Chapter 19

Repurposing a Benchtop Centrifuge for High-Throughput Single-Molecule Force Spectroscopy

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Abstract

We present high-throughput single-molecule manipulation using a benchtop centrifuge, overcoming limitations common in other single-molecule approaches such as high cost, low throughput, technical difficulty, and strict infrastructure requirements. An inexpensive and compact Centrifuge Force Microscope (CFM) adapted to a commercial centrifuge enables use by nonspecialists, and integration with DNA nanoswitches facilitates both reliable measurements and repeated molecular interrogation. Here, we provide detailed protocols for constructing the CFM, creating DNA nanoswitch samples, and carrying out single-molecule force measurements.

Key words Centrifuge Force Microscope, Single-Molecule Force Spectroscopy, DNA Nanoswitches

1 Introduction

Single-molecule force studies have led to significant insights in biology, including the regulatory role of mechanical force in systems ranging from bleeding to development [1–6]. While instruments such as optical tweezers, magnetic tweezers, and atomic force microscopes (AFM) have matured and gained acceptance as important scientific tools [2, 4], in general, force spectroscopy has found limited adoption among nonspecialists. The high cost of equipment and the required infrastructure (e.g. temperature-regulated, vibration-isolated rooms) is a significant barrier, as is the expertise required to build and operate most single-molecule instruments. Limited throughput poses another important challenge, which is starting to be addressed with multiplexed approaches [7–14]. Furthermore, instrumentation is only part of the equation limiting widespread adoption—the challenge of preparing and analyzing reliable single-molecule assays is an equal challenge, one which is exacerbated by the large volumes of data that result from parallel approaches. To enable the widespread use

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of single-molecule manipulation throughout biomedical and chemical research, all of these challenges must be met.

In 2010, we presented a proof-of-concept Centrifuge Force Microscope (CFM), and demonstrated the ability to carry out thousands of independent single-molecule force spectroscopy experiments in parallel by video-tracking tethered particles subject to centrifugal forces [15]. More recently, we introduced a benchtop CFM that builds upon an instrument that almost all biomedical researchers already have and use: the benchtop centrifuge [16]. This miniature microscope that fits into a standard centrifuge bucket was created to bring high-throughput single-molecule manipulation to nonspecialists. Furthermore, to solve significant issues of sample preparation and data analysis, we integrated modular nanoscale mechanical switches into this next-generation CFM. These nanoswitches serve as tethers that provide a distinct molecular signature, facilitating reliable and automated analysis of the resulting large data sets [16, 17]. In addition, these tethers enable the repeated interrogation of single receptor-ligand pairs, to both increase the throughput of measurements and to reveal heterogeneity at the single-molecule level.

In order to facilitate the adoption of these two techniques, we present detailed protocols here for both the construction of the benchtop CFM and the preparation, integration, and use of DNA nanoswitch samples for performing high-throughput force spectroscopy. We note that while these two technologies work well in tandem, readers interested solely in incorporating DNA nanoswitches into other assays, or in building a CFM for other purposes, will also find the protocols presented here invaluable.

2 Materials

The main component of the Centrifuge Force Microscope (CFM) is a compact optical microscope with a video acquisition system that can be integrated within a benchtop centrifuge. We are using a Thermo Fisher Scientific Heraeus X1R upgraded with computer control module provided by Thermo Fisher Scientific R&D, but any sufficiently-sized commercial centrifuge should be suitable. The microscope design is based on the Thorlabs lens tube system with a few custom parts. This first part of the protocol describes the construction of the optical microscope and the centrifuge modification required for real-time control and image acquisition through a fiber rotary joint. We note that if high-bandwidth, real-time data collection is not needed, video data could be stored locally or streamed via wireless networking [18]. This section concludes with a list of the materials and reagents needed to create the sample chamber and DNA nanoswitch assay.

