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Unravelling the Biophysical Properties of Chromatin Proteins and DNA Using Acoustic Force Spectroscopy

Szu-Ning Lin, Liang Qin, Kees-Karel H. Taris, Gijs J. L. Wuite, and Remus T. Dame

Abstract

Acoustic force spectroscopy (AFS) is a single-molecule micromanipulation technique that uses sound waves to exert force on surface-tethered DNA molecules in a microfluidic chamber. As large numbers of individual protein–DNA complexes are tracked in parallel, AFS provides insight into the individual properties of such complexes as well as their population averages. In this chapter, we describe in detail how to perform AFS experiments specifically on bare DNA, protein–DNA complexes, and how to extract their (effective) persistence length and contour length from force–extension relations.

Key words Acoustic force spectroscopy, Single-molecule manipulation, Protein–DNA interaction, DNA-binding protein, Bacterial chromatin protein

1 Introduction

The principle of applying sound waves to manipulate objects was first described in 2004 [1], when Hawkes and colleagues used an ultrasonic standing wave to drive bacteria spores in microfluidic systems. About 10 years later, this concept was modified such that controlled forces could be applied to molecules with sound waves, a method called acoustic force spectroscopy (AFS) [2]. The experimental layout for studying protein–DNA complexes with AFS is similar to that used for tethered particle motion (TPM) [3] and magnetic tweezers (MT) [4]: one end of a DNA substrate is labeled with DIG to bind the anti-DIG on the sample carrier or flow chamber surface, while the other end of the DNA is labeled with biotin to bind on silica or magnetic beads. In AFS, force is applied vertically to microspheres attached to surface-tethered DNA

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molecules as is the case for MT. The xy motion of the bead is monitored, and its diffraction pattern is used to determine its z -position from a lookup table (LUT) of radial profiles [2]. The difference in z -position compared to that of a reference bead directly attached to the surface corresponds to the end-to-end distance of the DNA tether. This method to study DNA–protein complexes with AFS has been successfully applied to study RNA polymerase activity [5], TFAM [6], and (artificial) virus compaction [7, 8]. The usage of DNA as a facilitator, e.g., as nanoswitches, allows to define the strength of protein bonds [9] or as a linker to characterize the unbinding of protein from cellulose [10] or antigen from antibody [2]. Of course, only DNA tethers can also be characterized, as has been demonstrated with DNA origami [11] and DNA hairpins [12].

Acoustic pressure is generated by a vibrating piezo element attached to the bottom or top of the flow cell. The acoustic pressure transfers potential energy into the medium in the flow cell chamber and forms a standing wave. Particles, in this case, polystyrene or silica microspheres 1–5 μm in diameter (with a volume V), are pushed to the nodes of the standing wave. By increasing the voltage (changing the amplitude of the wave), beads will experience a larger force (F) toward the wave node [13, 14]. The effective force applied on a bead is described by

$$F = -V\nabla\left[\frac{1-\kappa^*}{4}\kappa_m P^2 - \frac{(\rho^*-1)}{1+2\rho^*}\rho_m v^2\right] \quad (1)$$

in which P is the acoustic pressure (energy gradient), v is the velocity of particles, and ρ^* ($=\rho_p/\rho_m$) and κ^* ($=\kappa_p/\kappa_m$) are the density ratio and compressibility ratio between the particle and the fluid, respectively [14]. Thus, the magnitude of the force applied to a bead is determined by the material and size of the bead, the medium inside the flow cell, and the vibration of the piezo. One can routinely apply forces up to ~ 70 pN to achieve DNA overstretching and protein unfolding with 4.5 μm polystyrene microspheres.

Here, we describe the assembly of an acoustic force spectroscopy instrument around a commercial inverted microscope and explain how to perform experiments on DNA molecules and protein–DNA complexes. In addition, we demonstrate how structural and mechanochemical properties of protein–DNA complexes can be extracted from AFS data.

2 Materials

Prepare all solutions by using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M Ω -cm at 25 $^\circ\text{C}$; MilliQ). Prepare solutions at room temperature (RT) and store at 4 $^\circ\text{C}$.

2.1 Stock Solutions and Beads

2.1.1 Buffer for Analysis by Agarose Gel Electrophoresis (See Subheading 3.1)

TAE: 40 mM Tris–HCl (pH 7.6), 20 mM acetic acid, and 1 mM EDTA.

