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Dynamic organization of bacterial chromatin by DNA bridging proteins

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Citation

Qin, L. (2020, September 22). *Dynamic organization of bacterial chromatin by DNA bridging proteins*. Retrieved from https://hdl.handle.net/1887/136909

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Author: Qin, L.
Title: Dynamic organization of bacterial chromatin by DNA bridging proteins
Issue Date: 2020-09-22

Chapter 6

Unravelling the biophysical properties of chromatin proteins and DNA using Acoustic Force Spectroscopy

This chapter is based on the following chapter:

Lin, S. N., Qin, L., Wuite, G. J., & Dame, R. T. (2018). Unraveling the biophysical properties of chromatin proteins and DNA using acoustic force spectroscopy. In Bacterial Chromatin (pp. 301-316). Humana Press, New York, NY.

The chapter was jointly written by S.N.L. and L.Q..

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.

1. Introduction

Sound waves can be used to exert forces on objects; this concept is key to the application of controlled forces on surface-tethered microparticles using a method called acoustic force spectroscopy (AFS) [1,2]. By applying force on the microparticle, force is exerted on the tether, DNA or a protein-DNA complex [1,2]. The experimental layout for studying protein-DNA complexes 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 the silica or magnetic beads. In AFS, force is applied vertically to microspheres attached to surface-tethered DNA molecules as is the case for MT. The x-y motion of the bead is monitored and its diffraction pattern is used to determine its z-position from a look-up table (LUT) of radial profiles [1]. The z-position of the bead (minus the bead radius) corresponds to the end-to-end distance of the DNA tether.

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 forced to align at the nodes of the standing wave. By increasing the voltage (changing the amplitude of the wave), beads will experience a larger force (F) towards the wave node [5,6]. The effective force applied on each bead is described by Equation (1).

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

in which P is the acoustic pressure (energy gradient), v is the velocity of particles, and ρ^* (=ρ_p / ρ_m) and κ^* (= κ_p /κ_m) are the density ratio and compressibility ratio between the particle and the fluid, respectively [5]. 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. We routinely apply forces up to ~70 pN to achieve DNA overstretching and protein unfolding, with polystyrene microspheres, 4.5 μm in diameter.

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 MΩ-cm at 25 °C; MilliQ). Prepare solutions at room temperature (RT) and store at 4 °C.

2.1 Stock solutions and beads

2.1.1 Buffer for analysis by agarose gel electrophoresis (see section 3.1)

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

2.1.2 Solutions for reference bead preparation (see section 3.2)

1. Buffer A: 100 mM $Na_2B_4O_7$, 150 mM NaCl, 0.05 % w/v Pluronics (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% Pluronics.

2.1.3 Solutions for cleaning protocol (see section 3.4 and 4.5)

1. Bleach solution: 0.7 M NaClO.
2. Sodium thiosulfate solution: 10 mM $Na_2S_2O_3$.

2.1.4 Solutions for passivation of the flow cell (see section 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 in the buffer.
2. Anti-digoxigenin solution: 200 µg/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) Pluronics (Sigma-Aldrich) in PBS.
5. Buffer F: 0.02 % (w/v) Casein (Roche) and 0.02 % (w/v) Pluronics in PBS.

2.1.5 Solutions for passivation of the flow cell (see section 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' Digoxygenin-labeled primers [Note 1]. The length of DNA is designed to be in the range significantly shorter than the distance from the surface to the wave node. AFS is capable to measure DNA substrates as short as 1 kbp and as long as 45.5 kbps [10]. Table 6.1 and section 3.1.2 summarizes 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°C after purification and concentration determination.

