely with manipulation-based measurements. Recently, a hybrid technique [21–24] that combines MT with total internal reflection fluorescent microscopy (TIRF) was developed, which overcomes these limitations on understanding DNA-enzyme interactions by providing orthogonal information to the mechanical signal obtained with conventional magnetic tweezers.

TIRF is a frequently employed wide-field single-molecule fluorescent technique that generates a sub-wavelength localized excitation above the surface of the flow cell (evanescent wave) reducing the background noise and subsequently enabling single molecule fluorescent detection with a sensitive fluorescent detection camera. The physical principle of total internal reflection and the generation of an evanescent wave is well explained elsewhere [25,26]. Briefly, the evanescent wave is generated when the incident light is totally reflected at the interface between two media of different refractive indices, i.e., the interface between glass and water. The condition for total reflection is easily explained in terms of Snell's law at which the incident angle of light at the interface is larger than a critical angle, $\theta_c = \arcsin\left(\frac{n_2}{n_1}\right)$ where n_1 and n_2 are the refractive indices of the first and second medium respectively. The amplitude of the sine function cannot exceed 1, setting a condition for the total internal reflection to only occur when n_1 is larger than n_2 . The intensity of the evanescent wave decreases as $\sim \exp(-z/d)$ where z is the distance from the surface of the interface and d is a characteristic penetration depth expressed as:

$$d = \frac{\lambda}{2\pi} (n_1^2 \sin^2 \theta_1 - n_2^2)^{-\frac{1}{2}} \quad (1)$$

Here λ is the wavelength of the excitation light and θ_1 is the incident angle that should equal or exceed θ_c in order to achieve a total internal reflection. As shown in Eq. 1, the penetration depth, d , can be tuned to the optimal value for different applications. For Förster resonance energy transfer (FRET) measurements, the FRET signal provides a measure of the absolute distance between two dyes, thus the calibration of the evanescent wave is not strictly required as the short penetration depth serves primarily to reduce background noise.

On the other hand, a longer d may be useful for tracking out of plane (along the optical propagation, or z -, axis) motion, for example, to measure the motion of a fluorescently labeled DNA helicase moving along an extended DNA molecule, or to capture the full three-dimensional motion of a DNA-tethered particle [27]. The motion of the fluorescently labeled helicase can be deduced from the intensity change. However, in order to estimate the distance, it is necessary to calibrate the TIRF field intensity profile along the z -axis. In principle, the field profile can be estimated based on d calculated using eq. 1 by precisely measuring the incident angle [28]. Recently, the development of a hybrid MT - TIRF instrument [29,30] makes it feasible to directly obtain the TIRF field profile along the z -axis by measuring the auto-fluorescence intensity change of a magnetic bead as its height above the surface is decreased by supercoiling the DNA tether [31].

In this chapter, we provide a detailed description of how to incorporate micro-mirror based total internal reflection fluorescence microscopy (TIRF) into magnetic tweezers (Fig. 1). Instead of using a dichroic mirror, we utilize two micro mirrors to introduce the excitation laser into, and collect the reflected excitation light from, the objective lens (Fig. 1). This configuration simplifies multi-wavelength excitation and also reduces the effort and cost to separate the excitation light from the emission path [32,33]. Using the combined instrument, we employ DNA supercoiling as a way to calibrate the penetration depth of an evanescent wave similar to the method discussed in [31]. Previously, the autofluorescence of a magnetic bead was utilized to calibrate the penetration depth. In our method, we use biotinylated fluorescent nanodiamond (bio-FND) labelled magnetic beads that are tethered to DNA molecules since the auto-fluorescence of the magnetic beads is significantly reduced in our set-up (Excitation at 560 nm and emission >640 nm) (Fig. 2A). FNDs are fluorescent particles that are excited under broad range of excitation wavelengths ($\sim 500 - 600$ nm) and, importantly, are indefinitely optically stable [27,34]. The emission intensity of FNDs decrease in a magnetic field, but this effect saturates at $\sim 50\%$ for a applied field of ~ 500 Gauss or greater [34]. Thus, the FNDs are saturated for the majority of our force range (> 0.2 pN) and their fluorescent intensity remains constant. In order to set a desired d , we establish the relationship between d and the distance of the micro mirror (mm_1) to the center of the optical axis, r as in the micro mirror TIRF set-up, the incident angle, θ_1 is adjusted by changing r (Fig. 3A). We measure d for a range of r from subcritical to supercritical conditions by supercoiling the DNA to which the FND bound magnetic particle is attached as shown in Figs. 2 and 3. We also provide a complementary method to estimate d based on the intensity of surface immobilized fluorescent particles. The emission intensity from an immobilized fluorescent particle is expected to vary as $\exp(-z_{fixed}/d(r))$ for a particle at a fixed z position z_{fixed} . We show that the intensity of a fixed, non-bleaching, particle provides a complementary measurement of d as a function of r (Fig. 3B). To set d in situ, we characterize r vs d using a simple exponential function rather than using Eq. 1 (Fig. 3C). In addition, we characterize I_{fixed} vs d by fitting with $I_c \exp(-FND_f/d)$ using I_c and FND_f as free fitting parameters (Fig. 3C). Together these calibration routines provide a means to precisely define the intensity versus z -position relationship that affords quantitative fluorescent-based measurements of protein binding and translocation on DNA extended in the magnetic tweezers instrument.