2.1.2 Solutions for Reference Bead Preparation (See Subheading 3.2)

1. Buffer A: 100 mM $\text{Na}_2\text{B}_4\text{O}_7$, 150 mM NaCl, 0.05% w/v Pluronic (pH 8.3) (BASF), 30 μM Digoxigenin-NHS ester (Sigma-Aldrich).
2. Buffer B: 10 mM HEPES (pH 7.5).
3. Buffer C: 10 mM HEPES (pH 7.5) with 0.1% Pluronic.

2.1.3 Solutions for Cleaning Protocol (See Subheading 3.4)

1. Bleach solution: 0.7 M NaClO.
2. Sodium thiosulfate solution: 10 mM $\text{Na}_2\text{S}_2\text{O}_3$.

2.1.4 Solutions for Passivation of the Flow Cell (See Subheading 3.6)

1. Phosphate-buffered saline (PBS, pH 7.4): 150 mM NaCl and 10 mM phosphate. 1 mM EDTA and 10 mM NaN_3 are added to prevent bacterial growth.
2. Anti-digoxigenin solution: 200 $\mu\text{g}/\text{mL}$ anti-DIG (Roche) in PBS.
3. Buffer D: 0.2% (w/v) BSA (Sigma-Aldrich) in PBS.
4. Buffer E: 0.5% (w/v) Pluronic (Sigma-Aldrich) in PBS.
5. Buffer F: 0.02% (w/v) casein (Roche) and 0.02% (w/v) Pluronic in PBS.

2.1.5 Solutions for Passivation of the Flow Cell (See Subheadings 3.2 and 3.6)

1. 1.9 μm streptavidin-coated bead (Kisker Biotech) in PBS.
2. 4.5 μm streptavidin-coated bead (Kisker Biotech) in PBS.

2.2 DNA Substrates

DNA substrates for AFS experiments are generated via polymerase chain reaction (PCR) using 5' biotinylated and 5' digoxigenin-labeled primers (*see Note 1*). The length of DNA is designed to be significantly shorter than the distance from the surface to the wave node. AFS is capable to measure DNA substrates as short as 102 bp and as long as 45.5 kbps [12, 15]. Table 1 and Subheading 3.1, step 2 summarize oligonucleotides used to generate our toolbox of DNA of different lengths (2000–8000 bp) and sequence content (32% and 50%) by PCR. All DNA substrates are stored at $-20\text{ }^\circ\text{C}$ after purification and concentration determination. You will need the following:

Table 1
Primer sequences

Primer name	Sequence (5'–3')	Modification
32% GC AFS general forward primer	<u>G</u> TGTGTGTGTGTGGTGTGTGGTGG ATACATATGCAACTTGAACGGCG TAAAAGAGG	5' Digoxigenin
2000-bp 32% GC AFS reverse primer	GTGTGTGTGTGTGGTGTGTGGTGG TCCCTCACTAGTTTAGTACATGAACTG	5' Biotin
50% GC AFS general forward primer	CTCTCTCTCTCTTCTCTCTTCTCT GAATTCGCGGCCGCGCCTC	5' Digoxigenin
2000-bp 50% GC AFS reverse primer	CTCTCTCTCTCTTCTCTCTTCTCT CAGTGGGAACGATGCCCTC	5' Biotin
4000-bp 50% GC AFS reverse primer	CTCTCTCTCTCTTCTCTCTTCTCT CAGCGGTGGTTTGTGGCCG	5' Biotin
6000-bp 50% GC AFS reverse primer	CTCTCTCTCTCTTCTCTCTTCTCT CGATCCCCGGCAAACAGC	5' Biotin
8000-bp 50% GC AFS reverse primer	CTCTCTCTCTCTTCTCTCTTCTCT GGTACCAATGTTTTAATGGCGGATG	5' Biotin

T = Modified T

1. A DNA template contains the sequence of interest; plasmid pKYBI (8393 bp, New England Biolabs) and plasmid pRD227 are used as the templates for 8000-bp DNA substrate with 50% GC content and 2000-bp DNA substrate with 32% GC content, respectively.
2. 5' biotin-labeled reverse primer (*see* Table 1).
3. 5' digoxigenin-labeled forward primer (*see* Table 1).
4. 100% DMSO (New England Biolabs).
5. Recombinant *Taq* DNA polymerase (5 U/μL) (Thermo Scientific).
6. Deoxyribose nucleotide triphosphate (dNTP mix) (Thermo Scientific, 2 mM).
7. *Taq* DNA polymerase reaction buffer (Thermo Scientific, 10×).
8. GenElute™ PCR cleanup kit (Sigma-Aldrich).
9. Biorad T100 Thermocycler PCR or any other available PCR machine.
10. 1% agarose gel in 1× TAE.
11. Nanodrop® (Thermo Scientific).
12. GeneRuler DNA ladder (Thermo Fisher Scientific).