Table 1
Centrifuge Force Microscope instrumentation part list

Item no.	Vendor	Part number	Description	QTY.
1	Thorlabs	S1LEDM	SM1-Threaded Mount for LED	1
2	Thorlabs	SM1T1	SM1 (1.035″-40) Coupler	1
3	Thorlabs	SM05RR	Retaining Ring for diffuser	1
4	Thorlabs	DG05-220	Light Source Diffuser, Ø1/2″ N-BK7 Ground Glass	1
5	Thorlabs	SM1A6T	Diffuser and sample cell mount	1
6	SI Howard Glass Co	B-270	Ø 25 mm, 0.9 mm Thick	1
7	Kapton Tape	PPTDE-3	Sample Cell Assembly	1
8	VWR	63782-01	Gold Seal, #1 19 mm coverglass	1
9	Thorlabs	SM1L03	Sample Holder, SM1 Lens Tube, 0.3″ Thread Depth	1
10	Thorlabs	SM1V05	Focusing Ø1” SM1 Lens Tube	1
11	Edmund Optics	#86-815	40× Olympus Plan Achromat Objective, 0.65 NA, 0.6 mm WD	1
12	Thorlabs	SM1A3	Objective Adaptor with External SM1 Threads and Internal RMS Threads	1
13	Thorlabs	AC254-100	Tube Len, f = 100.0 mm, Ø1″ Achromatic Doublet, ARC: 400-700 nm	1
14	Thorlabs	SM1RR	Tube Lense SM1 Retaining Ring	1
15	Thorlabs	SM1M20	Objective SM1 Lens Tube Without External Threads, 2″ Long	1
16	Thorlabs	SM1A6T	Adaptor with External SM1 Threads and Internal SM05 Threads, 0.40″ Thick	2
17	–	–	Custom made turning block, aluminum	1
18	Thorlabs	PFE10-P01	Turning Mirror, 1″ Silver Elliptical Mirror, 450 nm to 20 μm	2
19	Thorlabs	SM1NT	Camera SM1 (1.035″-40) Locking Ring, Ø1.25″ Outer Diameter	1
20	Thorlabs	SM1A9	Camera Adaptor with External C-Mount Threads and Internal SM1 Threads	1
21	Allied Vision Technologies	Prosilica GC2450	Sony ICX625 CCD sensor, 2448 × 2050 resolution, 15 fps, 12 bit	1
22	IMC Network	855-10734	MiniMc-Gigabit Twisted Pair to Fiber Media Converter	1
23	PrinceTel	MJX	Fiber Optic Rotary Joint	1