2. Materials

2.1 PCR reaction for DNA substrate preparation

2.1.1 PCR templates

1. pET28b plasmid (EMD4Biosciences)
2. pBluescript II KS plasmid (Stratagene)

2.1.2 PCR primers

1. 5kb forward: 5' GCTGGGTCTCGCAAC-GAAACCCGACAGGACTATAAAG 3'
2. 5kb reverse: 5' GCTGGGTCTCGACCA-CTCTGATGCCGCATAGTTAAG 3'
3. Bio handle forward: 5' GCTGGGTCTCGGTTG-TTCCCTTTAGTGAGGGTTAATTC 3'
4. Dig handle forward: 5' GCTGGGTCTCGTGGT-TTCCCTTTAGTGAGGGTTAATTC 3'
5. Dig/Bio Handle Reverse primer: 5' TATAGTCCTGTCGGGTTTCG 3'

2.1.3 PCR enzymes, nucleotides and kits

1. Phusion High-Fidelity DNA polymerase (M0530, NEB) and supplied buffer.
2. *Taq* DNA polymerase (M0273, NEB) and supplied buffer
3. 100 µM Deoxynucleotide (dNTP) Solution
4. Biotin-16-dUTP (11093070910, Roche)
5. Digoxigenin-11-dUTP (11093088910, Roche)
6. DNA purification kit (QIAquick PCR purification kit, Qiagen)

2.1.4 Restriction enzyme digestion

1. BsaI-HF (R3535, NEB) and supplied buffer
2. Products from PCR reactions
3. DNA clean-up kit (11732668001, Roche)

2.1.5 DNA ligation

1. T4 DNA ligase (M1804, Promega) and supplied buffer
2. Products from restriction digestion reactions.

2.1.6 DNA tethering

1. Anti-digoxigenin, polyclonal (11333089001, Roche)
2. 1X Phosphate buffered saline (PBS)

3. Trizma base
4. NaCl
5. Products from DNA ligation reaction
6. Bovine serum albumin (BSA)
7. Tween-20
8. Magnetic beads (Dynabeads MyOne Streptavidin T1; 35601, Invitrogen)
9. Wash buffer (WB): 1x PBS, 0.1% w/v BSA, 0.01% v/v Tween-20
10. Bead wash buffer (BWB): 10 mM Tris (pH 7.5), 1 M NaCl

2.1.7 TIRF calibration—Biotinylated fluorescent nanodiamonds (*e.g.* Adamas Nanodiamonds)

2.1.8 General supplies

1. 200 μ l thin-wall PCR tubes
2. 1.5 ml Eppendorf tubes

2.2 Coverglass cleaning

1. #1½ cover glass (24 mm x 60 mm)
2. #1 cover glass (24 mm x 60 mm)
3. KOH pellets (3141, JT Baker)
4. 95% ethanol (3791, Macron)
5. Glass beaker, 1 L
6. Cover glass holder (custom made)
7. Sonicator

2.3 Sample cell assembly

1. Cleaned coverglasses
2. Nylon standoff (10SC008062; 10SC002037, McMaster Carr)
3. 58 μ m thickness adhesive transfer tape (9671LE, 3M)
4. CO₂ Laser engraver/cutter
5. Bead blaster cabinet
6. Epoxy
7. Vacuum grease
8. Acetone, HPLC grade
9. Ethanol, HPLC grade

10. 2 μ m Polystyrene beads (PS05N, Bangs Laboratories)

2.4 Magnetic tweezers and micro mirror TIRF components (Listed in Table 1)

3. Methods

3.1 Construction of Magnetic tweezers and micro mirror TIRF

The physical principle of magnetic tweezers and the details of the instrumentation have been extensively described in previously published chapters [1,17,3]. Here, we focus on updated information on the magnetic tweezers instrumentation and protocols. Briefly, the custom-built magnetic tweezers are comprised of two permanent magnets that are controlled by a linear translation motor and a rotary stepper motor. The hollow shaft of the stepper motor allows us to directly mount the magnet holder to the motor, which replaces the belt-driven systems used to control magnet rotation. This assembly is located above the sample cell centered along the z -axis of the illumination and imaging path. The sample cell is mounted on a custom-built cell holder that sits on a pair of stacked piezo-driven stages; one that provides nanometer scale position control in 3 dimensions, the other that provides large displacements in the x - y plane, which replaces a manual stage (Fig. 1B). The stages were mounted via four 1.5" posts to an optical bread board that was modified with additional mounting threads, a cut-out for the 95 mm structural rail and a 30 mm through-hole for the optical path. The magnetic tweezers instrument is assembled on a vibration isolation table and housed in a temperature controlled and vibration isolated clean room. The 95 mm structural rail supports the magnet and illumination assemblies (Fig. 1B). The illumination source is a 590 nm LED light that is weakly focused using four lenses (Fig. 1).