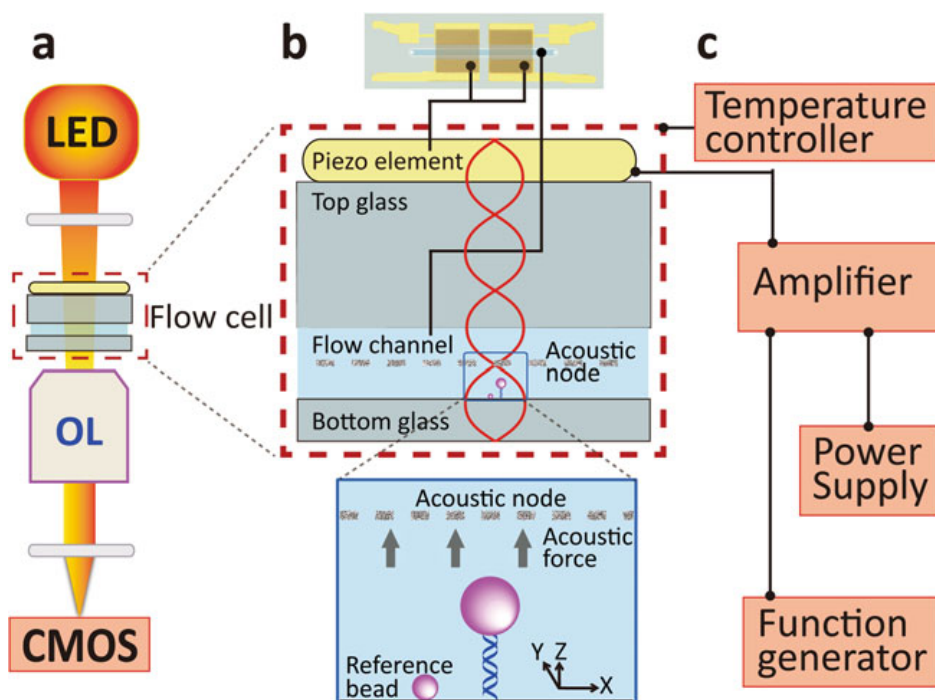


Fig. 1 Illustration of the principle of acoustic force spectroscopy. **(a)** The acoustic force spectroscopy instrument consists of a flow cell, an inverted microscope visualizing targets with objective lens (OL), temperature controller connected to the AFS chip holder, a CMOS digital camera, and 660-nm LED light source. **(b)** AFS flow cell consists of the piezo element and two glass slides with a fluidic channel in between. The acoustic wave generated by the piezo travels through the top glass to the bottom glass, and the bottom glass as a reflector reflects the acoustic wave, producing the standing wave over the flow cell. The acoustic standing waves carry pressure profile, which generate acoustic forces. The tethered particles in the flow channel exposed to the acoustic force are driven in the direction of the acoustic pressure node. In our setup, each flow cell has two piezo elements. **(c)** The temperature controller independently connects to the AFS chip metal holder. Both power supply and function generator connect to the amplifier, which drives the piezo element

2.3 AFS Instrument

The AFS system is built around a commercially available inverted microscope combined with commercially available electronics (*see* Fig. 1) and consists of the following:

1. Microscope: Inverted microscope (Nikon, TE200) with condenser (Nikon, LWD lens) and CFI Achromat 40 \times air objective (Nikon, NA = 0.65).
2. Illumination: Collimated LED (Thorlabs, 660 nm, 1200 mA) (*see* Note 2).
3. CMOS camera (Thorlabs, monochrome, pixel size 5.3 μm , 60 fps) connected to the computer.
4. Stage: Z-axis piezo translation stage (PI, MCLS03200), driven by Nano-Drive controller system (MCL, Nano-Drive, MCLC03200) connected to the computer.
5. Function generator (Keysight, 33220A).