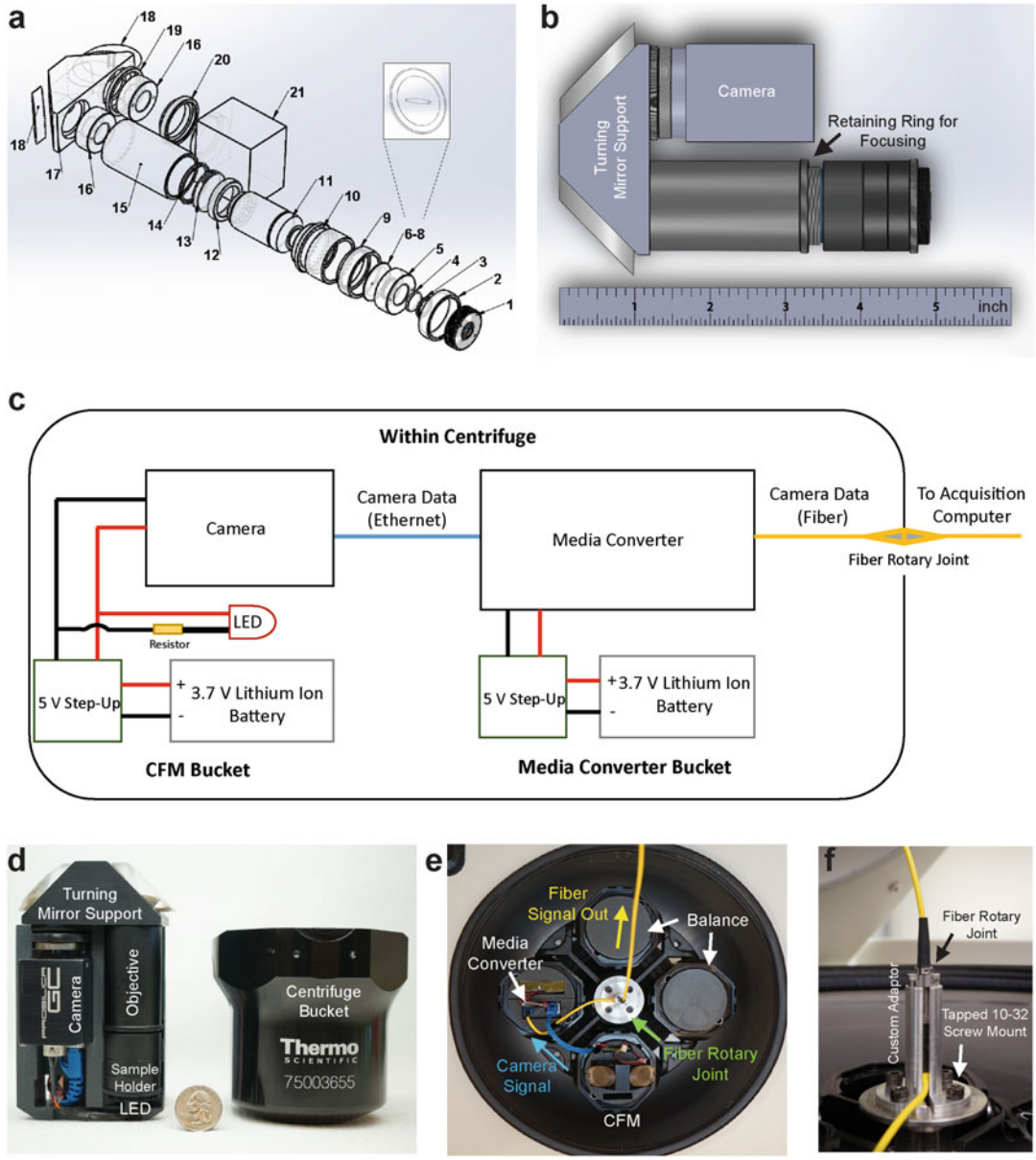

Fig. 1 Design and assembly of the benchtop CFM. (**a**) Exploded view of the CFM. The numbers correspond to the Item No. in Table 1. (**b**) Assembled model of the CFM. (**c**) Electrical and data wiring diagram. (**d**) The CFM module in part of the 3D printed adaptor next to the TX-400 400 mL centrifuge bucket. The LED, sample holder, and objective are housed within the lens tubes that are mounted to the turning mirror support. (**e**) Photograph of the benchtop CFM module inside of a centrifuge with batteries and wires connected. (**f**) Details of the fiber rotary joint mounted to a custom adaptor then onto the benchtop centrifuge. Panels (**a**), (**b**), (**d**), (**e**), and (**f**) adapted from [16]

2.1 Centrifuge Force Microscope

1. Obtain microscope components listed in Table 1. The custom-fabricated turning mirror support (Item No. 17) can be obtained from a machine shop. Use epoxy glue to secure the

mirrors (Item No. 18) to the turning mirror support. A technical drawing of the turning mirror support can be downloaded from https://goo.gl/bClf9J.

2. Assemble the centrifuge force microscope following the mechanical assembly diagram (Fig. 1a, b). This compact microscope is based on the Thorlabs SM1 threaded lens tube system. Turning the lens tubes clockwise or counter-clockwise for tightening and loosening, respectively. Mount a red LED (Thorlabs, LED630E) to the LED mount (Item No. 1 in Table 1).
3. Solder pin #1 and #2 of a 12-pin Hirose connector (HIROSE, HR10A-10J-12S) to the ground and power of a DC-to-DC step-up circuit board (SparkFun, PRT-08290), respectively. In parallel to this circuit, solder a LED socket (Thorlabs, 8060-2) with a 68 Ω resistor on the power wire. The Hirose connector and the LED socket are used to power the camera and illuminating LED, respectively, once a lithium ion battery (SparkFun, PRT-0034) is connected to the input of the step-up circuit (Fig. 1c).
4. Solder the power input of an Ethernet-to-Fiber gigabit media converter (IMC Networks, 855-10734) to another DC-to-DC step-up circuit (SparkFun, PRT-08290) (Fig. 1c).
5. Use a 3D-printer to fabricate the enclosure made of acrylonitrile butadiene styrene (ABS) to secure and integrate the imaging and acquisition system inside the centrifuge bucket (Thermo Fisher Scientific, 75003655) (Fig. 1d). Print the enclosure to secure the Ethernet-to-Fiber gigabit media converter in another bucket (Fig. 1e). Include data and power cables passages and battery housing in the enclosure design. Place the media converter containing bucket adjacent to the CFM containing bucket. A technical drawing of the enclosure can be downloaded from https://goo.gl/bClf9J.
6. Use a 6-in. Ethernet cable to connect the camera to the media converter across the buckets (Fig. 1c, e).