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 6.1)
3. 5' Digoxygenin-labeled forward primer (see Table 6.1)
4. 100% DMSO (New England Biolabs)
5. Recombinant *Taq* DNA polymerase (5 U/µl) (Thermo Scientific)
6. Deoxyribose Nucleotide Triphosphate (dNTP mix) (Thermo Scientific, 2mM)
7. *Taq* DNA polymerase reaction buffer (Thermo Scientific, 10X)
8. GenElute ™ PCR cleanup kit (Sigma-Aldrich)
9. Biorad T100 Thermocycler PCR or any other available PCR machine.
10. 1% agarose gel in 1x TAE
11. Nanodrop® (Thermo Scientific)
12. GeneRuler DNA ladder (ThermoFisher Scientific)

Table 6.1. Primer sequences

Primer name	Sequence (5' - 3')	Modification
32% GC AFS General forward primer	G**T**GTG**T**GTGTG**T**GGTG**T**GTGGTGG ATACATATGCAACTTGAACGGCGTAAAAGAGG	5' Digoxygenin
2000 bp 32% GC AFS reverse primer	G**T**G**T**G**T**G**T**GTGTGGTGTGTGGTGG TCCCTCACTAGTTTAGTACATGAACTG	5' Biotin
50% GC AFS general forward primer	C**T**CTC**T**CTCTC**T**TCTC**T**CTTCTCT GAATTCGCGGCCGCCGTC	5' Digoxygenin
2000 bp 50% GC AFS reverse primer	C**T**C**T**C**T**C**T**CTCTTCTCTCTTCTCT CAGTGGGAACGATGCCCTC	5' Biotin
4000 bp 50% GC AFS reverse primer	C**T**C**T**C**T**C**T**CTCTTCTCTCTTCTCT CAGCGGTGGTTTGTTTGCCG	5' Biotin
6000 bp 50% GC AFS reverse primer	C**T**C**T**C**T**C**T**CTCTTCTCTCTTCTCT CGATCCCCGGCAAAACAGC	5' Biotin
8000 bp 50% GC AFS reverse primer	C**T**C**T**C**T**C**T**CTCTTCTCTCTTCTCT GGTACCAATGTTTTAATGGCGGATG	5' Biotin
T = Modified T		

2.3 AFS Instrument

The AFS system is built around a commercially available inverted microscope combined with commercially available electronics and a commercially available AFS chip (see Fig. 6.1).

1 Microscope: Inverted microscope (Nikon, TE200) with condenser (Nikon, LWD lens), CFI Achromat 40x air objective (Nikon, NA= 0.65).

2 Illumination: Collimated LED (ThorLabs, 660 nm, 1200 mA). [Note2]

3 CMOS camera (Thorlabs, monochrome, pixel size 5.3 μm, 60 fps) connects to the computer.

4 Stage: Z-axis piezo translation stage (PI, MCLS03200), driven by Nano-Drive controller system (MCL, Nano-Drive, MCLC03200) which connects to the computer.