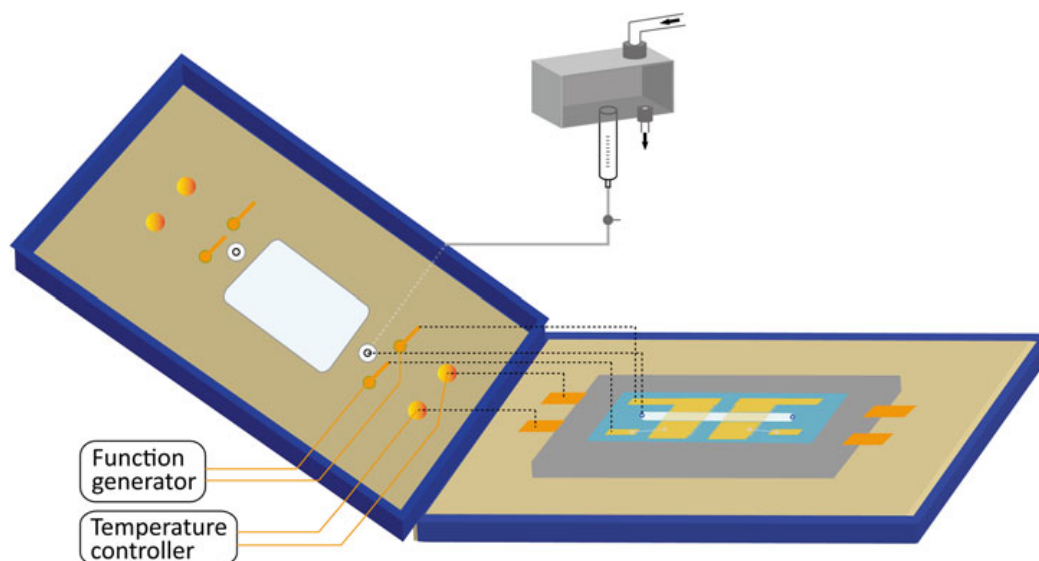


Fig. 2 Schematic representation of the temperature control holder. The AFS chip fits into the temperature control metal holder (gray), and this metal holder fits into the flow cell holder (light brown and navy blue) that consists of lid and base. When the lid closed, the metal points on the lid, connected to the function generator and temperature controller, will connect to their counterparts (metal regions) on the piezo and the metal holder (gray), and the holes on the lid, connected to the pressure can by thin tube, will connect to their counterparts (holes) on the AFS chip

6. Power supply (Votcraft, VSP 1410).
7. Amplifier (Mini-Circuits, LZY-22+).
8. TMC Vibracontrol clean top isolation table.
9. Computer: “Advanced AFS workstation” (LUMICKS B.V., AFS-CPU).
10. AFS chip (LUMICKS B.V., AFS-CH2; *see* Fig. 1b).
11. AFS chip temperature control holder (*see* Fig. 2).
12. Custom pressure system. Syringes contain samples, and buffers are connected to a gas pressure container (*see* Fig. 2).

Another example of a self-build system is Tsan et al. (2023) [16].

2.4 Particle Tracking, Control, and Analysis

Three programs, a bead tracking program (*see* Subheading 3.6), a data analysis program (*see* Subheading 3.7.1), and an extensible worm-like-chain (eWLC) fitting program (*see* Subheading 3.7.2), are used during the measurement and data analysis process.

1. The tracking program is written in LabVIEW [2] and available online http://figshare.com/articles/AFS_software/1195874. A detailed manual is also provided with the software. It is used to:
 1. Control output frequency and output power of vibration. Apply a linear force ramp mode, where the voltage is ramped with a square root function.

2. Create a template image of a bead (imaged via a LED with a camera) to select beads using a template-matching algorithm.
 3. Track the x and y movements of tethered beads and record a lookup table (LUT) in z direction for each bead. Routinely, the z -stage is moved in 80-nm steps through the LUT range of 0–8000 nm. The z -distance range of the LUT has to be larger than the maximal extension of the DNA molecule.
 4. Calibrate the force–voltage relationship.
 5. Perform real-time acquisition of three-dimensional bead positions in the flow cell.
2. The data analysis program is also written in LabVIEW [2] and available online via the same link as indicated under Subheading 2.4, step 1). It is used to:
 1. Determine anchor points of all the tracked beads.
 2. Correct for drift based on the positions of the surface attached reference beads.
 3. Generate force–extension (force–distance, FD) curves.
 3. The WLC model fitting program used here is written in MatLab [17] and available online at <https://github.com/onnodb/FDFIT/tree/AFSFitting>. An alternative program is discussed in [18]. It is used to:
 1. Fit FD curves exported from data analysis program to the eWLC model.
 2. Extract values of parameters reflecting the physical characteristics of bare DNA and protein–DNA complexes.

3 Methods

3.1 Generation of DNA Substrate Using PCR

1. DNA substrates for AFS experiments are generated by PCR. Carefully mix the reagents below in a PCR tube. Keep the enzymes in a $-20\text{ }^{\circ}\text{C}$ cold block and dNTP stocks on ice when taken outside the freezer.