2.2 Centrifuge Modification

1. Tap the four existing holes surrounding the center quick release button of the centrifuge rotor (Thermo Fisher Scientific, TX-400 4 × 400 mL Swinging Bucket Rotor, 75003181) to accept 10–32 screws (Fig. 1f). Custom fabricate fiber rotary joint adaptor mount and attach to the center of the centrifuge rotor utilizing the four threaded holes (Fig. 1f).
2. Remove the center viewing window on the centrifuge cover to allow optical data fiber pass through the centrifuge lid.
3. Attach the fiber optic rotary joint (PrinceTel, MJX-131-28-SC) to the custom adaptor mount (Fig. 1f). Connect the fiber cable

connector inside the centrifuge to the media converter (Fig. 1c, e). Connect the other end of the fiber optic cable connector to the computer through another media converter that converts the data signal into Ethernet connector.

4. Use counterweight to balance the mass and the center-of-mass of the counter centrifuge buckets. One option is to use metal coins stacked in a fitted 3D–printed enclosure (Fig. 1e).

2.3 DNA Nanoswitches

1. M13mp18 Single-stranded DNA, M13 ssDNA (New England Biolabs, N4040S).
2. Nuclease-free water: UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, 10977015).
3. 10× NEBuffer 2 (New England Biolabs, B7002S).
4. Synthetics oligonucleotide:
(Sequence: 5′-CTACTAATAGTAGTAGCATTAACATCCAA-TAAATCATACA-3′).

   BtsCI restriction enzyme (New England Biolabs, R0647S).

2.4 Sample Chamber Assembly

1. Coverslips: Gold Seal 19 mm circle #1 (VWR, 100502-528).
2. Cleaning solution: Hellmanex III (Sigma-Aldrich, Z805939-1EA) in Milli-Q water to a final concentration of 1% (v/v).
3. Nitrocellulose coating solution: nitrocellulose membranes paper (Bio-Rad Laboratories, 1620150) in amyl acetate to a final concentration of 0.2% (w/v).
4. Support cover glass: custom 25 mm diameter, 1 mm thick circular (S.I. Howard Glass, B-270).
5. Double-sided Tape: double-sided polyimide Kapton tape (Kapton Tape, PPTDE-3).

2.5 Sample Chamber Surface Functionalization and Beads Tethering

1. Anti-digoxigenin (Roche Diagnostics, 11333089001).
2. Phosphate buffered saline, PBS, pH 7.4 (Thermo Fisher Scientific, 10010023).
3. Blocking reagent (Roche Diagnostics, 11096176001).
4. Centrifuge tube top filter (Millipore, SCGP00525).
5. Streptavidin Dynabeads, M-270 (Thermo Fisher Scientific, 65305).
6. Silicone grease (Sigma-Aldrich, Z273554-1EA).
7. Blunt needle tip (Instech, LS22).

3 Methods

The first part of this section describes the construction of DNA Nanoswitches, self-assembled mechanical switches that provide a well-defined molecular signature to identify the force-induced molecular rupture of a ligand-receptor pair [16]. Specifically, ligand and receptor molecules of interest are attached to specific locations of a linear DNA scaffold, with binding between the ligand and receptor resulting in an internally looped structure with a shorter overall tethering length than the linear scaffold. The well-defined change in tethering length from the looped to the linear structure (or vice versa) provides the quantitative signature of a molecular rupture (or binding) event. Moreover, the DNA Nanoswitch scaffold allows repeated measurement of the same molecular pair, which enables characterization of population heterogeneity. Subsequent subsections describe chamber assembly, surface preparation and bead tethering, and data collection and analysis.