5 Function generator (Keysight, 33220A).

6 Power supply (Voltcraft, 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).

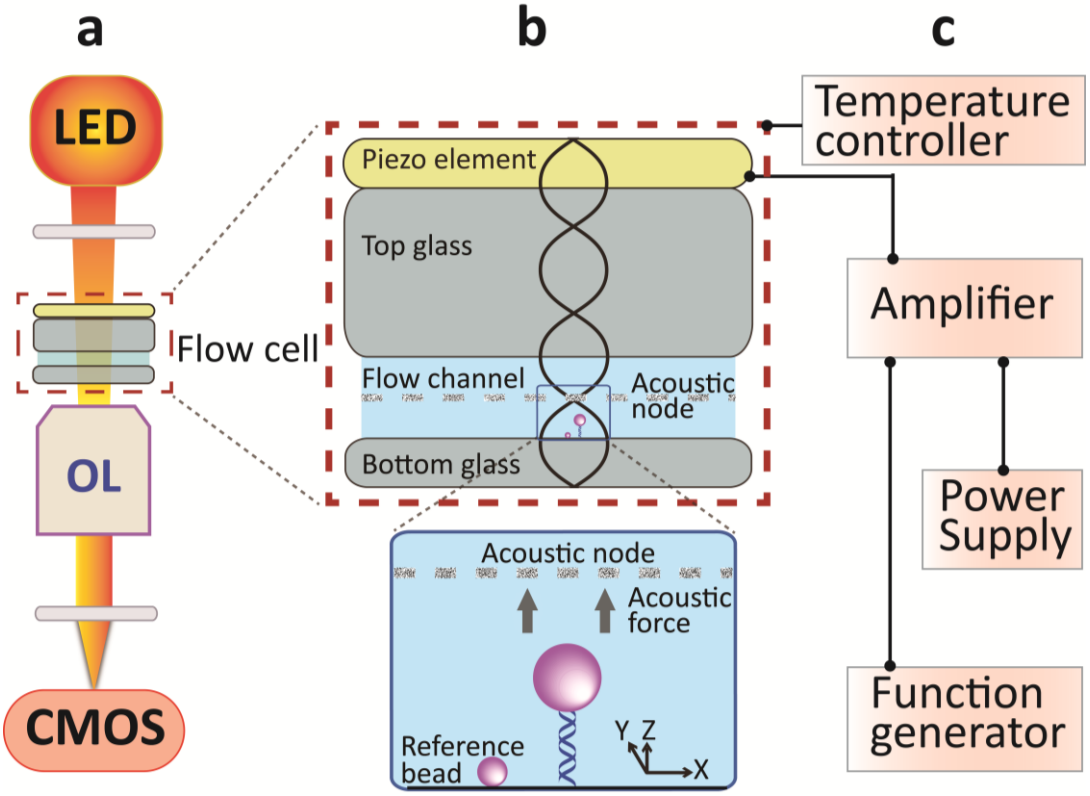

Figure 6.1: **Illustration of the principle of Acoustic Force Spectroscopy.** (a) The acoustic force spectroscopy instrument consists of a flow cell, an inverted microscope visualizing targets with objective lens (OL), temperature controller connected to the AFS-Chip holder, a CMOS digital camera and 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. (c) The temperature controller independently connects to the AFS-Chip metal holder. Both power supply and function generator connect to the amplifier, which controls the piezo element.

10 AFS-chip (LUMICKS B.V., AFS-CH2). (see Fig. 6.2)

11 AFS-chip temperature control holder. (see Fig. 6.3)

12 Custom pressure system. Syringes contain samples and buffers are connected to a gas pressure container. (see Fig. 6.3)

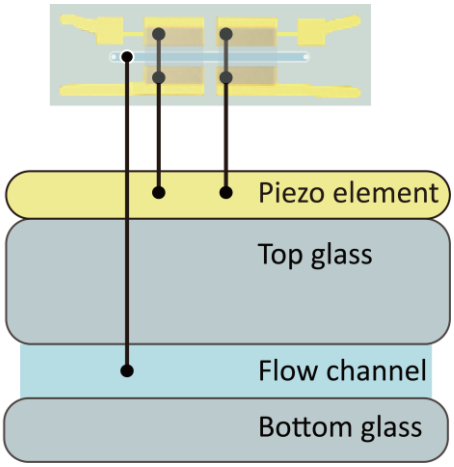

Figure 6.2: Schematic representation of an AFS-chip. The AFS flow cell consists of the piezo element, top glass slide and bottom glass slide with a flow channel in between.