Our micro mirror TIRF (mmTIRF) system was assembled following the protocol of Larson et al [32] with a few modifications (Fig. 1). In order to incorporate the mmTIRF system, a custom objective lens mount was machined and attached to a vertical stage that provided manual coarse focus control. Directly underneath the back aperture of the objective lens, two micro mirrors are glued to two individual 3/32" square machine key stocks that are each fitted to a key shaft on a 3-D axis stage (Fig. 1B). Two stacked cage cubes below the objective are centered on the optical axis. The top cube holds a dichroic that reflects the fluorescent emission from the objective onto a EMCCD camera with an imaging lens after passing through an emission filter. The bottom cube holds a turning mirror to steer the illumination light from the objective to an imaging lens, which projects an image onto a CCD camera.

3.2 Making DNA substrate

3.2.1 PCR reaction for a 5kb DNA

1. Prepare 1x 1.5 ml Eppendorf tube and 6x 200 μ l PCR tubes on ice.
2. Add the following components to the 1.5 ml tube and mix well by gently rocking the tube 10 times.

Component	50 µl volume	300 µl volume	Final Concentration
Nuclease-free water	to 50 µl	to 300 µl	
5X Phusion HF Buffer	10 µl	60 µl	1X
100 µM Forward Primer	0.25 µl	1.5 µl	0.5 µM
100 µM Reverse Primer	0.25 µl	1.5 µl	0.5 µM
pET28-b	5 ng	30 ng	<5 ng/µl
10 mM dNTPs	1 µl	6 µl	200 µM
Phusion DNA Polymerase	0.5 µl	3 µl	1.0 units/50 µl PCR

- Transfer 50 µl of the reaction mixture to each PCR tube and run PCR reaction using the following condition.

STEP	TEMP	TIME
Initial Denaturation	98°C	30 s
30 Cycles	98°C	10 s
	66°C	15 s
	72°C	2 min 30 s
Final Extension	72°C	30 min
Hold	4°C	

- Check the size of PCR products on a 1% DNA agarose gel.
- After PCR reaction, purify DNA using PCR purification kit. Follow the provided protocol but at the final step before adding the elution buffer, do an additional 5 min spin-down to remove any residual wash buffer. Additionally, increase the incubation time to 15–30 min before collecting the DNA sample after adding the elution buffer.
- Quantify DNA product and its purity.

3.2.2 PCR reaction for bio or dig labeled DNA handles

- Prepare 2x 1.5 ml Eppendorf tubes and 12 x 200 µl PCR tubes on ice.
- Add the components to 1.5 ml tube, depending on the reaction volume and labeling type (one for biotin and the other for digoxigenin) and mix well by gently rocking the tubes 10 times:

Component	50 µl Reaction	300 µl Reaction	Final Concentration
Nuclease-free water	to 50 µl	to 300 µl	
10 X standard Taq Buffer	5 µl	30 µl	1X
100 µM Forward Primer (Either bio or dig)	0.25 µl	1.5 µl	0.5 µM

Component	50 µl Reaction	300 µl Reaction	Final Concentration
100 µM Reverse Primer	0.25 µl	1.5 µl	0.5 µM
pBluescript II KS	1 ng	6 ng	
low dTTP-dNTP mixture (10 mM of dATP, dCTP, dGTT and 7.0 mM of dTTP)	1 µl	6 µl	200 µM except for dTTP (140 µM)
Either Biotin-16-dUTP or Digoxigenin -11-dUTP [*]	3 µl	18 µl	60 µM
Taq DNA pol	0.5 µl	3 µl	1.0 units/50 µl PCR

^{*} Lower this amount if PCR is not working, particularly for digoxigenin.

- Transfer 50 µl of the reaction mixture to 6 individual PCR tubes and run the following PCR reaction.

STEP	TEMP	TIME
Initial Denaturation	95°C	30 s
30 Cycles	95°C	30 s
	60°C	30 s
	68°C	2 min
Final Extension	68°C	30 min
Hold	4°C	

- Check the size of PCR products on a 1% DNA agarose gel.
- After PCR reaction, purify DNA using PCR purification kit. Follow the provided protocol but at the final step before adding the elution buffer, do an additional 5 min spin-down to remove any residual wash buffer. Additionally, increase the incubation time to 15–30 min before collecting the DNA sample after adding the elution buffer.
- Quantify DNA product and calculate the molar concentration.

3.2.3 Restriction digestion

- Add the following components in a 200 µl PCR tube:

Component	50 µl Reaction
Nuclease-free water	to 50 µl
10x Buffer 4	5 µl
PCR product	<1 µg
BsaI-HF	2 µl

- Restriction digestion reaction

STEP	TEMP	TIME
Incubation	37°C	6 hrs
Add additional 2 µl BsaI-HF to the reaction sample	37°C	
Incubation	37°C	6 hrs
Stop reaction	65°C	20 min
Hold	4°C	

3. Purify the digestion product using purification kit (Roche). Follow the provided protocol but at the final step before adding the elution buffer, spin down the tube for 5 min to remove any residual wash buffer. Additionally, incubate the sample tube after adding the elution buffer at 65°C for 5 min before elution.
4. Quantify the purified DNA and calculate the molar concentration.

