Chapter 18

Probing Chromatin Structure with Magnetic Tweezers

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Abstract

Magnetic tweezers form a unique tool to study the topology and mechanical properties of chromatin fibers. Chromatin is a complex of DNA and proteins that folds the DNA in such a way that meter-long stretches of DNA fit into the micron-sized cell nucleus. Moreover, it regulates accessibility of the genome to the cellular replication, transcription, and repair machinery. However, the structure and mechanisms that govern chromatin folding remain poorly understood, despite recent spectacular improvements in high-resolution imaging techniques. Single-molecule force spectroscopy techniques can directly measure both the extension of individual chromatin fragments with nanometer accuracy and the forces involved in the (un)folding of single chromatin fibers. Here, we report detailed methods that allow one to successfully prepare in vitro reconstituted chromatin fibers for use in magnetic tweezers-based force spectroscopy. The higher-order structure of different chromatin fibers can be inferred from fitting a statistical mechanics model to the force-extension data. These methods for quantifying chromatin folding can be extended to study many other processes involving chromatin, such as the epigenetic regulation of transcription.

Key words Magnetic tweezers, Force spectroscopy, Single molecule, Chromatin, Nucleosome

1 Introduction

Chromatin is a DNA-protein complex that organizes and compacts DNA in the nucleus of eukaryotic cells. The basic unit of a chromatin is a nucleosome, which consists of 147 base pairs of DNA turning 1.65 times around a histone protein octamer. The histone octamer is composed of a $(H3\text{-}H4)_2$ histone tetramer and two H2A-H2B histone dimers [1, 2]. Nucleosomes interact with each other via histone tails and form higher-order chromatin structures [3–5].

Eukaryotic DNA is subject to many processes such as replication and transcription. To regulate these processes, the structure and composition of chromatin fibers must be very dynamic [6]. Previous studies have shown how the nucleosome positioning, post-translational histone modifications, and chromatin remodelers can

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alter the stability of nucleosomes and may modulate the structure of chromatin [7–10]. Using X-ray crystallography [11], electron microscopy [12–15], or atomic force microscopy [16], researchers have established detailed structures of the nucleosome and small arrays of nucleosomes. However, as the structure of chromatin fibers becomes more complex and disordered as their size increases, these techniques have their limits in contributing to an examination of higher-order chromatin structure. Moreover, they do not typically address the dynamical rearrangements of chromatin fibers that are essential for the genomic transactions occurring in eukaryotic nucleus. In contrast, single-molecule force spectroscopy techniques are capable of tracking and manipulating the extension of chromatin fibers in real time and under physiological conditions, providing unique means to study their structure, assembly, and disassembly.

Magnetic tweezers represent an especially powerful tool for the study of chromatin folding, as they permit the straightforward application of both tension and torsion [17, 18] as well as multiplexing [19, 20]. Precise control of the force combined with nanometer accuracy measurements of the extension of individual fibers will help to elucidate the molecular mechanisms that control chromatin condensation and the regulation of processes involving chromatin.

In our assay, chromatin fibers are tethered between a glass slide and a paramagnetic bead. As the distance between a pair of magnets and the beads reduces, the beads experience an increasing force, which is directly applied to the tethered fibers. In this methods chapter, we describe how a DNA substrate containing an array of nucleosome positioning sequences is prepared (Subheading 3.1) and functionalized for magnetic tweezers experiments (Subheading 3.2). This involves labelling of one end of the DNA with digoxigenin and the other end with biotin. Subsequently, the DNA substrate is loaded with histone proteins resulting in well-defined reconstituted chromatin fibers (Subheading 3.3). Though protein chaperones (i.e., NAP-1) can load histones on DNA into arrays of nucleosomes [21], we employ salt-dialysis reconstitution in which positively charged histones assemble onto the negatively charged DNA as the salt concentration is slowly decreased [22]. To ensure a well-defined composition and spacing of the nucleosomes in the chromatin fiber, we use a DNA template with tandem repeats of Widom-601 positioning sequences [23]. The quality of chromatin assembly is assessed using agarose gel electrophoresis (Subheading 3.4) which shows different mobility of incomplete and overcomplete chromatin fibers compared to stoichiometric assemblies of chromatin fibers.

We use a homebuilt magnetic tweezers microscope and custom flow cells to immobilize and manipulate individual chromatin fibers. The flow cell design constitutes an aluminum holder that fixes two cover slides and PDMS (polydimethylsiloxane) layer that seals the channel between the two slides. The holder has two

channels for a buffer exchange and a large aperture for illumination of the sample (Subheading 3.5). After functionalizing the chamber (Subheading 3.6), chromatin fibers are injected into a flow cell and tethered between a glass slide and a magnetic bead. The chromatin fiber is manipulated by changing the force on the beads by moving the magnets (Subheading 3.7). The resulting changes in tether end-to-end distance are measured by a bead-tracking algorithm and are subsequently analyzed with a statistical mechanics model (Subheading 3.8). Following these procedures yields a detailed characterization of the mechanical properties of single-folded chromatin fibers.

2 Materials

2.1 DNA Cloning

1. Ampicillin-resistant pUC18 plasmid with tandem repeats of the Widom-601 sequence (*see* **Note 1**).
2. XL1-blue *E. coli* competent cells stored at −80 °C (*see* **Note 2**).
3. ThermoMixer® C Eppendorf™ 5424R Microcentrifuge (Eppendorf™).
4. Shaker KS 130 (IKA KS).
5. Luria-Bertani (LB) medium and LB agar plate.
6. NucleoBond® Xtra Midi kit (Macherey-Nagel).

2.2 DNA Digestion and Labelling

1. Restriction enzymes: BseYI, BsaI (New England Biolabs).
2. NEBuffer 3.1 (with bovine serum albumin) –10× concentrated (New England Biolabs).
3. Promega Wizard SV Gel and PCR cleanup kit (Promega).
4. BioDrop μLITE spectrophotometer (Isogen Life Science).
5. 1× Tris/Borate/EDTA buffer (TBE).
6. dNTPs (100 mM) (Roche).
7. GeneRuler DNA Ladder Mix (Thermo Fisher Scientific).
8. Klenow Fragment (2 U/μL), LC (Thermo Fisher Scientific).
9. Reaction buffer for Klenow Fragment (10×) (Thermo Fisher Scientific).
10. Digoxigenin-11-dUTP (1 mM) (Roche).
11. Biotin-16-dUTP (1 mM) (Roche).
12. UV transilluminator ChemiDoc™ XRS+ (Bio-Rad).

2.3 Chromatin Reconstitution

1. Slide-A-Lyzer mini dialysis tubes, 10,000 MWCO (Thermo Scientific).
2. Low-binding pipette tips (VWR).
3. Magnetic stirrer with a heat plate (VWR).

4. Glass beaker (1 L).
5. Econo gradient pump (Bio-Rad).
6. Histone Octamer, Recombinant Human (stored at −20 °C) (EpiCypher).
7. 1.5 L of miliQ H_2O (4 °C).
8. 50× Tris/EDTA (TE), pH 7.5.
9. 5 M NaCl.
10. High salt buffer (80 mL of 5 M NaCl +4 mL of 50× Tris/EDTA (TE) + miliQ H_2O up to 200 mL).
11. Low salt buffer (980 mL of miliQ H_2O + 20 mL of 50× Tris/EDTA (TE)).

2.4 Electrophoretic Band Shift Assay

1. Agarose Standard (Roth).
2. PowerPac™ Basic Power Supply (Bio-Rad).
3. KuroGEL Midi 13 Electrophoresis Horizontal (VWR).
4. GeneRuler DNA Ladder Mix (Thermo Fisher Scientific).
5. 5× Tris/boric acid (TB).
6. Loading buffer (20% glycerol, 20 mM Tris, 1 mM EDTA, bromophenol blue).
7. Ethidium bromide (10,000×) (Thermo Fisher Scientific).

2.5 Assembly of the Flow Cell Chamber

1. Aluminum flow cell frame, custom-built (Fig. 1).
2. Perspex mold, custom-built (Fig. 1).
3. Cover slip 24 × 40 mm, # 1.5 thickness (Menzel Gläser).
4. Cover slip 24 × 60 mm, # 1.5 thickness (Menzel Gläser).
5. Sylgard® 184 silicone elastomer kit PDMS (Sylgard).
6. FEP tubing (1/16″ OD × 0.020″ ID) (Upchurch Scientific).
7. Electrical wire (~0.02″ OD) (Nexans).
8. Embossing tape (Dymo).
9. N_2 Spray Gun Assembly (NCI).
10. Vacuum controller CVC 3000 and Diaphragm pump MZ 2 NT (Vacuubrand).
11. M4 screws.
12. Ultrasonic cleaning bath USC-TH (VWR).
13. Microscope slide (75 × 26 × 1 mm) (Menzel Gläser).
14. Glass beakers (100 mL + 250 mL).
15. Cover slip holder (custom-made).
16. 2-Propanol.