Reagent	Quantity
dNTP mix (2 mM)	5 μL
Forward primer (10 pmol)	1 μL
Reverse primer (10 pmol)	1 μL
Taq polymerase buffer (10 \times)	5 μL
DNA template (10 ng)	1 μL
Taq DNA polymerase (5 U/ μL)	0.2 μL

(continued)

Reagent	Quantity
DMSO (100%)	1.25 μ L
MilliQ	Add to 50 μ L total volume

- Use the program below to perform PCR (optimized for use in a Biorad T100 Thermocycler).

	Temperature	Duration	Cycle
Initialization	95	5 min	1
Denaturation	95	30 s	35
Annealing	65	30 s	
Extension/elongation	72	4 min	
Final elongation	75	10 min	1
Final hold	15	∞	1

- Load 5 μ L of each PCR product on a 1% agarose gel in TAE buffer, alongside a DNA marker for size estimation of the PCR product. Purify the PCR products with GeneElute PCR cleanup kit; *see* Fig. 3 for an example of the purified PCR products.
- Use Nanodrop® to measure the concentration of PCR products. Store the purified DNA products in MilliQ at -20 °C.

3.2 Preparation of Reference Beads

- Mix 8 μ L of 0.5% (w/v) 1.9 μ m polystyrene beads into 1 mL of buffer A.

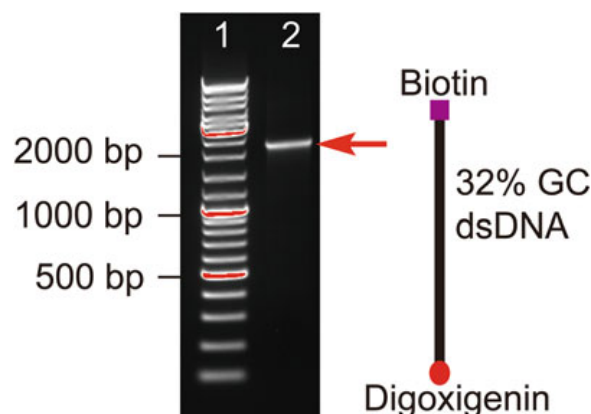


Fig. 3 Visualization of PCR product size by agarose gel electrophoresis. (1) 2 μ L of the GeneRuler DNA marker. (2) 2 μ L of the purified PCR product, and it is ready for use in acoustic force spectroscopy experiments. The schematic representation of PCR-generated 5' digoxigenin and 3' biotin-modified DNA

2. To coat polystyrene beads with DIGs, incubate the bead solution with DIG-NHS at RT for 3 h with tumbling.
3. Centrifuge the bead solution at $2000 \times g$ for 1 min and discard the supernatant.
4. Remove the free DIG-NHS by washing the bead solution with 1 mL buffer C. Centrifuge at $2000 \times g$ for 1 min and discard the supernatant. Repeat the wash twice.
5. Resuspend the beads in 1-mL buffer B. The beads can be used immediately or stored at 4 °C.

3.3 Preparation of Tether Beads

1. To exchange the storage buffer of the commercial beads with PBS, dilute 20 μ L of the bead solution in 500 μ L PBS.
2. Vortex the sample thoroughly.
3. Centrifuge at $2000 \times g$ for 2 min and discard the supernatant, but avoid discarding beads. Resuspend the beads in 500 μ L PBS.
4. Repeat **steps 2** and **3** and carefully discard \sim 450 μ L of supernatant, leaving \sim 50 μ L in tube.
5. Add 4 μ L of the reference beads to the 50 μ L solution from **step 5** and resuspend by vortexing.

3.4 Flow Cell and Tubing Cleaning

The tubing and the flow cell are used repeatedly in our system; we replace the syringe tubing connectors before each experiment.

1. Install the flow cell chip into its holder. The holder is connected to tubing (*see* Fig. 2).
2. Introduce 0.2 mL of bleach into the flow cell through the tubing and incubate for 10 min.
3. Rinse the tubing and the flow cell with MilliQ.
4. Introduce 0.2-mL $\text{Na}_2\text{O}_2\text{N}_3$ into the flow cell via the tubing and incubate for 10 min.
5. Flush 0.5 mL MilliQ into flow cell through the tubing.

3.5 Preparation of Flow Cell and Bead Tethers

To minimize waste of materials, in **steps 1**, **5**, and **7** below, the sample is manually pipetted into the flow cell without using the syringe. However, all the buffers for passivation are introduced through the syringe so the syringe and tubing are also passivized. All preparations are at RT.