3.2.4 T4 DNA ligation

1. Add the following components in a 200 µl PCR tube:

Component	50 µl Reaction	Final concentration
Nuclease-free water	to 50 µl	
10x T4 DNA ligase Buffer	5 µl	1x
5 kb	~0.50 pmol	10 nM
Biotinylated DNA handle *	~0.50 pmol	10 nM
Digoxigenin DNA handle *	~0.50 pmol	10 nM
T4 DNA ligase	1 µl	1 unit

* Typical concentration.

2. T4 DNA ligation reaction

STEP	TEMP	TIME
Incubation	25°C	3 hrs
Incubation	16°C	15 hrs
Hold	4°C	

3. Run the ligated product on 1% DNA agarose gel to check the ligation efficiency. If the ligation efficiency is less than 80%, redo the restriction digestion and repeat ligation.

3.3 Preparing and assembling sample cells

3.3.1 Cover glass cleaning

1. Place cover glasses in a Teflon cover glass holder that holds the cover glass vertically.

2. Prepare enough KOH-ethanol solution (6 % w/v) in a 1 L glass beaker to submerge cover glass holder (~500 ml).
3. Sonicate the cover glass in the KOH solution for 30 min.
4. Remove the holder and place it in a 1 L glass beaker containing deionized water.
5. Sonicate for 30 min.
6. Repeat steps 4 and 5 twice.
7. Take out the holder and rinse each cover glass 2–3 times with deionized water to remove remaining KOH.
8. Squirt 100 % ethanol over each cover glass.
9. Dry the cover glass either by microwaving for three minutes or baking for 15 min at 150° C.
10. Store the cover glasses in an air-tight container.

3.3.2 Sample cell assembly

1. Assemble a sample cell by attaching a cleaned top glass (#1 coverglass with two inlet holes) to a bottom glasses (#1-1/2 coverglass) with double-sided tape with a central 40 mm x 4 mm channel (Fig. 4). The channel is cut in the double-sided adhesive using a CO₂ laser engraver/cutter. Two 2mm diameter through-holes are drilled on the top coverslip using a bead blaster and a soft plastic template.
2. Two different sizes of nylon screw inserts are attached at the centers of the individual holes for the buffer reservoir (larger one) and the outlet (small one) channels by spreading vacuum grease on the bottom surface and applying epoxy around the edge of the inserts.
3. Cure the epoxy for an hour.

3.3.3 Stuck bead generation (8 – 10 sample chambers)

1. Add 200 µl of deionized water in a 1.5 ml Eppendorf tube.
2. Add 2 µl of 2 µm polystyrene beads (4% w/v) to the tube and mix by quickly vortexing.
3. Sonicate the bead solution for 5 min.
4. Add 200 µl of Acetone to the bead solution and mix by quickly vortexing.
5. Introduce ~40 µl (slightly larger than a sample chamber volume) of the bead solution to each sample chamber immediately after step 4.
6. Incubate for 10 – 20 min. The distribution of stuck beads on the surface can be checked on a microscope.
7. Wash the sample cell with 200 µl of deionized water followed by 300 µl of 100% ethanol (HPLC grade).

8. Completely remove ethanol from chamber using a vacuum line.
9. Bake sample cells on a hot plate at 125 °C for 15 s.

3.4 Preparing a sample for TIRF calibration

1. Mix 3 μ l of anti-digoxigenin solution (0.2 mg/ml in 1X PBS), 1 μ l of 0.2 nM coilable DNA (Sec 3.1 above), 10 μ l of 5X PBS-0.5 mM EDTA, 1 μ l of bioFND for surface immobilization (0.5 mg/ml), and water up to 50 μ l.
2. Incubate for 15–20 min at room temperature.
3. Pipette 40 μ l into a sample chamber.
4. Incubate the sample chamber overnight at 4° C or 1 hour at RT.
5. Wash the sample chamber twice with 200 μ l of wash buffer and incubate for 10 min.
6. While waiting, pipette 2 μ l of a 0.1% w/v 1 μ m streptavidin magnetic particle suspension and put it in an Eppendorf tube.
7. Pull beads to the bottom of the tube by placing it on top of a permanent magnet.
8. Gently pipette out the supernatant.
9. Add 200 μ l of bead wash buffer (BWB) and vortex the tube quickly to mix.
10. Repeat steps 7, 8 and 9 three times.
11. After the final wash, instead of adding BWB, add 50 μ l of WB.
12. Introduce the bead solution into the flow-cell and wait for 30 min.
13. Remove unbound beads by washing 1 ml of WB through the sample chamber.
14. Add 5 μ l of 0.5 mg/ml bioFND in WB and incubate overnight.
15. Before use, remove unbound FND by washing 1 ml of WB through the sample chamber.