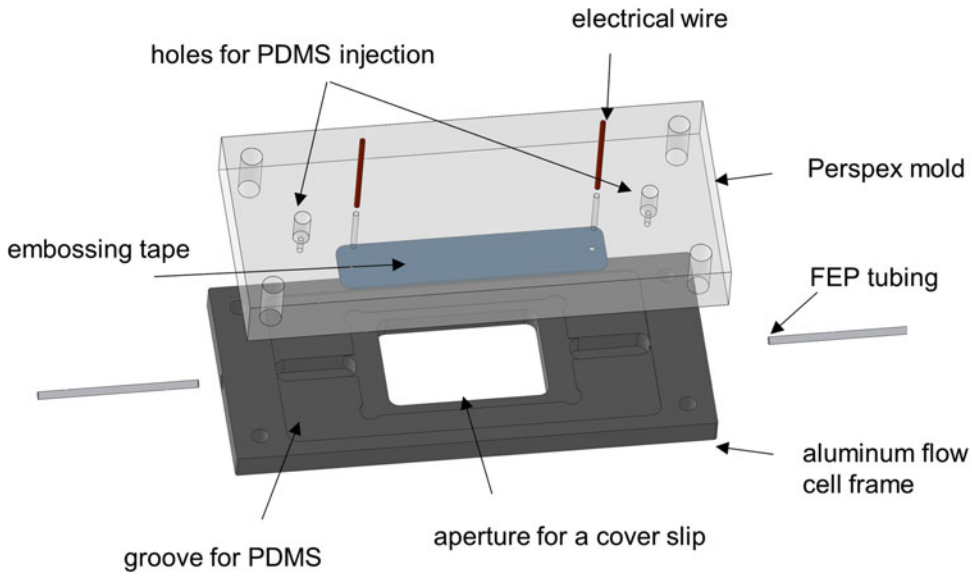


Fig. 1 Components of the flow cell chamber. A custom-built Perspex mold is mounted onto a custom-built aluminum flow cell frame that holds a 24 × 40 mm cover slip. Subsequently, PDMS is injected into the chamber between the mold and the groove of the metal frame

2.6 Flow Cell Functionalization

1. Dynabeads™ M-270 Streptavidin or MyOne Streptavidin (Invitrogen).
2. Microscope slide (75 × 26 × 1 mm).
3. Pentyl acetate, puriss. p.a., ≥98.5% (GC) (Sigma-Aldrich).
4. Nitrocellulose – 1% in pentyl acetate (Ladd Research Industries).
5. Anti-digoxigenin – from sheep (Sigma-Aldrich).
6. Bovine serum albumin (BSA) heat shock fraction, pH 7, ≥98% (Sigma-Aldrich).
7. Dynal® Invitrogen Bead Separations (Invitrogen).
8. Tween-20 (Sigma-Aldrich).
9. Sodium azide (NaN_3) (Merck).
10. 1 M HEPES, pH 7.5.
11. 2 M KCl.
12. 20 mM $MgCl_2$.
13. 1× Tris/EDTA (TE).
14. Plastipak 1 mL syringe (BD).
15. Sterican 18G single-use needle (B. Braun).
16. Tygon tubing (0.8 mm ID/0.8 mm wall).

2.7 Hardware

1. 25 Mpix Condor camera (CMOS Vision GmbH).
2. Frame grabber Camera Link PCIe-1433 (National Instruments).
3. NIKON CFI Plan Fluor objective (NA = 1.3, 40×, oil, Nikon Corporation).
4. Infinity-corrected tube lens ITL200 (Thorlabs).
5. 100 μW, 20 mA LED collimator emitting at 645 nm (IMM Photonics GmbH).
6. Computer T7610 (Dell) with a 10 core Intel Xeon 2.8 GHz processor (Intel) and 32GB DDR3 memory.
7. Multi-axis piezo scanner P-517.3CL (Physik Instrumente GmbH & Co.).
8. 5 mm cube magnets N50 (Supermagnete, Webcraft GmbH).
9. Two-phase hollow shaft stepper motor (Casun).
10. M-126.2S1 Translation Stage for a magnet (Two Physik).
11. M-126.2S1 Translation Stage for objective (Two Physik).
12. Syringe pump (Prosense B. V.).

3 Methods

3.1 DNA Cloning

1. *Cell transformation with pUC18 plasmid*. Thaw about 100 μL of XL1-blue competent cells on ice and add ~1 μL of a plasmid. Mix gently and incubate on ice for at least 30 min. Induce a 42 °C heat shock of about 90–120 s with the ThermoMixer. Place the sample back on ice for 1 min and add 900 μL of LB medium. Shake gently (240 rpm) and incubate for 30–60 min at 37 °C. Centrifuge the sample for 1 min at 14,000 rpm (18,500 × *g*), remove the supernatant, and then resuspend the sample in 100 μL LB medium. Spread the sample on an LB agar plate with ampicillin, and incubate upside-down at 37 °C overnight.
2. *Cell culture*. Select a single colony and inoculate in 500 mL flask with 250 mL LB medium with ampicillin. Incubate overnight at 37 °C while shaking (320 rpm). Next day, centrifuge the culture (14,000 rpm [18,500 × *g*] at 4 °C for 10 min). Discard the supernatant and keep the cell pellet.
3. *Plasmid DNA isolation*. Isolate and purify the plasmid DNA using NucleoBond® Xtra Midi kit using the manufacturer's protocol.

3.2 DNA Digestion and Labelling

1. *Plasmid digestion with BsaI and BseYI*. Linearize the plasmid DNA with two restriction enzymes in a buffer that contains maximally 50 ng/μL of the substrate in 1× NEBuffer 3.1 (*see*

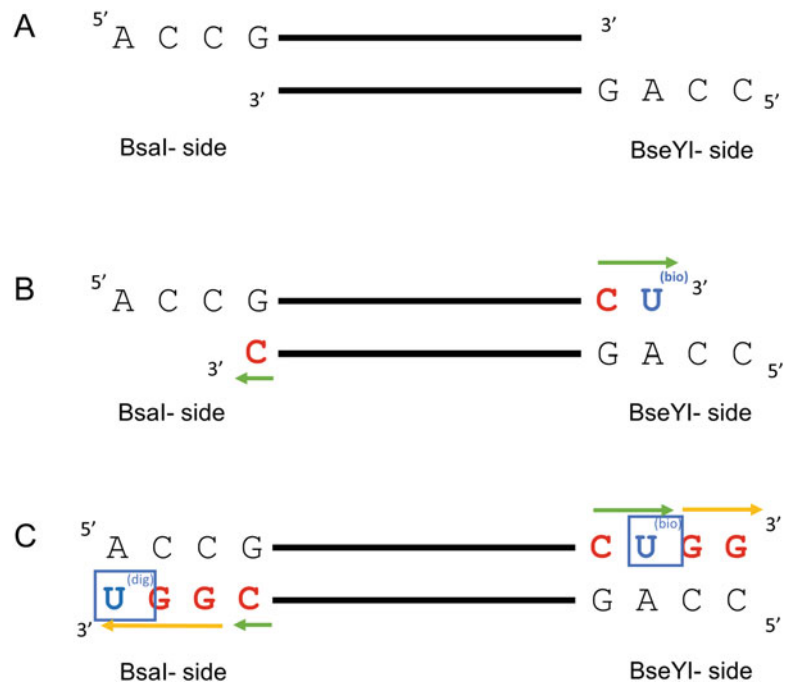


Fig. 2 Labelling of the DNA with a single biotin and digoxigenin tag. (**a**) The pUC18 plasmid digested with BsaI and BseYI has single-stranded overhangs at both sides. (**b**) A Klenow reaction with biotin-dUTP and free nucleotides other than dGTP (here dCTP) results in incorporation of a biotin only at the BseYI side. (**c**) Subsequently, the DNA is purified and a second Klenow reaction with dGTP and digoxigenin-dUTP results in binding of a digoxigenin at the BsaI side

Note 3). Digest overnight at 37 °C. Subsequently, inactivate the enzyme by incubating the reaction at 80 °C for 20 min. Purify the digested DNA with the Promega Wizard SV Gel and PCR cleanup kit (*see* **Note 4**).

2. *DNA labelling with biotin tag.* Mix the purified DNA, the Klenow fragment (5 units per 100 μL of a reaction volume), and a regular, non-tagged dNTP complementary to the first nucleotide at one side of the digested DNA, here CTP (*see* **Note 5** and Fig. 2b), together with biotin-16-dUTP (final concentration of dNTPs is 20 μM). Incubate for 2 h at 37 °C. Purify the DNA with the Promega Wizard SV Gel and PCR cleanup kit.
3. *DNA labelling with digoxigenin tag.* Mix the biotinylated DNA; the Klenow fragment (5 units per 100 μL of a reaction volume); a regular, non-tagged dNTP complementary to the first single nucleotide at the other side of the digested DNA (Fig. 2c); and digoxigenin-11-dUTP (final concentration of dNTPs is 20 μM). Incubate 2 h at 37 °C. Purify the DNA with the Promega Wizard SV Gel and PCR cleanup kit.