3.1 Construction of DNA Nanoswitch

1. Linearize circular M13 ssDNA to make the DNA Nanoswitch scaffold. Mix 10 μL of 100 nM circular M13 ssDNA with a 38 μL mixture consisting of 32 μL of nuclease-free water, 5 μL of 10× NEBuffer 2, and 1 μL of 100 μM synthetic oligonucleotide complementary to the BtsCI restriction site.
2. Use a thermal cycler to bring the above mixture to 95 °C, hold for 30 s and then cool to 50 °C. Add 1 μL of the BtsCI enzyme and mix thoroughly. Incubate the mixture further at 50 °C for 1 h and then bring the temperature up to 95 °C for 1 min to heat deactivate the enzyme. This will result in 50 μL of 20 nM linearized DNA Nanoswitch scaffold in NEB Buffer 2. Make 5 μL aliquots of the linearized M13 and store at −20 °C till needed.
3. Based on the sequence map of M13mp18, obtain a set of 120 60-nt oligonucleotides and one 49-nt oligonucleotide that are complementary to the linearized M13 DNA scaffold. Among those, select the first and last 60-nt regions at the ends of the scaffold to be the tethering handle attachments and two additional specific regions that are at least 3000 nt apart to attach the ligand and receptor molecules (*see* **Note 1**). A more detailed protocol is described previously in the supplemental protocol of ref. 19.
4. Leave out the handle attachments, ligand, and receptor oligonucleotides. Create a complementary tiling oligonucleotide mixture consisting of the remaining 117 oligonucleotides, at equal molar concentration and total oligonucleotide concentration of 100 μM. Store the mixture at −20 °C till needed. A more detailed protocol is described previously in the supplemental protocol of ref. 19.

5. Create a handle attachment oligonucleotide mixture by mixing 1 μL of 100 μM of digoxigenin- and biotin-functionalized tethering oligonucleotides and diluting to 100 μL with nuclease-free water. Store the mixture at −20 °C till needed.
6. Mix 5 μL of 20 nM linearized DNA Nanoswitch scaffold (from **step 2**) with 1.17 μL of the complementary tiling oligonucleotide mixture (from **step 4**) and 0.5 μL of the handle attachment oligonucleotide mixture (from **step 5**). Use a thermal cycler to heat the mixture to 90 °C for 1 min then cool the sample 1 °C/min until it reaches 20 °C. This will result in 6.67 μL of ~15 nM biotin and digoxigenin-functionalized M13 scaffold with two single-stranded regions for ligand and receptor attachment. The mixture can be stored at 4 °C for up to 2 weeks or −20 °C for long term storage.
7. Covalently attach the molecules of interest to the ligand and receptor oligonucleotides using chemical attachment strategies such as SMCC, click chemistry, EDC, sortase-based conjugation [20] etc., or utilizing commercially available oligonucleotide conjugation kits (*see* **Note 1**).
8. Hybridize ligand- and receptor-coupled oligonucleotides at a 1.25 M excess to the M13 scaffold at a final M13 concentration of 250 pM in the experimental buffer at room temperature for 1–2 h (*see* **Note 2**).

3.2 Sample Chamber Assembly

1. Submerge coverslips loaded in a coverslip rack in cleaning solution. Use the microwave oven to heat up the solution to a gentle boil, then use a bath sonicator to sonicate for 30 min. Rinse the coverslips thoroughly with Milli-Q water then dry with nitrogen gas flow.
2. Coat one side of the cleaned and dried coverslips with 1 μL of nitrocellulose coating solution using the side of a pipette tip. Bake the nitrocellulose-coated coverslips in an 80 °C oven for 5 min.
3. Drill two 0.75 mm diameter holes, 10 mm apart, on support cover glass for flow chamber inlet and outlet ports (*see* **Note 3**).
4. Cut a rectangle (1×10 mm^2) of flow cell pattern with rounded edges centered in a 25 mm diameter circle on a double-sided tape using cutting plotter (*see* **Note 4**).
5. Sandwich the tape between the nitrocellulose-coated coverslip and support cover glass with the fluid ports aligned to the edges of the flow channel. Use a soft plastic pipette tip or cotton swap to seal the tape. Be gentle pressing over the flow channel region which can be easily cracked. The assembled flow chamber can be stored under vacuum at room temperature for up to a month.