3.5 TIRF field calibration

1. Add 1 drop of oil immersion oil on top of the objective lens and place a prepared sample cell on the sample cell holder.
2. Adjust the objective lens focus on a surface immobilized FND based on the fluorescent image of FND.
3. Find a coilable DNA molecule and a surface immobilized FND in the same field of view.
4. Adjust the micro mirror position, r , (Fig 3A) to maximize the fluorescent intensity of the FND, which corresponds to an incident angle close to the critical angle for total internal reflection (Fig. 3B).
5. Set the force to 1 pN and perform supercoiling of DNA by rotating the magnet from 0 to 52 turns. Do the following measurements each turn:

- Measure the fluorescent intensity of FND that is immobilized on the surface, I_{fixed} .
 - Measure the magnetic bead position by video tracking [1,3] and the intensity of FND attached to the bead simultaneously (Fig. 2).
6. Repeat the measurements (step 4 and 5) for increasing r .
 7. Estimate d by fitting the fluorescent intensity as a function of z -position (Fig. 2B) for all r positions.
 8. Obtain the relationships between I_{fixed} and d as well as r and d by fitting an exponential function that will be used as a calibration to adjust r to obtain a desired d (Fig. 3C).

4. Notes

1. Use the ethanol and KOH from the listed vendors as we have found that KOH pellets do not dissolve well and/or the solution becomes cloudy if KOH and ethanol from different sources are used, which results in poor cleaning efficiency. Alternatively, if KOH or ethanol from other sources are used, the cleaning efficiency should be carefully evaluated.
2. KOH-ethanol solution can be reused at least 3–4 times.
3. The profile of $I_{fixed}(r)$ and subsequently the relationship between I_{fixed} and d depends on the size of the surface immobilized FND. When using this calibration to set d , make sure that the maximum intensity of the surface immobilized FND and the profile of $I_{fixed}(r)$ are similar to those of the FND used in the initial measurement of the relationship between I_{fixed} and d .

Acknowledgments

We thank Ganesh Shenoy, Haksung Jung, and Rolf Swenson for providing biotinylated fluorescent nanodiamonds. We thank Gary Melvin for machining. This research was supported by the Intramural Research Program of the National Heart Lung and Blood Institute, National Institutes of Health.

References

1. Lionnet T, Allemand J-F, Revyakin A, Strick TR, Saleh OA, Bensimon D, Croquette V. Single-Molecule Studies Using Magnetic Traps. *Cold Spring Harbor Protocols*. 2012; 2012(1) pdb.top067488. doi: 10.1101/pdb.top067488
2. Neuman KC, Nagy A. Single-molecule force spectroscopy: optical tweezers, magnetic tweezers and atomic force microscopy. *Nat Meth*. 2008; 5(6):491–505.
3. Seol, Y., Neuman, KC. Magnetic Tweezers for Single-Molecule Manipulation. In: Peterman, EJ., Wuite, G., editors. *Single Molecule Enzymology*, vol 783. *Methods in Molecular Biology*. Humana press; 2011. p. 265-293.
4. Vlamincx ID, Dekker C. Recent Advances in Magnetic Tweezers. *Annual Review of Biophysics*. 2012; 41(1):453–472. DOI: 10.1146/annurev-biophys-122311-100544
5. Fulconis R, Bancaud A, Allemand J-F, Croquette V, Dutreix M, Viovy J-L. Twisting and Untwisting a Single DNA Molecule Covered by RecA Protein. *Biophysical Journal*. 2004; 87(4):2552–2563. doi:<http://dx.doi.org/10.1529/biophysj.104.043059>. [PubMed: 15454450]