3.3 Chromatin Reconstitution

The DNA is mixed with histone octamers in a high salt buffer [24]. Subsequently, the salt concentration is slowly reduced by dialysis to first assemble $(H3\text{-}H4)_2$ tetrasomes on the 601 DNA. At low salt concentration, histone dimers H2A-H2B bind to the tetrasomes, and full nucleosomes are formed. The nucleosome reconstitution depends critically on the histone: DNA ratio. Excess histones yield additional tetrasomes and nucleosomes on the DNA handles, and excess DNA yields undersaturated fibers. Therefore, each reconstitution is done in a titration series.

1. *Preparation of histone : DNA titrations.* Prepare at least five batches with increasing histone : DNA ratios. Each batch contains at least 500 ng of DNA in a total volume of 50 μL. The minimal DNA concentration (m/v) is then 10 ng/μL, and the minimal molar concentration of the 601 DNA (excluding the DNA handles) is ~0.1 μM. Center the titrations at 1 : 1 molar ratio between 601 segments and histone octamer (Table 1). *See* **Note 6** for an explanation of the calculation method.
2. *Salt gradient dialysis.* Cool down all the buffers before starting the assembly (*see* **Note 7**). Insert a magnet rod into a beaker that contains 200 mL of high salt buffer. Put the dialysis tubes on a floater, and incubate for 15 min in this buffer to allow the membranes to soak. Transfer 50 μL of histone : DNA mixtures into the dialysis tubes using the low-binding pipette tips (*see* **Note 8**). Rinse the Econo gradient pump with miliQ H_2O and subsequently with the low salt buffer. Connect the pump as in Fig. 3. Set the flow rate at 0.9 mL/min to ensure a gradual

Table 1
The composition of individual titrations used for chromatin reconstitution

15 * 601 Chromatin assembly						
Component	**I**	**II**	**III**	**IV**	**V**	**VI**
180 ng/μL DNA template (μL)	7	7	7	7	7	7
200 ng/μL histone octamer (μL)	2	3	4	5	6	7
330 ng/μL competitor DNA (μL)	1	1	1	1	1	1
Final 601-DNA molar conc. (μM)	*0.179*	*0.179*	*0.179*	*0.179*	*0.179*	*0.179*
Final histone octamer molar conc. (μM)	*0.074*	*0.111*	*0.148*	*0.185*	*0.222*	*0.259*
High salt buffer (μL)	40	39	38	37	36	35
Total volume (μL)	50	50	50	50	50	50
Octamer: DNA ratio	**0.4**	**0.6**	**0.8**	**1.0**	**1.2**	**1.4**

Samples with increasing histone: DNA ratio are prepared to account for lower reproducibility while pipetting small volumes

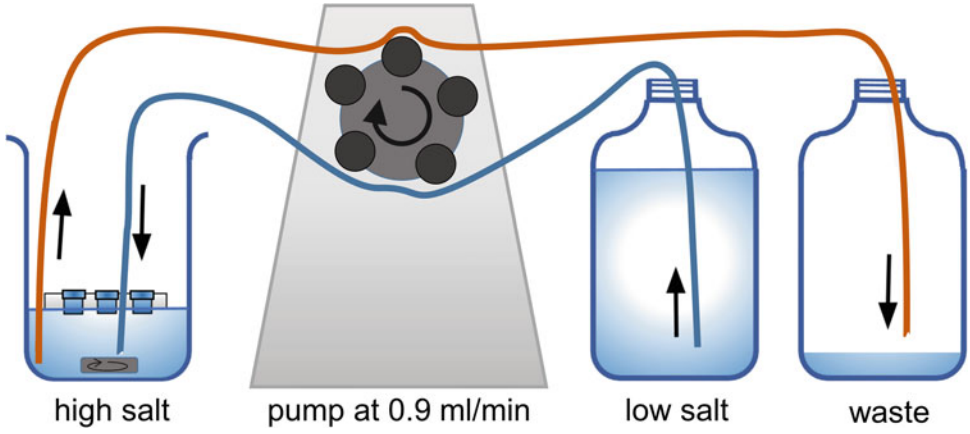


Fig. 3 Schematic representation of the dialysis system controlled by a peristaltic pump. Samples are dialyzed against a high salt buffer. Subsequently, a low salt buffer is pumped into the beaker with dialyzed samples, while an equal volume is flown out into a waste bottle to ensure a constant volume and a gradual decrease of salt concentration in the dialysis tubes

decrease of the salt concentration in the dialysis tubes. Switch on the magnet plate to properly mix the incoming low salt buffer with the high salt buffer (*see* **Note 9**).

3. *Collecting the reconstituted samples.* After approximately 18 h, 900 mL of the buffer is pumped to the waste bottle. Transfer the content of the dialysis tubes into separate low-binding tubes. Store the chromatin samples at 4 °C for maximally 5 weeks. Rinse the tubing of the pump with miliQ H_2O.

3.4 Electrophoretic Band Shift Assay

Quality assessment using agarose gel electrophoresis helps to select chromatin fibers with the optimal histone: DNA ratio prior to a single-molecule experiment. Due to their size and charge, reconstituted chromatin fibers migrate slower through a gel than a bare DNA template (Fig. 4).

1. *Gel preparation.* Dissolve 0.7 g of agarose gel in 100 mL of 0.2× TB buffer by heating the solution in a microwave (700 W, 1 min). Pour the melted agarose solution in a large gel tray. Place a 20 μL well comb in the gel. Let the agarose solidify for ~45 min.
2. *Sample loading.* Put the gel together with its tray into an electrophoresis chamber. Fill it with the 0.2× TB buffer. Gently remove the comb. Load 3 μL of GeneRuler DNA Ladder into the first and the last well. Pipette the DNA-only substrate (~200 ng) mixed with a 10× loading dye (final concentration 1×) into the second well. Load 10 μL of reconstituted chromatin samples mixed with the 10× loading dye (final concentration 1×) to subsequent wells.