3.3 Sample Chamber Surface Functionalization and Beads Tethering

1. Dissolve 200 μg of anti-digoxigenin antibody with 1 mL of PBS to the final concentration (0.2 mg/mL). Centrifuge the reconstituted anti-digoxigenin at 13,000 × *g* for 5 min. Make 100 μL aliquots of the supernatant and store at 4 °C for up to 6 months.
2. Add 500 mg of blocking reagent to 50 mL of PBS to make the blocking solution. Autoclave (121 °C, 20 min) the mixture to dissolve the blocking reagent. Use a centrifuge tube top filter to remove the undissolved particles. Add sodium azide (0.02%, w/v) to prevent bacterial growth. Make 1.5 mL aliquot and store at −20 °C for up to 1 year. Use a fresh aliquot for each experiment.
3. Incubate the flow channel with anti-digoxigenin by adding in 20 μL of anti-digoxigenin solutions (0.1 mg/mL in PBS) for 30 min. Wash the channel with 20 μL of PBS two times. All incubation steps are done at room temperature under humidified conditions, i.e. in a glass petri dish with wet Kimwipes. Avoid pipetting air-bubble into the flow channel. Throughout the protocol, use Kimwipes to soak up the fluid from the outlet.
4. Incubate the flow channel with blocking solution for 1 h by flowing in 20 μL of blocking solution every 20 min.
5. Wash the flow channel with 20 μL of the experimental buffer two times after the blocking step. If the channel contains air bubbles flush the channel with 30–60 μL of experimental buffer (*see* **Note 5**).
6. Incubate the flow channel with 100 pM of functionalized DNA Nanoswitch construct for 30 min. Wash the channel with 20 μL of experimental buffer two times after the DNA Nanoswitch attachment (*see* **Note 6**).
7. Prepare 5 μL of Streptavidin Dynabeads per sample. First wash the beads with 50 μL of blocking solution, vortex for 10 s, and use a magnetic sample rack to exchange solution. Carry out this step twice. Then wash the beads with 50 μL experimental buffer five times. Resuspend the beads with 5 μL of experimental buffer per sample (*see* **Note 7**).
8. Pipette ~1–2 μL of the washed beads into the flow channel. Stop the pipetting flow immediately when the channel is filled with beads. Seal the flow inlet and outlet by injecting silicone grease using a syringe with a blunt needle tip (*see* **Note 8**).
9. Let the beads contacting the DNA-functionalized glass surface for 1 min. If the sample is to be measured in more than 5 min, flip and orient the flow chamber such that the beads are being pulled from the DNA-functionalized glass surface by gravity force. The nontethered beads will be pulled away from the surface as well (*see* **Note 9**).

3.4 Data Collection and Analysis

Here we describe how to carry out experiments using the CFM instrumentation and DNA Nanoswitch samples, and how to analyze the resulting microscopy videos to identify and validate force-induced molecular rupture events by using the molecular signature afforded by the Nanoswitch constructs.

3.4.1 Sample Mounting and CFM Operation

1. Mount the sealed sample chamber (shown as Item No. 6–8 in Table 1 and *see* Fig. 1a) in the sample holder (Item No. 5 and 9 in Table 1 and *see* Fig. 1a). Attach the LED illumination assembly (Item No. 1–3 in Table 1 and *see* Fig. 1a) to the sample holder.
2. Mount the sample holder with the LED illumination assembly to the focusing tube (Item No. 10 in Table 1 and *see* Fig. 1a).
3. Attach the LED socket to the LED. Connect the battery to the step-up circuit that powers the camera and LED. Connect another battery to the media converter in the adjacent bucket.
4. With the LED and camera powered and the signal connected to the computer through the fiber-optic rotary joint, use the focusing tube (Item No. 10 in Table 1 and *see* Fig. 1a) to bring the tethered beads to focus. Live images can be acquired using the VIMBA software (Allied Vision) or customized LabVIEW software (National Instrument). Use the retaining ring (Fig. 1b) to secure the focus.
5. Place the CFM within the 3D printed adaptor and carefully place it in the centrifuge bucket.
6. Spin the rotor by hand to make sure centrifuge spinning is not obstructed by electrical wiring and no sign of imbalance. Close the centrifuge lid.
7. If your centrifuge includes the option for computer control, use the software provided by the centrifuge manufacturer to set the desired acceleration and rotational speed profile. Our Heraeus X1R centrifuge can be controlled using WinMass (Thermo Fisher Scientific), which also records the measured speed of rotation. If computer control is not available, simply control the operation of the centrifuge manually using the standard front-panel interface. Record the camera-acquired images during centrifugation using software provided by the camera manufacturer or customized LabVIEW (National Instrument) software.