6. Kemmerich FE, Kasaciunaite K, Seidel R. Modular magnetic tweezers for single-molecule characterizations of helicases. *Methods*. 2016; 108:4–13. doi:<http://dx.doi.org/10.1016/j.ymeth.2016.07.004>. [PubMed: 27402355]
7. Koster DA, Crut A, Shuman S, Bjornsti M-A, Dekker NH. Cellular Strategies for Regulating DNA Supercoiling: A Single-Molecule Perspective. *Cell*. 2010; 142(4):519–530. doi:<http://dx.doi.org/10.1016/j.cell.2010.08.001>. [PubMed: 20723754]
8. Lionnet T, Dawid A, Bigot S, Barre F-X, Saleh OA, Heslot F, Allemand J-F, Bensimon D, Croquette V. DNA mechanics as a tool to probe helicase and translocase activity. *Nucleic Acids Research*. 2006; 34(15):4232–4244. DOI: 10.1093/nar/gkl451 [PubMed: 16935884]
9. Manosas M, Xi XG, Bensimon D, Croquette V. Active and passive mechanisms of helicases. *Nucleic Acids Research*. 2010; 38(16):5518–5526. DOI: 10.1093/nar/gkq273 [PubMed: 20423906]
10. Seol Y, Strub M-P, Neuman KC. Single molecule measurements of DNA helicase activity with magnetic tweezers and t-test based step-finding analysis. *Methods*. 2016; 105:119–127. doi:<http://dx.doi.org/10.1016/j.ymeth.2016.04.030>. [PubMed: 27131595]
11. Charvin G, Strick TR, Bensimon D, Croquette V. Tracking Topoisomerase Activity at the Single-Molecule Level. *Annual Review of Biophysics and Biomolecular Structure*. 2005; 34(1):201–219. DOI: 10.1146/annurev.biophys.34.040204.144433
12. Neuman KC. Single-molecule Measurements of DNA Topology and Topoisomerases. *Journal of Biological Chemistry*. 2010; 285(25):18967–18971. DOI: 10.1074/jbc.R109.092437 [PubMed: 20382732]
13. Seol, Y., Neuman, K. Single-Molecule Measurements of Topoisomerase Activity with Magnetic Tweezers. In: Mashanov, GI., Batters, C., editors. *Single Molecule Enzymology*, vol 778. *Methods in Molecular Biology*. Humana Press; 2011. p. 229–241.
14. Dulin D, Cui TJ, Cnossen J, Docter Margreet W, Lipfert J, Dekker Nynke H. High Spatiotemporal Resolution Magnetic Tweezers: Calibration and Applications for DNA Dynamics. *Biophysical Journal*. 2015; 109(10):2113–2125. doi:<http://dx.doi.org/10.1016/j.bpj.2015.10.018>. [PubMed: 26588570]
15. Huhle A, Klaue D, Brutzer H, Daldrop P, Joo S, Otto O, Keyser UF, Seidel R. Camera-based three-dimensional real-time particle tracking at kHz rates and Ångström accuracy. *Nature Communications*. 2015; 6:5885. <http://www.nature.com/articles/ncomms6885#supplementary-information>. doi: 10.1038/ncomms6885
16. Lansdorp BM, Tabrizi SJ, Dittmore A, Saleh OA. A high-speed magnetic tweezer beyond 10,000 frames per second. *Review of Scientific Instruments*. 2013; 84(4):044301. doi:<http://dx.doi.org/10.1063/1.4802678>. [PubMed: 23635212]
17. Lipfert J, Hao X, Dekker NH. Quantitative Modeling and Optimization of Magnetic Tweezers. *Biophysical Journal*. 2009; 96(12):5040–5049. doi:<http://dx.doi.org/10.1016/j.bpj.2009.03.055>. [PubMed: 19527664]
18. te Velthuis AJW, Kerssemakers JWJ, Lipfert J, Dekker NH. Quantitative Guidelines for Force Calibration through Spectral Analysis of Magnetic Tweezers Data. *Biophysical Journal*. 2010; 99(4):1292–1302. doi:<http://dx.doi.org/10.1016/j.bpj.2010.06.008>. [PubMed: 20713015]
19. Yu Z, Dulin D, Cnossen J, Köber M, van Oene MM, Ordu O, Berghuis BA, Hensgens T, Lipfert J, Dekker NH. A force calibration standard for magnetic tweezers. *Review of Scientific Instruments*. 2014; 85(12):123114. doi:<http://dx.doi.org/10.1063/1.4904148>. [PubMed: 25554279]
20. Lansdorp BM, Saleh OA. Power spectrum and Allan variance methods for calibrating single-molecule video-tracking instruments. *Review of Scientific Instruments*. 2012; 83(2):025115. doi:<http://dx.doi.org/10.1063/1.3687431>. [PubMed: 22380133]
21. Guo Q, He Y, Lu HP. Manipulating and probing enzymatic conformational fluctuations and enzyme-substrate interactions by single-molecule FRET-magnetic tweezers microscopy. *Physical Chemistry Chemical Physics*. 2014; 16(26):13052–13058. DOI: 10.1039/C4CP01454E [PubMed: 24853252]
22. Kemmerich FE, Swoboda M, Kauert DJ, Grieb MS, Hahn S, Schwarz FW, Seidel R, Schlierf M. Simultaneous Single-Molecule Force and Fluorescence Sampling of DNA Nanostructure Conformations Using Magnetic Tweezers. *Nano Letters*. 2015; doi: 10.1021/acs.nanolett.5b03956