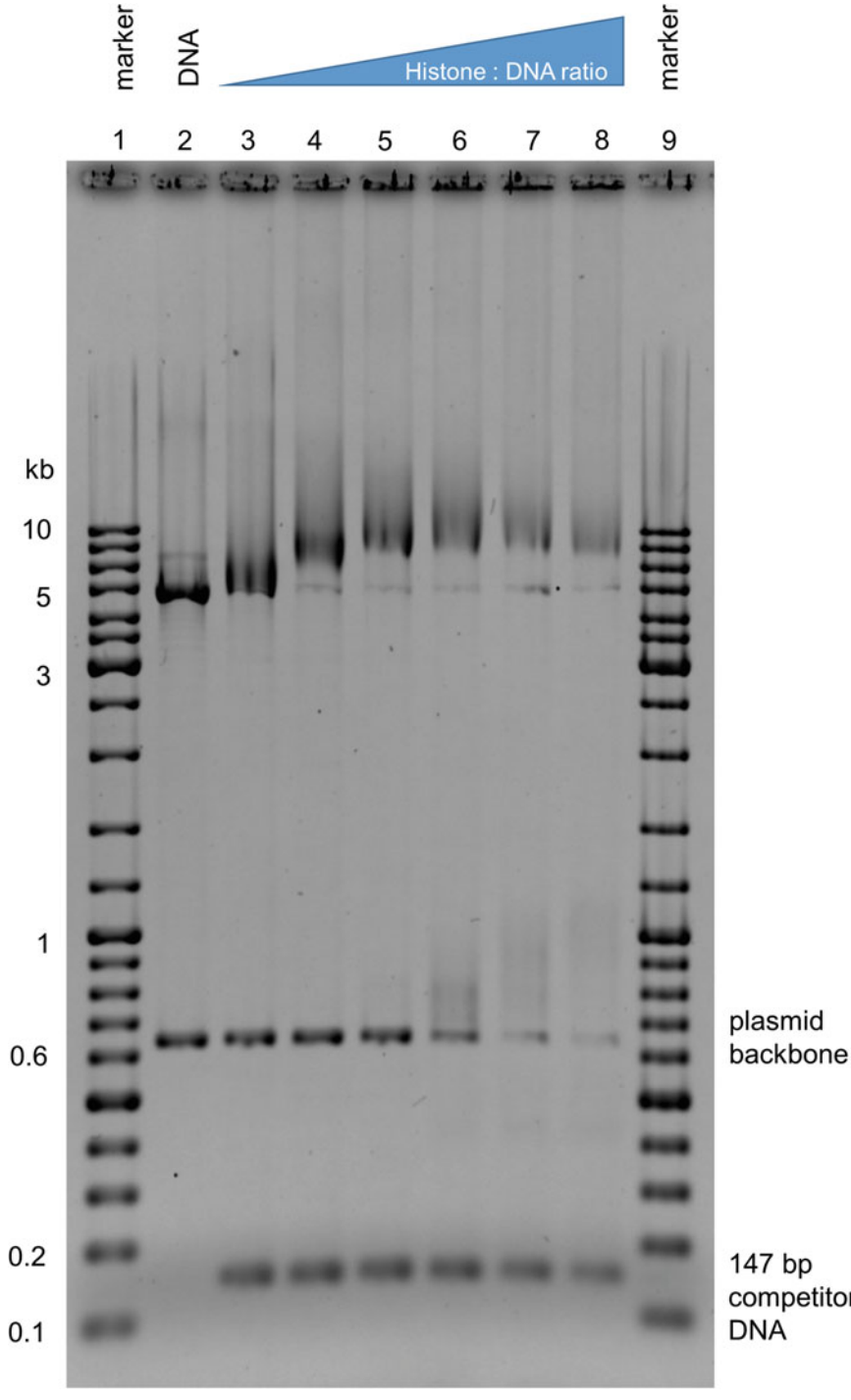


Fig. 4 Electrophoretic mobility shift assay of reconstituted chromatin fibers. With the increasing histone concentration, chromatin fibers migrate slower through the agarose gel. The consecutive bands on the gel (lanes 3–8) are gradually shifted with respect to the bare DNA band (lane 2). The plateau in the band shift reflects a full saturation of chromatin fibers and indicates that excessive histones start to assemble on the

3. *Running the electrophoresis*. Put the lid on the electrophoresis chamber, and connect to the power source. Run at 100 V for 2.5 h to ensure a good separation of bands.
4. *Staining with EtBr*. Remove the tray with the gel and place it carefully in a container with 1000 mL of 1× EtBr. Incubate while shaking for 30 min.
5. *Destaining*. Move the gel on its tray to a container with a distilled water and incubate for 15 min.
6. *Gel imaging*. Visualize the bands on the ChemiDoc UV. Select the optimal histone: DNA titration for single-molecule experiments. Choose the titration ratio with a smaller shift than the bare DNA that does not exhibit any excessive assembly on a competitor DNA (lane 5 in Fig. 4, *see* **Note 10**).

3.5 Assembly of the Flow Cell Chamber

A properly assembled flow cell chamber can be reused for numerous experiments when it is handled gently and is not contaminated during an assembly. Only the top 24 × 60 mm cover slip, with a functionalized surface, needs to be replaced.

1. *Cleaning*. Remove the top glass slide from the flow cell and any leftovers of PDMS from the Perspex molds and the aluminum holders. Wash thoroughly with miliQ H_2O and 2-propanol. Make sure that no PDMS remains inside the inlets of the molds and the holders. Dry under N_2 gas stream (Fig. 5a).
2. *PDMS preparation*. Mix PDMS (polydimethylsiloxane) base and curing agent in a 10:1 ratio (viscous and nonviscous component, respectively) (Fig. 5b). Prepare at least 2–3 mL per flow cell. Mix vigorously with a pipette tip for 2 min (*see* **Note 11**). Put under a vacuum chamber for 2 h in order to degas the viscous polymer.
3. *Assembling a flow cell chamber*. Insert a stiff electric wire into two 10-cm-long pieces of FEP tubing. Insert the tubes into a metal frame such that the electric wire protrudes out of the inner outlet of the holder, near the central aperture of the flow cell. Do the same on the other side of the flow cell (Fig. 5c, left). Put a clean glass cover slip (24 × 40 mm) onto the aperture. Mount the Perspex mold tightly onto the flow cell with M4 screws (Fig. 5c, right). Make sure that the electric wires protrude through the holes in the top of this mold and that the cover slip remains in its place. The end of the FEP tubing must remain inside the outlet near the aperture of the aluminum holder.

Fig. 4 (continued) non-601 DNA, resulting in additional band shift of the plasmid backbone and the competitor DNA (lanes 6–8). The sample in lane 5 exhibits a full saturation of the 601 arrays and no excessive assembly on the competitor DNA; therefore it was chosen for single-molecule experiments

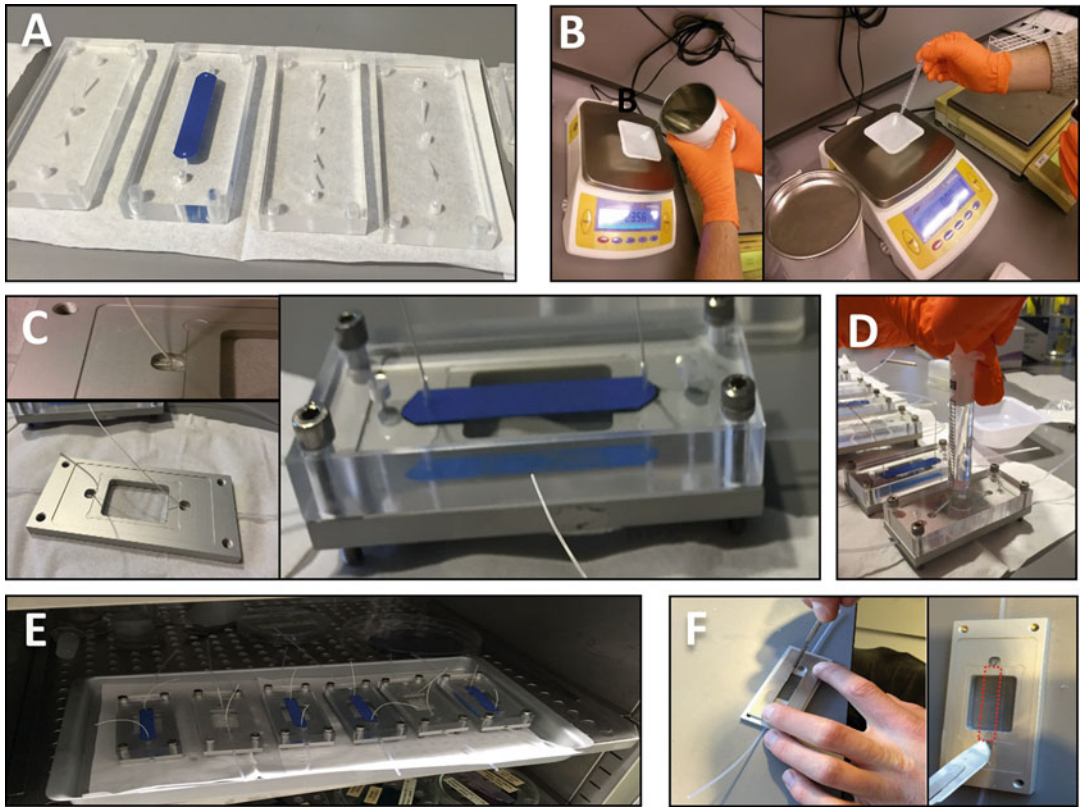


Fig. 5 Assembly of flow cell chambers. (**a**) Perspex molds are cleaned and dried. (**b**) The PDMS base and the curing agent are weighted, mixed, and degassed. (**c**) During the degassing process, aluminum flow cell holders, cover glasses, wiring, and Perspex molds are mounted. (**d**) The PDMS solution is injected into chambers. (**e**) Flow cells are cured at 65 °C. (**f**) Perspex molds are removed and a channel is cut out from a flat, cured PDMS layer (red dotted line indicates the part of PDMS that was cut out)

4. *Casting PDMS.* Gently inject the degassed PDMS with a syringe into the assembled flow chambers through one of the holes on top of the Perspex molds. Push the syringe slowly until the whole chamber is filled with the PDMS and the excess of polymer starts to leak out of the second hole in the mold (Fig. 5d). Prevent incorporating air bubbles into the chamber. Cure the assembly in an oven at 65 °C for at least 2 h to initiate the polymerization of PDMS (Fig. 5e).
5. *Cutting the channel in a cured PDMS.* Loosen the screws and gently remove the electric wires and the Perspex mold such that the FEP tubing and the cured PDMS remain intact. The large groove of the aluminum holder should be filled with a transparent, smooth, and solid layer of PDMS that covers its whole surface and, as a result, immobilizes the 24 × 40 mm cover slip firmly on the aluminum holder. Carefully cut a rectangular

channel in the PDMS layer above the cover slip (Fig. 5f) (*see* **Note 12**). The channel connects the two holes in the PDMS that are left after removing the electric wire from the FEP tubes.

3.6 Flow Cell Functionalization

A cover slip (24 × 60 mm) coated with anti-digoxigenin is put on top of the cured PDMS layer, closing the cut out channel. Buffer can be flushed in and out through the FEP tubes that connect the two sides of the flow channel. After an experiment, the cover slip can be replaced, and the flow cell may be reused for another experiment.