3.4.2 Data Analysis of DNA Nanoswitch Rupture

With this particular CFM design based on the TX-400 rotor, the angle of the centrifuge swing bucket does not swing up to the full 90° with respect to the axis of rotation, resulting in a component of the centrifugal force directed in the x–y image plane (Fig. 2a). Furthermore, the off-radial orientation of the CFM will also result in a force component in the x–y plane (Fig. 2d). As the rotational

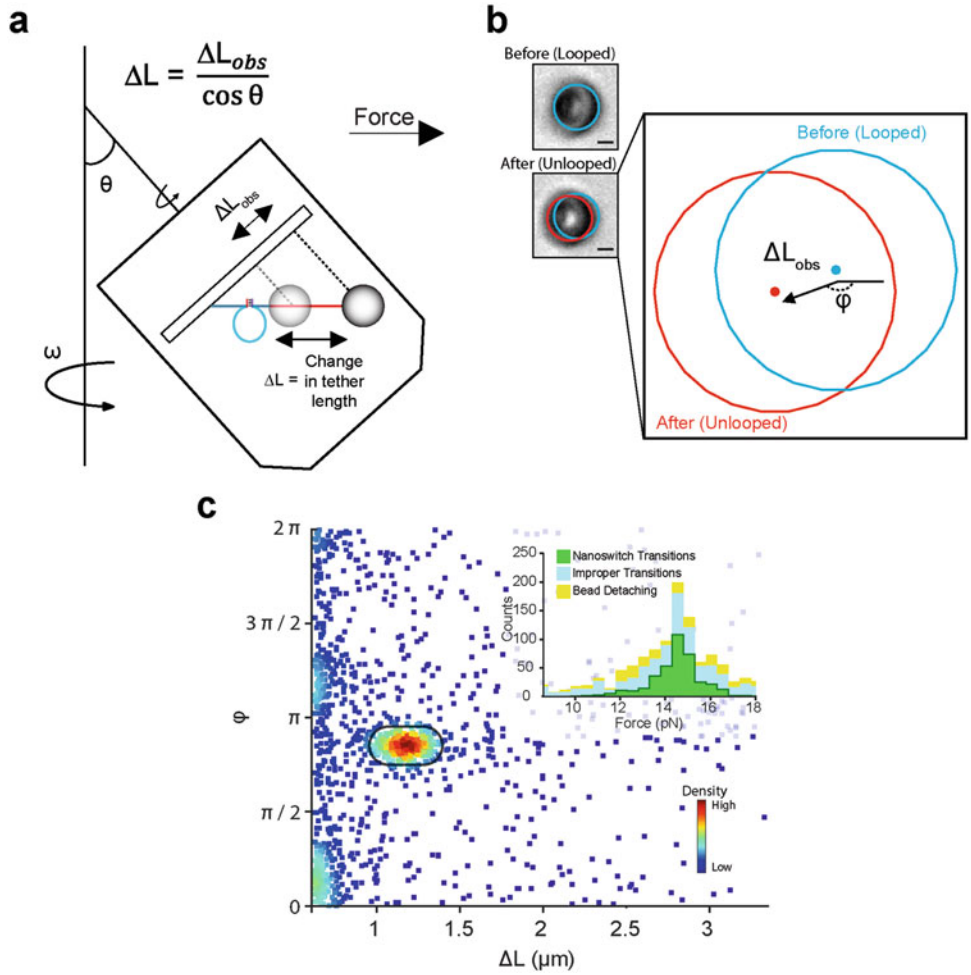

Fig. 2 Analysis of DNA Nanoswitch unlooping. (**a**, **b**) When the centrifuge bucket is at angle θ, the centrifugal force has components in both perpendicular and parallel directions to the sample surface (*x–y* plane). When the bond is ruptured, the DNA Nanoswitch goes from looped (*blue*) to unlooped (*red*) that leads to a change in length ΔL. This is identified by measuring the projected change in length in the *x–y* plane $\Delta L_{obs} = \Delta L/\cos\theta$. The orientation of the miniCFM in the bucket also sets the direction of ΔL_{obs}. (**c**) A scatter plot of all contour length changes detected for all directions. The transitions within the boxed region are accepted as nanoswitch transitions. A histogram of transition forces for three different types of transitions: beads which leave the surface (*yellow*), beads which display discontinuous transition with (*green*) and without (*light-blue*) correct direction and magnitude is shown as insert. Figure adapted from [16]