23. Lebel P, Basu A, Oberstrass FC, Tretter EM, Bryant Z. Gold rotor bead tracking for high-speed measurements of DNA twist, torque and extension. *Nat Meth.* 2014; 11(4):456–462. <http://www.nature.com/nmeth/journal/v11/n4/abs/nmeth.2854.html#supplementary-information>. DOI: 10.1038/nmeth.2854
24. Swoboda, M., Grieb, M., Hahn, S., Schlierf, M. Measuring Two at the Same Time: Combining Magnetic Tweezers with Single-Molecule FRET. In: Toseland, CP., Fili, N., editors. *Fluorescent Methods for Molecular Motors*, vol 105. *Experientia Supplementum*. Springer; Basel: 2014. p. 253–276.
25. Axelrod D. Evanescent Excitation and Emission in Fluorescence Microscopy. *Biophysical Journal*. 2013; 104(7):1401–1409. DOI: 10.1016/j.bpj.2013.02.044 [PubMed: 23561516]
26. Martin-Fernandez ML, Tynan CJ, Webb SED. A ‘pocket guide’ to total internal reflection fluorescence. *Journal of Microscopy*. 2013; 252(1):16–22. DOI: 10.1111/jmi.12070 [PubMed: 23889125]
27. Bumb A, Sarkar SK, Billington N, Brechbiel MW, Neuman KC. Silica Encapsulation of Fluorescent Nanodiamonds for Colloidal Stability and Facile Surface Functionalization. *Journal of the American Chemical Society*. 2013; 135(21):7815–7818. DOI: 10.1021/ja4016815 [PubMed: 23581827]
28. Burghardt TP. Measuring incidence angle for through-the-objective total internal reflection fluorescence microscopy. *BIOMEDO*. 2012; 17(12):126007–126007. DOI: 10.1117/1.JBO.17.12.126007
29. Long X, Parks JW, Stone MD. Integrated magnetic tweezers and single-molecule FRET for investigating the mechanical properties of nucleic acid. *Methods*. 2016; 105:16–25. doi:<http://dx.doi.org/10.1016/j.ymeth.2016.06.009>. [PubMed: 27320203]
30. Swoboda, M., Grieb, MS., Hahn, S., Schlierf, M. Measuring Two at the Same Time: Combining Magnetic Tweezers with Single-Molecule FRET. In: Toseland, CP., Fili, N., editors. *Fluorescent Methods for Molecular Motors*. Springer; Basel, Basel: 2014. p. 253–276.
31. Duboc C, Graves ET, Strick TR. Simple calibration of TIR field depth using the supercoiling response of DNA. *Methods*. 2016; 105:56–61. doi:<http://dx.doi.org/10.1016/j.ymeth.2016.03.028>. [PubMed: 27038746]
32. Larson J, Kirk M, Drier EA, O’Brien W, MacKay JF, Friedman LJ, Hoskins AA. Design and construction of a multiwavelength, micromirror total internal reflectance fluorescence microscope. *Nat Protocols*. 2014; 9(10):2317–2328. DOI: 10.1038/nprot.2014.155 [PubMed: 25188633]
33. Friedman LJ, Gelles J. Multi-wavelength single-molecule fluorescence analysis of transcription mechanisms. *Methods*. 2015; 86:27–36. doi:<http://dx.doi.org/10.1016/j.ymeth.2015.05.026>. [PubMed: 26032816]
34. Sarkar SK, Bumb A, Wu X, Sochacki KA, Kellman P, Brechbiel MW, Neuman KC. Wide-field in vivo background free imaging by selective magnetic modulation of nanodiamond fluorescence. *Biomedical Optics Express*. 2014; 5(4):1190–1202. DOI: 10.1364/BOE.5.001190 [PubMed: 24761300]

Figure 1.

A) Schematic of magnetic tweezers combined with micro-mirror total internal reflection fluorescence microscopy. The bright-field imaging path is indicated by a solid yellow line. The 590 nm light emitting diode (LED) is collimated and weakly focused by multiple lenses (L5, L6, L7, and L8) onto the sample via a turning mirror (M0.5). The sample is imaged on to a CCD with a 60X oil immersion objective lens (OL) and a 150 mm achromatic lens (L9). The illumination light passes through a 590 nm bandpass filter (F4). The excitation light path is indicated by a solid green line. 560 nm excitation is obtained by passing a supercontinuum laser source through a heat absorbing colored glass filter (F1) to remove the IR region and a 560 nm bandpass filter (F2). The laser beam is expanded 10-fold via two lenses (L1 and L2) with a pinhole placed at the focus of L1 to spatially filter the excitation light. The excitation beam is re-focused and directed into the objective lens via a lens (L3) and multiple mirrors (two M1 and mm₁). The totally internally reflected light emerging from the objective is directed by mm₂ to a beam block. The emission light is reflected by a dichroic mirror (DC) and imaged onto an EMCCD via OL and a 400 mm focal length lens

(L4). The emission light is filtered with a 640 nm long-pass filter (F3). **B)** Picture of magnetic tweezers combined with micro-mirror TIRF. The micro mirrors (highlighted with red dashed circle) located under the objective back aperture are shown in the inset.

Figure 2.

Calibrating the evanescent wave penetration depth by simultaneously measuring FND emission intensity and the z-position of DNA tethered magnetic particle as a function of DNA supercoiling. **A)** Rotation of magnetic bead by turning the magnet assembly results in overwinding (positive turn) or underwinding of DNA (negative turn). At 1 pN, underwinding of DNA results in no significant change in DNA extension due to melting of DNA duplex (inset left) whereas overwinding of DNA leads to reduction of DNA extension because of plectoneme formation after the buckling transition (inset right). As DNA extension (left axis) decreases, the magnetic bead labeled with a FND approaches the surface and the fluorescence emission intensity increases (right axis, FND). **B)** FND emission intensity is plotted as a function of DNA extension and fitted with an exponential to estimate the evanescent field penetration depth, d .

Figure 3.

A) A simple description of the relationship between the incident angle, θ_1 and the micro-mirror lateral displacement r . The lateral movement of the micro-mirror under the objective lens (r) results in a change of the incidence angle, $\theta_1 = \text{asin}(r/n_1 f)$ at the interface between glass and the medium (buffer). Here f is the effective focal length of the objective lens and n_1 is the refractive index of glass. r_c indicates the critical displacement at which total internal reflection occurs. **B)** d and the intensity of FND immobilized on surface, I_{fixed} , are plotted as a function of r . The characteristic penetration depth, d , and I_{fixed} decrease as r increases beyond r_c (dashed vertical line). I_{fixed} is normalized by its maximum intensity. The calculated d from eq. 1 (blue solid line) is overlaid with the experimentally measured d . **C)** r and I_{fixed} are plotted as a function of d and individually fitted with exponential functions.