3.6.1 Cover Slip Functionalization

1. *Cleaning*. Put several 24 × 60 mm cover slips in a holder, and sonicate in 2-propanol for 15 min in an ultrasonic cleaner. Dry the cover slips under N_2 gas stream.
2. *Coating with nitrocellulose*. Clean a 100 mL beaker and a 1 mm microscopy slide with 2-propanol. Cover the bottom of the beaker with 5 mL of a 0.1% nitrocellulose in pentyl acetate. Put the slide vertically into the beaker, immersing its bottom edge. Gently bring a 24 × 60 mm cover slip into the liquid, parallel to the microscopy slide, and make use of capillary forces to fill the gap in between the two glasses with nitrocellulose solution. Remove the cover slip after 30 s and dry the glasses under a stream of N_2. Store the slides in a closed container with the coated side up.

3.6.2 Flow Cell Assembly Prior to an Experiment

1. *Cleaning*. When recycling a used flow cell, remove and discard the 24 × 60 mm cover slip with a scalpel. Clean the PDMS surface, the bottom 24 × 40 mm cover slip, and the FEP tubing with miliQ H_2O and subsequently with 2-propanol.
2. *Sealing the flow cell*. Put a new functionalized 24 × 60 mm cover slip in the middle of the PDMS layer, covering the channel, with the nitrocellulose-coated side facing inward. Gently press the glass to seal the flow cell channel. Connect one side of the FEP tubing to a 1 mL syringe. Insert the tubing on the opposite side of the flow cell into an Eppendorf tube containing 1 mL of miliQ H_2O, and suck the liquid into the flow cell. Prevent any air to get inside the flow channel.
3. *Incubation of anti-digoxigenin*. Insert 300 μL of 10 ng/μL anti-digoxigenin (dissolved in miliQ H_2O) into a flow cell the same way as in the previous step. Incubate for 2 h at 4 °C.
4. *Passivation of the glass slide surface*. Mix 1 mL of 4% BSA (dissolved in miliQ H_2O) with 50 μL of 2% Tween-20. Rinse the flow cell with the solution as previously. Seal the inlet and outlet tubes and incubate overnight at 4 °C. At this stage, the flow cell can be stored for maximally 1 week.

3.6.3 Tethering Chromatin Fibers

1. *Preparation of the measurement buffer*. Use a 10× concentrated solution of the stock buffer (Table 2), and prepare 10 mL of the measurement buffer (Table 3).
2. *Washing the flow cell*. Flush out the passivation buffer with 1 mL of the measurement buffer.
3. *Introducing DNA/chromatin*. Dilute 1 μL of reconstituted chromatin fibers (~20 ng/μL) in 500 μL of the measurement buffer. Gently flush in the chromatin into the flow cell with a 1 mL syringe. Incubate for 10 min at room temperature.
4. *Washing the magnetic beads*. Add 20 μL of magnetic beads from the stock to 20 μL of 1× TE in a low-retention Eppendorf tube, and mix by filling and releasing the pipette a couple of times. Pull down the beads from the solution using a magnet stand. Discard the supernatant. Resuspend the beads in 20 μL of 1× TE. Repeat the washing **step three** times.
5. *Attaching beads*. Dilute 1 μL of washed M270 or 0.5 μL of MyOne beads in 500 μL of the measurement buffer. Gently flush the beads into the flow cell with a syringe. Incubate for 10 min at room temperature.

Table 2
The composition of the 10× measurement buffer

Component		Final concentration
KCl	37.28 g	1 M
NaN_3	3.25 g	100 mM
Tween-20	5 mL	1%
1 M HEPES pH 7.5	50 mL	100 mM
mili Q	Fill up to 500 mL	

Table 3
The composition of the measurement buffer

Component		Final concentration
10× Measurement buffer	1 mL	100 mM KCl, 10 mM NaN_3, 10 mM HEPES pH 7.5, 0.1% Tween-20
20 mM $MgCl_2$	1 mL	2 mM
4% BSA	0.5 mL	0.2%
miliQ H_2O	7.5 mL	

3.7 Dynamic Force Spectroscopy on Chromatin Fibers

1. *Magnetic tweezer initialization*. Start up the magnetic tweezers microscope, including motor controllers, CMOS camera, and LED (Fig. 6). Move the magnet to its highest position relative to the objective.
2. *Mounting the functionalized flow cell on the microscope stage*. Put the flow cell on the stage such that the topside of the 24 × 60 mm cover slip is in focus (add immersion oil if an oil objective is used). Tighten the flow cell with screws. Connect one of the tubes to the pump and the other to a reservoir containing the measurement buffer. Rinse the flow chamber with the measurement buffer to remove loose beads. Use a minimal flow rate (<0.1 mL/min) to prevent sample degradation by excessive drag forces.

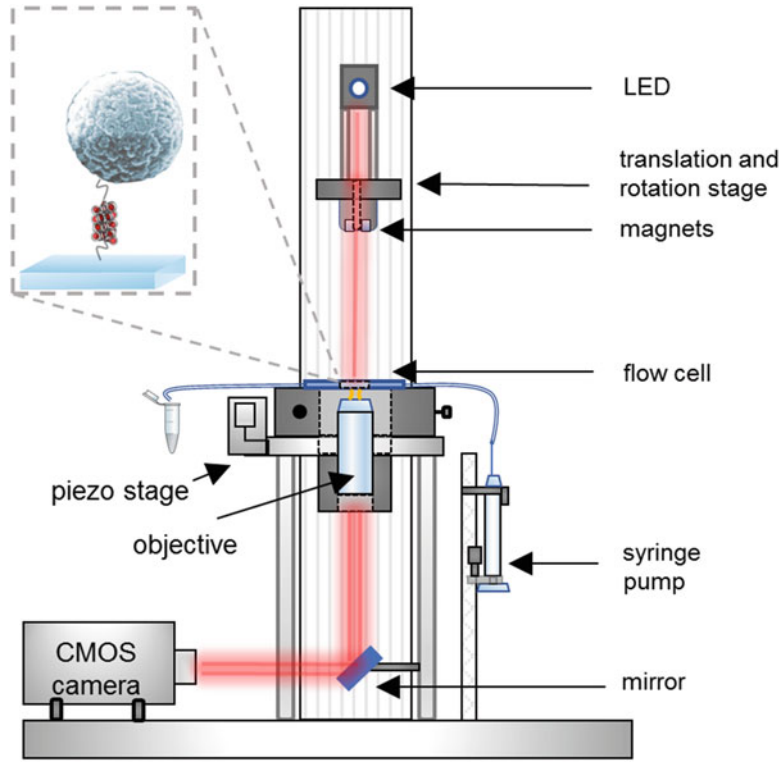


Fig. 6 Schematic representation of the magnetic tweezers setup (a front view with the cross section of the microscope stage). The sample is illuminated by a collimated LED light beam that is guided through the objective and reflected by the mirror toward the CMOS camera. Forces and torques are generated by two cubic magnets mounted on a translation and rotation stage. A piezo stage allows controlling the focus of the microscope. A syringe pump is used to exchange buffers in the flow cell during an experiment. Inset: a chromatin fiber flanked by short DNA handles, tethered in a flow cell between a cover slip and a magnetic bead (not to scale)

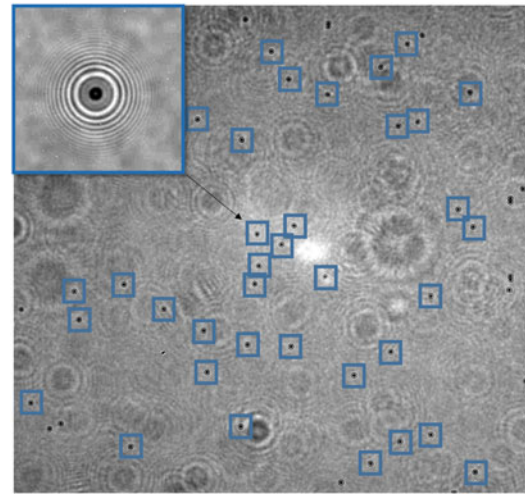


Fig. 7 Selection of beads before tracking. Distribution of tethered magnetic beads on the imaged region of a flow cell. Small regions of interest (ROIs) were selected to track the diffraction rings of individual magnetic beads. Inset: a zoom of one of the selected ROIs

3. *Adjusting the objective position*. Lower the objective until the beads appear out of focus and multiple diffraction rings are visible.
4. *Finding a field of view with tethered beads*. Move the microscope table manually to a region of the flow cell where multiple beads are distributed throughout the field of view (Fig. 7). Select regions of interest (ROIs) around the beads manually or using an automatic bead finder algorithm (Fig. 7, inset). Set a size of the ROIs such that multiple diffraction rings are included, depending on the bead size, objective magnification, and pixel size of the camera.
5. *Tracking calibration*. Using the piezo stage, move the sample through the focus, and record the diffraction ring pattern of a bead. Subsequent image analysis relates the objective position to the changes of the diffraction profile. Correct for the difference in refraction index between the aqueous flow channel and the immersion medium of the objective. For oil objectives, this is a factor 0.88 (*see* **Note 13**).
6. *Force calibration*. Calibrate the force as a function of the magnet height by measuring the lateral thermal fluctuations of a bead and the tether height at several magnet positions. Using equipartition theorem, the force can be calculated as the product of the bead height, Boltzmann's constant, and absolute temperature divided by the variance of the lateral position [25, 26] (*see* **Note 14**).