speed of the CFM increases, the looped DNA tethers will be continuously extended. The opening of the loop is identified as a discontinuous change in the extension (Fig. 2b).

1. Use the function "imfindcircles" in Matlab (Mathworks) to identify the beads.
2. Store a template image for each bead. To identify the bead position in the subsequent frame, use the template image to

scan in the *x*–*y* plane to find the position of maximum correlation.

3. Take the median position change in *x* and *y* for all beads being tracked from frame to frame as the reference to correct drift in the *x*–*y* plane during the course of the experiment. This drift correction is sufficient for identifying the DNA Nanoswitch looped-to-unlooped transition.
4. Identify the DNA Nanoswitch transition by filtering out all bead trajectories except those that contain a discontinuous change in extension with both the correct magnitude and direction (Fig. 2c).
5. Convert the frame at which the DNA Nanoswitch transition occurs into time or force for subsequent data analysis.

4 Notes

1. Ligand and receptor molecules localized on the DNA scaffold should be at least 3000 nt apart so that an internal loop around 1 μm in length is made when the ligand binds to the receptor. This will provide a very clear signature for identifying bond rupture events.
2. The efficiency of the hybridization process of the ligand- and receptor-coupled oligonucleotides to the M13 scaffold can be improved by incubation at a higher temperature that melts the internal secondary structure of the oligonucleotides. If the ligand or receptor molecule is sensitive to the elevated temperature, the oligonucleotide and its complementary target region should be free of internal secondary structure so that the hybridization can be carried out at room temperature or below.
3. Mechanical drilling of the inlet and outlet ports using a drill press and diamond-coated bit can often crack the glass or produce uneven cylindrical port. One solution is to bind the circular support glass to a 1 mm thick microscope slide (VWR, 16005-106) with a thin layer of melted natural rosin (D'Addario, VR200) using a heat plate. The drilling should also be carried out slowly with a layer of water between the drill bit and glass. After the holes are drilled, the support glass can be removed from the microscope slide by soaking in acetone solution.
4. We use a Graphtech cutting plotter, model CE5000, to cut out a flow channel on the double-sided Kapton tape. The cutting can also be done using smaller desktop size cutting plotter or by hand. Secure the double-sided Kapton tape using other tapes on

a cutting mat (VWR, 14222-832) with one adhesive side facing up during cutting.

5. To avoid pipetting air-bubbles into the flow channel, use the reverse pipetting technique. Load an excess amount of solution into the pipette tip by press the knob to the second stop to load. Dispense the solution into the flow channel by pressing the pipette knob only to the first stop. Discard the tip containing the excess solution.
6. Control experiments should be carried out to verify the surface passivation and tethering efficiency. Negative controls such as sample preparation without anti-digoxigenin surface activation, DNA Nanoswitch incubation, biotin- and digoxigenin-DNA functionalization, etc., should be performed to ensure that the beads are tethered through specific interaction and not nonspecifically sticking. To limit multiple tethers on each bead, either utilize the DNA as the limited component to ensure that the spacing of each immobilized DNA is larger than the bead size, or perform a DNA concentration series to measure the concentration range in which the number of tethered beads is linearly dependent.
7. Adjust the resuspension volume if necessary to achieve 60–70% bead surface coverage during the bead tethering step.
8. Avoid creating air-bubbles at the fluid–grease interface. Make sure the fluid ports are filled with solution and create a small dab of vacuum grease at the needle tip prior to injecting the grease into the channel.
9. The sample can also be stored at 4 °C overnight with the tethered beads hanging down under gravitational force to avoid beads from nonspecifically sticking to the surface. Avoid any strong magnetic fields and mechanical impacts on the sample.

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