1. Inject 20 μ L of anti-digoxigenin solution into the flow chamber and incubate for 20 min.
2. To prevent air bubbles flowing into the flow chamber, introduce 0.5 mL of buffer D into the syringe and flush out the air present in the tubing before connecting the tubing to the

holder. Place the chip in holder, leave drops at the two holes in the flow cell, and clamp the holder gently [19].

3. Inject 0.1 mL of buffer D and incubate for 30 min for the first passivation. Discard the residual of buffer D in syringe.
4. Add 0.5-mL buffer E into the syringe, flush in 0.1 mL of buffer E, and incubate for 30 min for the second passivation. Discard the residual of buffer E in the syringe.
5. Take out the chip to introduce 30 μL of DNA solution in the flow chamber and incubate for 20 min. Place the chip back into the holder and clamp the holder gently.
6. Add 0.5-mL buffer F into the syringe. Flush in 0.1-mL buffer F to remove free DNA.
7. Take out the chip to introduce 20 μL bead solution in the flow chamber. Place the chip back into the holder, clamp the holder gently, and incubate for 30 min.
8. Add 0.5-mL measuring buffer in the syringe.
9. The flow cell is ready for measurement.

3.6 Measurements

1. Switch on the illumination and bring the bottom-tethered beads into focus (*see* Fig. 1).
2. Start the bead tracking program in LabView.
3. Set the frequency for the piezo. The piezo frequency is given by provider (LUMICKS). Each piezo has a specific impedance; in other words, there is deviation of vibration frequency from chip to chip. More details have been described previously [15].
4. Remove untethered beads by flushing the flow cell with the measurement buffer at a flow rate of 0.2 $\mu\text{L}/\text{min}$ until such beads are no longer observed in the region of interest (ROI).
5. Select the tethered beads in the ROI.

Generate a LUT for each ROI, applying a constant force (≈ 10 pN) to the tethers to minimize bead motion. The “Create LUT” bottom in the program starts to move the sample stage (or objective) and record the ring patterns at different z-positions. We collect the LUT at 60 Hz with a camera exposure time of 16.6 ms; each stage’s moving step is 80 nm throughout a range of 0–8000 nm.

6. Start to record the tracking of the selected tethered beads.
7. Record the xy motions of the tethered beads in the absence of force; the xy motions are used for determination of the single-tethered beads in data analysis. The time required to sample all conformations depends on bead size; for beads with a diameter of 4.5 μm , a 10-min recording is sufficient.
8. To calibrate the force–voltage relationship of each tethered bead, apply a series of different forces on the tethered particles. To collect sufficient data for power spectra fitting (*see*

Subheading 3.7.1), perform 2 min of z-position recording for each force.

9. Apply low force (~10 pN) and slow flowing rate (0.2 $\mu\text{L}/\text{min}$) while introducing protein solution. The flow in the flow chamber will result in a drag force on the tethered beads.
10. Apply a constant rate force ramp (120 ms between each force step) to generate FD curves of bare DNA and protein–DNA complexes.

3.7 Data Analysis

3.7.1 Generate FD Curves

1. Load the data into the analysis program in LabView.
2. Determine the single-tethered beads and reference beads data by the root mean squared (RMS) displacement values and anisotropic ratio (s) from the xy motions of each tether.

In Subheading 3.6, step 7, tethered beads randomly move around their anchor points without force applied. In AFS, RMS is used to quantify the degree of the tether motions in two dimensions over a period of time, t :

$$\text{RMS} = \sqrt{\langle (x - \bar{x})^2 + (y - \bar{y})^2 \rangle_t} \quad (2)$$

In which \bar{x} and \bar{y} are the average positions of the tethered bead over time t .

Usually, not all tethered microspheres are attached to the surface via a single tether. Calculation of the anisotropic ratio (s) allows separation of single-tethered microspheres from stuck and multiple-tethered microspheres.

$$s = \frac{\lambda_{\text{major}}}{\lambda_{\text{minor}}} \quad (3)$$

In which λ_{major} and λ_{minor} represent the maximum and minimum value along the axes of the xy scatterplot, respectively.

Single-tethered microspheres are expected to exhibit a perfectly symmetrical motion and to have an anisotropic ratio of 1. In our studies we use $s < 1.3$ as a threshold to discard multiple-tethered or poorly tracked particles. The particles that match the selection criterium are used for further analysis.