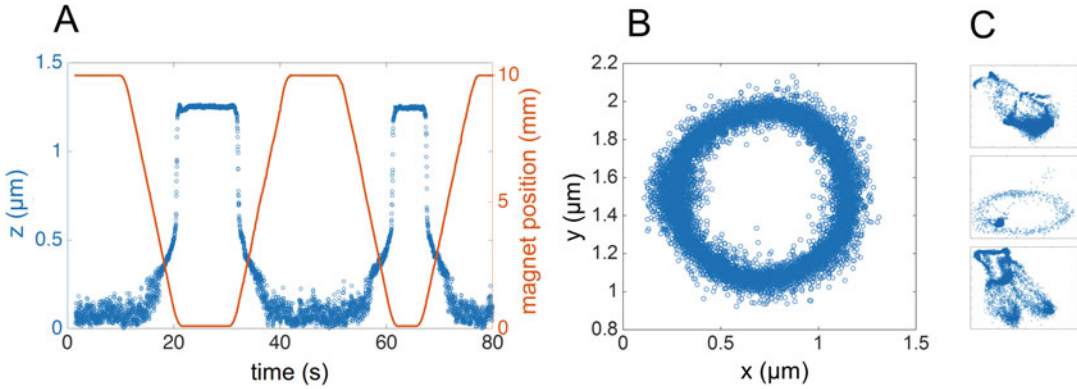


Fig. 8 Quality assessment of a selected tether. (**a**) Force-induced unfolding and refolding of a chromatin fiber tethered with a MyOne bead. Multiple stretching and refolding cycles with low forces (<10 pN) are not destructive to the sample. (**b**) A bead follows a circle in the X, Y plane upon magnet rotation unless more than one chromatin fiber is attached to a single bead. Note that the thicker spot on the left side of the circle results from an extended period before and after rotation of the magnets. (**c**) Anomalous XY tracks upon rotation of the magnets that indicate multiple tethers on a bead

7. *Setting the magnet trajectory to define a force ramp.* Define a trajectory of the magnet from a position where the force is close to 0 pN to a force up to ~7 pN during the first measurement. Prevent sample degradation due to excessive exposure to force. To maintain fiber integrity, minimize the duration of the high-force part in the force trajectory, so the fibers can refold to its initial state before histones dissociate (*see* **Note 15**). We use a magnet velocity of ~0.5 mm/s in both directions. Figure 8a shows the extension of a chromatin fiber tethered with a MyOne bead upon stretching twice with low forces.
8. *Rotation experiment for checking for individually tethered DNA molecules.* Subsequently, set the magnet position to a force of approximately 0.5 pN, and define a new trajectory that controls the magnet rotation. Turn the magnet at least 15 times in both directions. Because the tether is rarely attached to the very bottom of the bead, each bead trajectory should describe a circle in the X, Y plane, even when the DNA is not rotationally constrained (Fig. 8b). However, when more than one tether is attached to the same bead, the trajectory will deviate from a circle (Fig. 8c).
9. *Selection of good tethers.* Discard beads that exhibit too large extensions, indicating incompletely reconstituted chromatin fibers or bare DNA. Also discard too short tethers and beads that have irregular X, Y fluctuations caused by nonspecific sticking of the bead or the chromatin to the surface. The extension of the tether should be close to the contour length of the DNA substrate minus the length of the 601 array.

10. *Probing chromatin folding*. Fiber folding is reversible up to forces of ~7 pN. Multiple force trajectories can be applied to the same tethers.
11. *Complete chromatin unfolding*. Perform a high-force stretching experiment to induce the complete unpeeling of histone octamers/tetramers from the DNA template. This is informative as the unwrapping of the final wrap of DNA from the histone core results in distinctive 25 nm steps that are indicative for each nucleosome or tetrasome. Define a magnet trajectory to apply a force ramp from 0 to 50–60 pN (*see* **Note 16**) to observe these discrete steps in extension. The number of steps should correlate with the number of the 601 sequence repeats on the DNA template. Analyze the results (Subheading 3.8).
12. *Select another field of view*. Move to another field of view in the same flow cell. Make sure that the displacement is large enough to ensure that the beads in the new field of view have not been exposed to high forces during the previous experiment. Displacements should typically be larger than the magnet size, i.e., more than 5 mm.

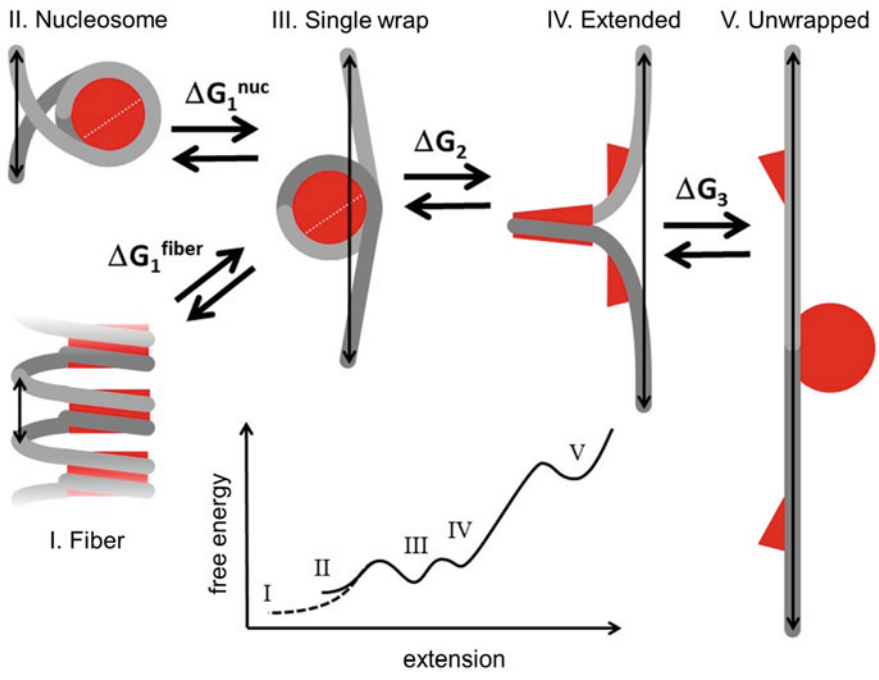
3.8 Data Analysis

The force-extension graphs are characterized by transitions from a folded fiber through an array of partially unwrapped nucleosomes to fully unwrapped nucleosomes (Fig. 9a). We fit the force-extension curves with a statistical mechanics model developed by Meng et al. [27] (Fig. 9b). The model quantifies all conformational changes of DNA and chromatin fibers upon stretching. By fitting the data to the model, we obtain parameters like fiber's stiffness, unfolding energy, number of nucleosomes, and number of tetrasomes (Table 4).

The model contains a large number of parameters that capture the complexity of the fiber. It includes a degeneracy factor that is not easily implemented in analytical formulae, but is straightforward to implement numerically. We typically observe discrete variations of fiber compositions, indicating one or more missing or additional nucleosomes, despite best efforts to reconstitute the fibers. Moreover, the high-force transitions are not in equilibrium, whereas the model is only valid for equilibrium transitions. For these reasons, one cannot simply fit the curves using a standard Levenberg-Marquardt algorithm. Instead, model parameters are fit sequentially, using only the parameters which are relevant in a limited force regime. Subsequent fits on the same fiber in different force regimes use the thus obtained parameters as fixed constants.