Figure 4.

Sample cell assembly. A typical sample cell is composed of a #1-1/2 coverslip as a bottom surface and a #1 coverslip with two holes separated by 40 mm as a top surface. The two coverslips are attached by double-sided adhesive film with a rectangular slot that results in a sample channel in the assembled flow cell. Two different sized nylon standoffs are attached to the centers of the two holes. The larger one is used as a buffer reservoir and the smaller one is used as an outlet to remove buffer from the sample channel.

Table 1

List of components for magnetic tweezers and micro-mirror TIRF instrument

Item	Description	Company	Catalogue number
Excitation components			
Laser	Super continuum laser	NKT Photonics	ETW-12
F1	Infra-red filter	Newport	FSR-KG2
F2	560 nm bandpass filter	Semrock	FF01-560/14-25
L1	Objective lens, f = 16.5 mm	Newport	M-10x
L2	Collimating lens, f = 150 mm, AR=14	Newport	PAC058AR.14
L3	Focusing lens, f = 250 mm, AR=14	Newport	PAC067AR.14
mm	45° Rod mirror	Edmunds Optics	54-092
M1	Silver mirror, Ø=1"	Thorlabs	PF10-03-P01
OL	Objective lens, 60X, N.A.=1.45, Oil	Olympus	PLAPON60XOTIRFM
Emission components			
DC	Dichroic filter, Ø=2"	Semrock	FF611-SDi01-50.8D
F3 *	642 nm long-pass filter	Semrock	FF01-642/LP
L4	Imaging lens, f = 400 mm, AR=14	Newport	PAC090AR.14
EMCCD	Electron Multiplying Charge Coupled Device Camera	Andor	iXon+DU-879E
Magnetic tweezers illumination and imaging components			
F4	590 nm long-pass filter	Newport	FSR-OG590LP
LED	Mounted light emitting diode (LED), Amber, 1000 mA, 160 mW	Thorlabs	M590L3
L5	Aspheric lens, f = 3.1 mm	Thorlabs	C330TMD -A
L6	Aspheric Condenser Lens, f = 20.1 mm	Thorlabs	ACL2520U-A
L7	Collimating lens, f = 80 mm	Thorlabs	
L8	Focusing lens, f = 250 mm	Thorlabs	
L9	Imaging lens, f = 150 mm	Newport	PAC086AR.14
MS	Mounted silver mirror	Thorlabs	CM1-P01
M0.5	Silver mirror, Ø=0.5"	Thorlabs	PF05-03-P01
M2	Silver mirror, Ø=2"	Thorlabs	PF20-03-P01
CCD	Charge Coupled Device camera	Prosilica	GE-680
Magnetic tweezers and microscope components			
Structural rail	Mount illumination and linear/rotary motors	Thorlabs	XT95-50
Sample cell platform	Aluminum breadboard, 12" x 18" x 1/2", ¼-20 Threaded		MB1218
Mounting Posts and post bases	6" height mounting posts and bases for supporting a sample cell platform 1.5" height posts for mounting PILine stage		P6; PB2; P1.5
Cage cube system	Build 3-D optical path		LC6W LB1C, LB5C1, CPVM

Item	Description	Company	Catalogue number
Rail carrier for illumination assembly	Illumination assembly height control		XT95P12
Rail carrier for magnet assembly	Mount for Linear and rotary stages to the structural rail	Newport	CXL95-120
Objective lens coarse focus stage	Micrometer Z-Stage	Edmunds Optics	66-495
Optical table	Vibration isolation table (29" x 35" x 4")	Technical Manufacturing Corporation	Micro-g Laboratory table 63-533
Micro mirror control			
Micro mirror mount and extension	3/32" square machine key stock	Grainger	5UU68
	Key shaft	McMaster	1570K21
Shaft holder	Plate Holder	Thorlabs	FP01
3-D position control of mms	3 axis stage	Thorlabs	DT12XYZ
1-D position control of mm ₁	linear stage	New Focus	9062-X
	a nanopositioning actuator and controller		8301NF 8751-CL
Custom parts			
Objective lens holder		Custom machined	NA
Flow cell holder			
Magnet holder			
Magnets	Rectangular magnets N52; NdFeBr; Ni-plated; 5x8x3 mm	Pacific PAC	NA
Stages, computer and software			
Computer	Computer for hardware control and data analysis	HP	HP workstation Z820
Stages	PILine® Piezo Motor XY open frame stage system	Physik Instrumente	M-686.XYPM C-867
	PIMars™ XYZ piezo nanopositioning/ scanning Stage and controller		P-561.3CD/E-710
	Linear motor for the magnet assembly and controller	Newport	LTA-HS/ESP300
	Stepper motor for the magnet assembly	Lin Engineering	ZH-417
Software	NI developer suite	National Instruments	Labview and NI vision

* Discontinued. Alternative choice: BLP01-635R-25 (Semrock)