2. Remove the drift from the measured data by calculating the average drift of the stuck beads. Stuck beads are selected based on their xy motions. In our studies we use $\text{RMS} < 200/s \approx 1$ (see Note 3).
3. Determine the anchor points of the tethers by selecting the xy motion trace without force applied during the measurement. Use the “Anchor point” function in the program to determine the end-to-end length of the DNA molecule by Pythagoras calculation and the anchor point in data analysis program.

4. Calibrate the force–voltage relationship by selecting the voltage–time plot in the program where constant voltages were applied. Generate and fit a power spectrum (*see Note 4*).
5. Generate FD curves by selecting the time period which force ramp was applied. Export FD curves in text file with “Export function” in the program for eWLC fitting (*see Subheading 3.7.2*).

3.7.2 Extensible Wormlike Chain (eWLC) Model Fitting

The extensible wormlike chain model in Eq. 4 describes the behavior of elastic polymers such as DNA and protein–DNA complexes [20].

$$\frac{z}{L_c} = 1 - \frac{1}{2} \left(\frac{k_B T}{FL_p} \right)^{\frac{1}{2}} + \frac{F}{K_0} \quad (4)$$

In which z is the extension and F is the external force, k_B is the Boltzmann constant, T is the absolute temperature, K_0 is the stretch modulus, L_p is the persistence length, and L_c is contour length. A typical value for the stretch modulus of double-stranded DNA is about 1000 pN [19].

1. Run the fitting program in MatLab software.
2. Import FD curves in MatLab program.
3. Select the data point of the FD curves which are taken below 30 pN.
4. Determine persistence length (L_p), contour length (L_c), and stretch modulus (K_0).

4 Analysis of Protein–DNA Complexes Using AFS

Architectural proteins bind to DNA via minor or major groove interactions and result in wrapping, bending, or bridging of the DNA. By applying force to protein–DNA complexes, the effect of proteins on DNA conformation and the binding behavior of these proteins can be investigated. Here we discuss the effects of two types of DNA binding proteins, HU and H-NS.

4.1 Force–Extension Curves of HU–DNA Complex

HU protein compacts and stiffens DNA in a protein concentration-dependent manner. In our experimental system, DNA was compacted at concentrations below 400 nM. Above 400 nM, DNA was extended by the filament of HU proteins (*see Fig. 4*).

4.2 Pulling of DNA and DNA–H-NS Complex

Histone-like nucleoid-structuring (H-NS) protein is a bacterial protein that plays a key role in chromosome organization and regulation. DNA and DNA–H-NS complex were studied by using AFS system (*see Fig. 5*). It shows that DNA was stiffened by H-NS at 2000 nM, which is in agreement with previous studies [15, 21].

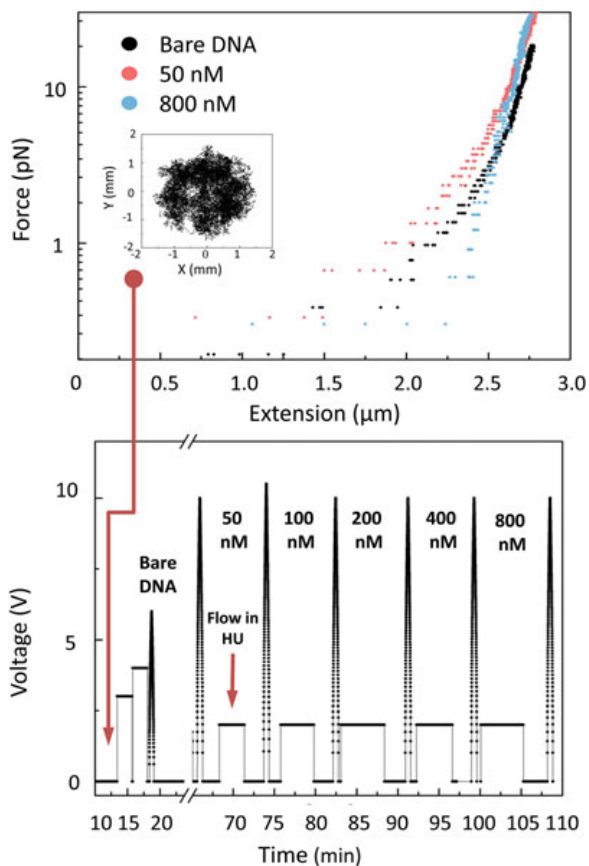


Fig. 4 Stretched HU–DNA complex and the flowchart of force application. The top figure shows three force–extension curves of 8000-bp DNA at HU absent (black), low HU concentration (red), and high HU concentration (blue). Top inside is the free movement of a tethered bead (symmetry in 1.2) under 0 pN force. Bottom plot shows the applied voltage vs time. Low force ~ 2 pN was applied while introducing proteins. The measuring buffer contains 20 mM HEPES pH 7.5, 60 mM KCl, 1 mM DTT, and 0.2% (w/v) BGB