1. *Correction for drift*. A difference in the extension between the beginning and the end of the time trace (Fig. 10a) may result from mechanical drift of the microscope (*see* **Note 17**). We use

A



B

	extension	energy
DNA	$z_{DNA}(f,L) = L\left[1 - \frac{1}{2}\sqrt{\frac{k_BT}{fA}} + \frac{f}{S}\right]$	$G_{DNA}(f,L) = -L\left[f - \sqrt{\frac{fk_BT}{A}} + \frac{f^2}{2S}\right] + fz_{DNA}$
fiber	$z_{fiber}(f) = \frac{f}{k} + z_0$	$G_{fiber}(f) = \frac{f^2}{2k}$
total	$z_{tot}(f) = \sum_i n_i z_i(f) + z_{DNA}(f)$	$G_{tot}(f) = \sum_i n_i G_i(f) + G_{DNA}(f)$

degeneracy	$D(\text{state}) = \prod_{i<j} \binom{n_i + n_j}{n_i}$
mean equilibrium extension	$\langle z_{tot}(f) \rangle = \frac{\sum_{states} z_{tot}(f) D(state) e^{-(G_{tot} - Fz_{tot})/k_BT}}{\sum_{states} D(state) e^{-(G_{tot} - Fz_{tot})/k_BT}}$

Fig. 9 Quantification of force-induced structural transitions in chromatin fibers using a statistical mechanics model (figure adapted from Meng et al. [27]). (**a**) Fiber stretching proceeds through four stages of nucleosome unfolding. (**b**) Analytical formulas that describe the extension of each nucleosome state as a function of force. *A* persistence length (nm), *f* force (pN), *G* free energy (k_BT), *k* stiffness (pN/nm), k_B Boltzmann's constant, *z* tether extension (nm), *L* contour length (nm), *S* DNA stretching modulus (pN), *T* temperature (K), *D* degeneracy factor, n_i number of nucleosomes in a particular state *i*

Table 4
Fixed parameters of the statistical mechanics model used in data analysis

Parameter	
DNA persistence length	50 nm
DNA stretch modulus	1100 pN
Contour length	Length of the entire DNA substrate (bp)
Nucleosome Repeat Length (NRL)	Length of the 601 repeat (bp)
Folded length of a single nucleosome	1.5 nm
Unwrapped base pairs of the 1st (outer) nucleosomal turn	56 bp
Unwrapped length in the intermediate transition	5 nm

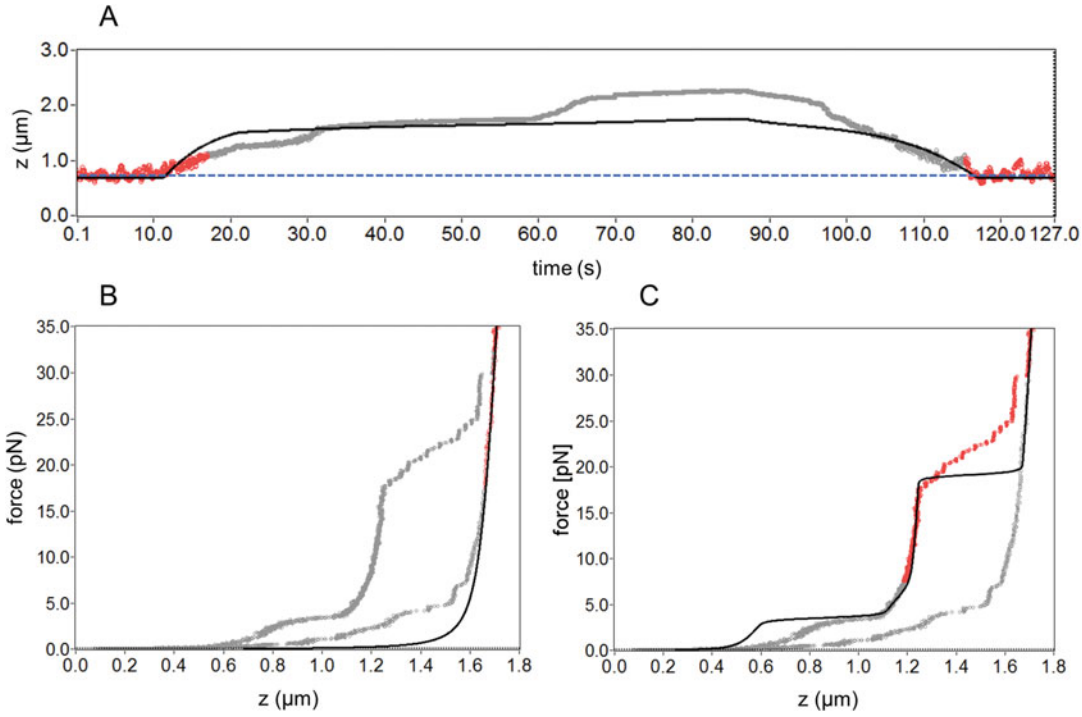


Fig. 10 Initial procedures of the data analysis. (**a**) Quantifying the mechanical drift. Data points from the beginning and the end of the time trace (red) are recorded at the same magnet position and should align with an arbitrary straight, horizontal line. The difference in height of selected regions before and after the alignment is divided by the duration of the measurement to estimate the linear drift factor (nm/s). (**b**) Finding the z-offset. Data points with the highest z-coordinate (red) are aligned with the fitted WLC model with a persistence length of 50 nm and a given contour length (Table 4). (**c**) Quantifying the number of nucleosomes. The staircase-like curve is selected (red), and the distance between the first and the last step is measured. The obtained extension is divided by 25 nm to estimate the number of nucleosomes and tetrasomes assembled on the tether

a linear drift term to enforce maximal overlap and a horizontal trend in the extension versus time plot.

2. *Determining the offset*. Determine the offset in the z-direction by fitting an extensible WLC with fixed contour length, persistence length, and stretch modulus (Table 4) to the most extended part of the curve, i.e., at forces above 30 pN (Fig. 10b).
3. *Fitting the number of nucleosomes*. Start with the number of nucleosomes equal to the number of tandem repeats of the Widom-601 sequence on the DNA used for chromatin assembly. Adjust or fit the number of nucleosomes to obtain the best fit in the range between 8 and 15 pN (Fig. 10c) (*see* **Note 18**). When fitting, the number of nucleosomes should approximate an integer number.
4. *Fitting the unfolding steps*. Check that the number of steps in the force range between 8 and 40 pN agrees with the number of nucleosomes. A Student's t-test is used to compare the 3–6 neighboring data points to identify discrete steps (Fig. 11a). The step size corresponds with the difference between the extension of the last partially wrapped nucleosome and the fully unwrapped nucleosome conformation, i.e., states IV and V in Fig. 9a (*see* **Note 19**).
5. *Fitting the low-force regime*. Fit the part of the trace between 0.5 and 8 pN to obtain the fiber stiffness, unstacking free energy and the free energy of the intermediate transition (k_BT) (Fig. 11b). The plateau width at 3–5 pN depends on the number of nucleosomes that stack and form a folded fiber. We observed that the plateau width is not always consistent with the number of nucleosomes as determined from the high-force region, described in the previous step [27]. In all experiments, the plateau size indicated that the number of stacked nucleosomes is less or equal to the number of high-force unwrapping events. The difference is attributed to tetrasomes that feature the same unwrapping steps at high force but lack the ability to fold into more condensed structures [5]. The number of tetrasomes is also fitted from the low-force region.

4 Notes

1. We use plasmid constructs with multiple repeats of the Widom-601 sequence (tggagaatcccggtgccgaggccgctcaattggtcgtagacagc tctagcaccgcttaaacgcacgtacgcgctgtcccccgcgttttaaccgccaaggggat tactccctagtctccaggcacgtgtcagatatatacatcctgt) that are spaced with 20 bp or 50 bp of non-601 linker DNA (nucleosome repeat length = 167 bp or 197 bp, respectively). In addition

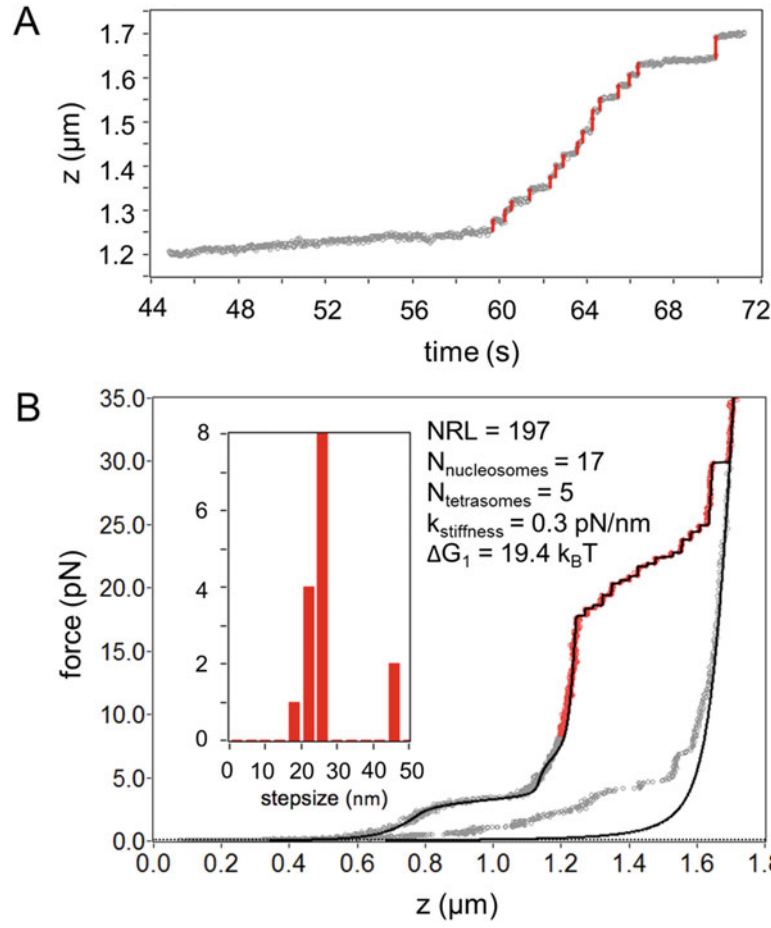


Fig. 11 Fitting the data to the statistical mechanics model. (**a**) Step size analysis. The same part of the trace remains selected as in Fig. 10c. A Student's t-test is performed to quantify the sizes of steps that occur in the 10–40 pN force regime. (**b**) Fitting the plateau of the force-extension curve at the low-force regime to characterize the chromatin fiber folding. Inset: histogram of the measured step sizes and fitted parameters – *NRL* nucleosome repeat length, $N_{\text{nucleosomes}}$ fitted number of assembled nucleosomes and tetrasomes, $N_{\text{tetrasomes}}$ fitted number of tetrasomes, $k_{\text{stiffness}}$ stiffness of a folded fiber (pN/nm), ΔG_1 free energy of the first transition ($k_B T$)

to the 601 repeats, we keep about 1 kbp of flanking DNA on both sides, yielding the following DNA substrates:

15 × 601_167–5185 bp

15 × 601_197–5635 bp

30 × 601_167–7690 bp

25 × 601_197–7605 bp

The flanking DNA helps to keep the chromatin fiber away from the surface of the flow chamber or the bead and facilitate selecting tethers that are not stuck to one of the surfaces. On the downside, additional nucleosomes can form on this DNA, putting high demands on the reconstitution stoichiometry.

2. When handling frozen competent cells, it is important to keep them cold. Frozen cells are very sensitive to temperature fluctuations, and therefore plasmid transformation may not work if the cells are not incubated on ice.
3. The reaction volume for digestion of 1 μg of isolated plasmid DNA is 20–50 μL. A minimum of 5–10 units of each enzyme is required for complete digestion of 1 μg of DNA in 1 h. For an overnight incubation, the amount of enzyme can be therefore lower. Enzyme should not exceed 10% of the total reaction volume.
4. The maximum binding capacity is approximately 40 μg per column. Multiple elutions from the spin columns and longer incubation time are recommended to improve the yield of DNA purification.
5. Selection of nucleotides is based on the sequences of the "sticky end" resulting from an enzymatic digestion. A regular, non-tagged nucleotide other than UTP should be complementary to the first nucleotide at one side of the digested DNA substrate. Absence of this nucleotide in the first Klenow reaction prevents incorporation of tagged UTP on the other side of the DNA substrate. The presence of other nucleotides could result in labelling the DNA with the same tag on both sides. This would not allow for DNA tethering in a flow cell as the bead and the surface have orthogonal binding proteins.
6. Typically, we use a 4535 bp DNA construct containing about 2200 bp of 601 sequence repeats. The rest is a random sequence of the plasmid backbone. A typical DNA concentration after labelling is ~250 ng/μL. Since the nucleosome contains roughly equal weights of protein and DNA, one can easily set up a good titration scheme:
   (a) Use 4 μL of DNA per dialysis tube (in total 1000 ng of DNA).
   (b) DNA handles are 50% of the total DNA; therefore in total there is 500 ng of 601 DNA that should fold into nucleosomes. This amount divided by the molar mass of a single 601 repeat (~10^5 g/M) and then by the total volume ($5*10^{-6}$ l) results in ~ 0.1 μM molar concentration of the DNA substrate that is supposed to form nucleosomes.

(c) We use 200 ng/μL aliquots of histone octamer, so 2.5 μL of this aliquot needs to be added to end up with a solution with 500 ng of protein.

(d) Add high salt buffer for samples to obtain a final volume of 50 μL.

(e) Prepare tubes with the same amount of DNA, and vary the volume of histones (1.5, 2, 2.5, 3, 3.5 μL) to accommodate a loss of histones by sticking to tubes and pipette tips and uncertainties in histone and DNA concentrations.

7. Keep all the buffers at 4 °C and perform the dialysis in a cold room.
8. Low-binding (siliconized) pipette tips and tubing prevent sticking of histone octamers and reconstituted chromatin fibers. This is especially important when small volumes are used.
9. To enforce that the volume in the beaker with dialysis tubes remains constant, make sure that the same amount of the low salt buffer is pumped into the beaker as the high salt buffer is pumped out. This can conveniently be achieved using a peristaltic pump with a double head.
10. Competitor DNA starts to reconstitute into nucleosomes when all 601 sequences are occupied with histones. A band shift of the competitor DNA therefore indicates saturation of the chromatin fibers. Fibers with a minimal oversaturation, i.e., having a small excess of histones assembled on the flanking DNA around 601 repeats, are preferred for magnetic tweezers experiments over subsaturated, incompletely folded fibers.
11. Mix the viscous and nonviscous components of PDMS intensively for at least 2 min. Incomplete mixing will impede homogenous polymerization.
12. Make sure that the FEP tubing does not get loose while removing the Perspex molds and the electrical wires. After its removal, the flow cell should contain two fixed pieces of the FEP tubing and a flat PDMS surface that completely covers and immobilizes the 24 × 40 mm cover slip in the central aperture of the holder. The cured PDMS is a flat, transparent layer that fills the cavity between the aluminum holder and the Perspex mold. Some Perspex molds have a blue embossing tape stripe attached to its bottom side. The void that is left by the embossing tape after curing the PDMS and the removal of the Perspex mold forms the actual flow channel. This channel can be increased in height by cutting out the remaining layer of cured PDMS that was left underneath the embossing tape, with a scalpel. When no embossing tape is used, the entire channel can be cut out at the location where the embossing

tape would be. The channel dimension should be smaller than the dimensions of the cover slip in order to keep the glass in its place. The aluminum holder remains an intrinsic part of the flow cell for convenient mounting on the microscope and connections with tubing.

13. Various methods have been published to quantify changes in the diffraction pattern of the bead [28–30]. We use the 3D FFT algorithm to correlate the measured ROIs with a computer-generated model image (Brouwer et al., in prep.). The empirical relation between the objective position and the diffraction pattern is interpolated for tracking the bead in the z-direction.

14. For a particular magnetic tweezer setup and magnets, the force calibration only needs to be performed once. As chromatin features large changes in extension as a function of force, which impedes thermal fluctuations, the calibration is done with a bare DNA molecule, prior to chromatin experiments. Typically, a double exponential decay of the force with magnet height is obtained, which is characteristic for a given batch of beads and a particular pair of magnets with a fixed configuration. In force spectroscopy experiments on chromatin, the force-magnet position relation for the appropriate bead size is used to convert magnet position to force.

    The Invitrogen beads feature a rather constant magnetic content. The variation is sufficiently small to use the same force calibration for all beads from the same batch. Other beads may have larger variations in magnetic strength.

    Our flow cells have a reproducible height. The offset of the magnet position can be obtained from measuring the height at which the magnets touch the top of the flow channel. This is easily recognized as beads will abruptly move out of focus.

15. A first experiment involves checking the quality of tethers. Some beads are directly stuck to the surface. Others feature multiple tethers or nonspecific interactions between the chromatin and surface. An initial force ramp is used to select tethers that feature force-extension relation that is indicative of a proper chromatin fiber.

    In the buffer conditions used in our method and at the pulling rate lower than 0.2 pN/s, the structural transitions in the fiber remain in equilibrium. Unstacking of the nucleosomes in the chromatin fiber can be reversed upon releasing the force. This may not be the case when a chromatin fiber is not properly folded (a hysteresis is observed between a pulling and a release curve) or in other experimental conditions. Importantly, extended exposure to forces above 3 pN should be avoided, as histone H2A/H2B dimers tend to dissociate above this threshold.

16. The pulling rate is higher in the high-force regime (above 10 pN) compared to the low-force regime. Depending on the pulling rate, unwrapping steps occur at different forces.
17. Mechanical drift can be measured more precisely by tracking the z-extension of a bead stuck to the flow cell surface. When the z-position of such a stuck bead does not remain constant, its position can be subtracted from other extension traces to correct for drift.
18. Individual chromatin fibers may have more or less nucleosomes than the number of 601 repeats on the DNA template. The measured change in extension between the first unwrapping step and the last unwrapping step, divided by 25 nm, should correspond with the fitted number of nucleosomes. A force plateau at 3–5 pN that is smaller than expected indicates the presence of tetrasomes, next to nucleosomes.
19. The average step size should be close to 25 nm. Occasionally, two unwrapping events can occur simultaneously, resulting in a double-sized step. When the number of nucleosomes obtained in the previous step does not match the number of steps observed in this force region, the amount of wrapped DNA and/or the extension of the extended conformations can be adjusted. Typically, step sizes and extensions are within 0.5 nm of the average values obtained from multiple experiments. In this force regime, the non-equilibrium steps in the force-extension curves and all points in between are assigned to discrete levels. Each level corresponds to a WLC with a contour length that is reduced by an integer number times the amount of DNA that is wrapped in a single-wrapped nucleosome or tetrasome.

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