5 Notes

1. DNA substrates can also be prepared by other approaches, e.g., by filling in digoxigenin/biotin at two ends of a cut plasmid or by ligating modified oligos/dsDNAs.
2. Diffraction ring patterns are required for accurate bead tracking. The light source needs to be monochromatic and aligned in parallel. To obtain collimated light, either a point source or an iris conjugated to your condenser is required. For monochromatic illumination, a LED of defined wavelength or band-pass filter in the illumination path is suggested.
3. Movement of the machine or heat created by piezo vibration causes drift in the flow cell. A highly efficient piezo results in minimal heating of the system. To correct drift signal, the

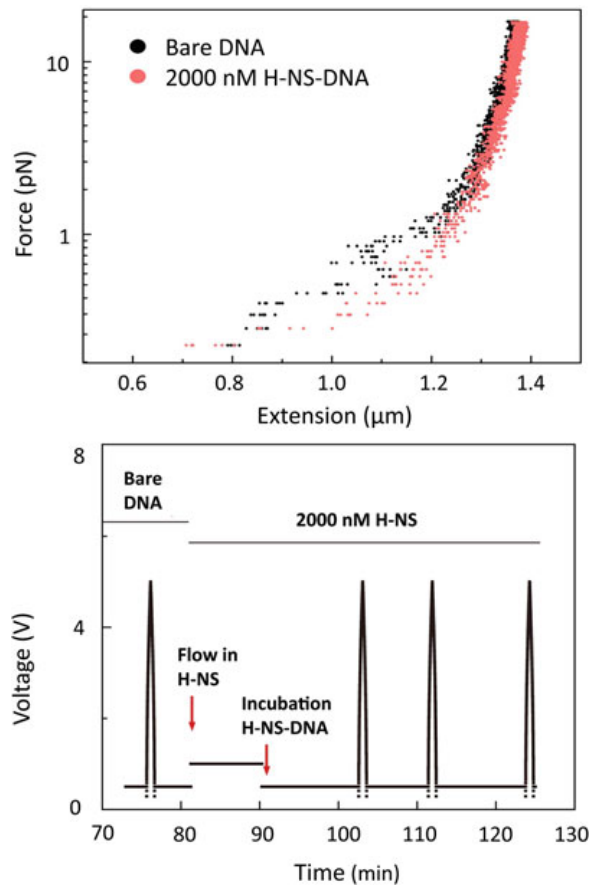


Fig. 5 Stretched H-NS–DNA complex and the flowchart of force application. Top plot shows force–extension curves of 4000-bp bare DNA (red) and H-NS–DNA complex (blue). The free movement of the tethered bead (symmetry in 1.22) is incorporated in the top plot. Bottom plot shows the applied voltage vs time. Low force of 0.8 pN was applied while introducing proteins, and 0.2 pN was applied while incubating H-NS–DNA complexes. The measuring buffer contains 10 mM Tris–HCl pH 8.0, 50 mM KCl, 10 mM EDTA, 5% glycerol, and 1 mM DTT

program subtracts the displacements of x , y , and z on the average traces of the selected stuck beads from each trace. Then the average displacement of the stuck beads is as a starting point, 0.

4. The force power spectrum fitting is described by Nørrelykke and Flyvbjerg [22]. Two important parameters that are obtained from the fitting are the frequency at the corner of the spectrum and the diffusion coefficient. The diffusion coefficient dependence can also be predicted from the bead size and the distance from the surface [23]. Nevertheless, the microsphere size varies in the same batch. Checking whether the theoretical value overlaps with the fitted value gives a reference of the error. This function is already included in the AFS data analysis program. Lorentzian equation and importance-weighted least squares fitting generate the force power spectra fitting [24, 25]:

$$P(f) = \frac{D/(2\pi^2)}{f^2 + (k/(2\pi \times \gamma_{\text{fax}}))^2}$$

where $D = k_B T / \gamma_{\text{fax}}$ is the microsphere diffusion constant, k_B is the Boltzmann constant, T is the temperature, γ_{fax} is the effective drag coefficient, f is the frequency, and k is the stiffness of a Hooke spring acting on the microsphere. In the expression of k : $k = F / (L_{\text{ext}} + R)$, F is the force, L_{ext} is the measured extension of the DNA, and R is the radius of the microsphere.

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