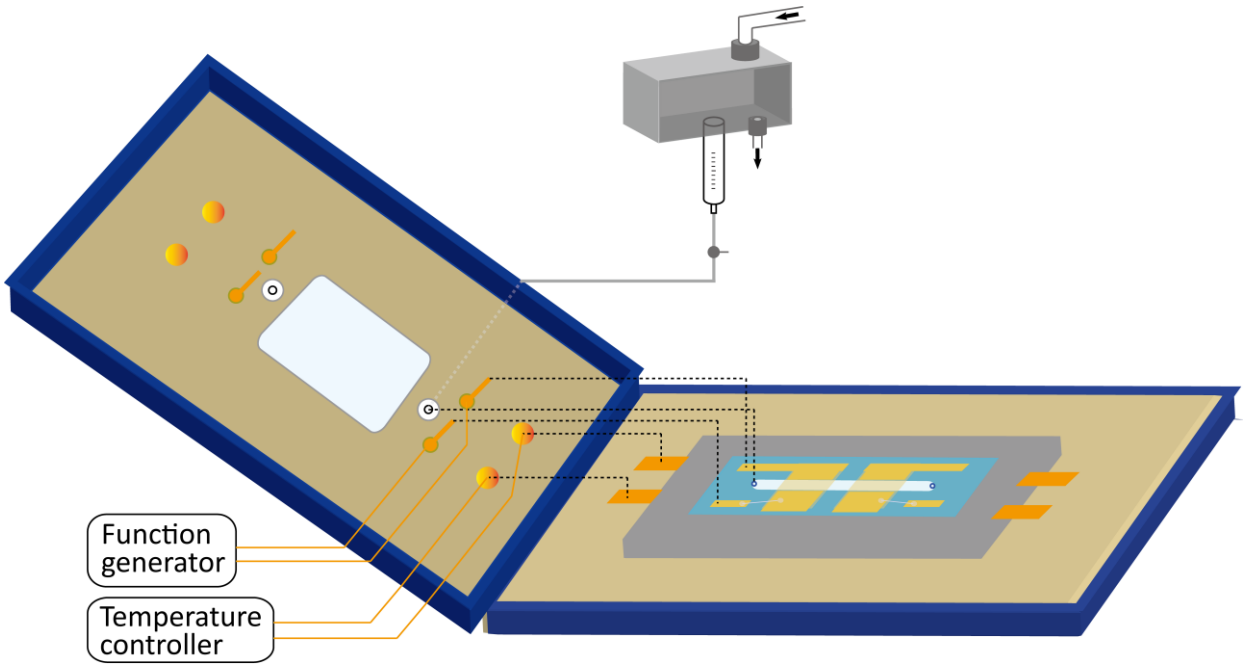

Figure 6.3: Schematic representation of the temperature control holder. The AFS-chip fits into the temperature control metal holder (grey) and this metal holder fits into the flow cell holder (light brown and navy blue) that consists of lid and base. When the lid closed, the metal points on the lid, connected to the function generator and temperature controller, will connect to their counterparts (metal regions) on the piezo and the metal holder (grey), and the holes on the lid, connected to the pressure can by thin tube, will connect to their counterparts (holes) on the AFS-chip.

2.4 Particle tracking, control and analysis

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

1. The tracking program is written in LabVIEW [1] 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, 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 track bead position using a template-matching algorithm.
3) Track the x, y movements of tethered beads and record a look-up-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 force-voltage relationship.

2. The data analysis program is also written in LabVIEW [1] and available online via the same link as indicated under 2.4.1). It is used to:

1) Perform real-time acquisition of three-dimensional bead position in the flow cell.
2) Determine anchor points of all the tracked beads.
3) Correct for drift (see **note 3**) based on positions of the surface attached reference beads.
4) Generate force-extension (force-distance, FD) curves.

3. The WLC model fitting program is written in MatLab [7] and available online https://github.com/onnodb/FDFIT/tree/AFSFitting. 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˚C cold block and dNTP stocks on ice when taken outside the freezer.

Reagent	Quantity
dNTP mix (2mM)	5 µL
Forward primer (10 pmol)	1 µL
Reverse primer (10 pmol)	1 µL
Taq Polymerase buffer (10X)	5 µL
DNA template (10 ng)	1 µL
Taq DNA Polymerase (5U/ µL)	0.2 µL
DMSO (100%)	1.25 µL
MilliQ	Add to 50 µL total volume

2. 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 sec	35
Annealing	65	30 sec	
Extension/Elongation	72	4 min	
Final Elongation	75	10 min	1
Final hold	15	∞	1

3. Load 5 µL 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 figure 6.4 for an example of the purified PCR products.
4. Use Nanodrop® to measure the concentration of PCR products. Store the purified DNA products in MilliQ at -20°C.

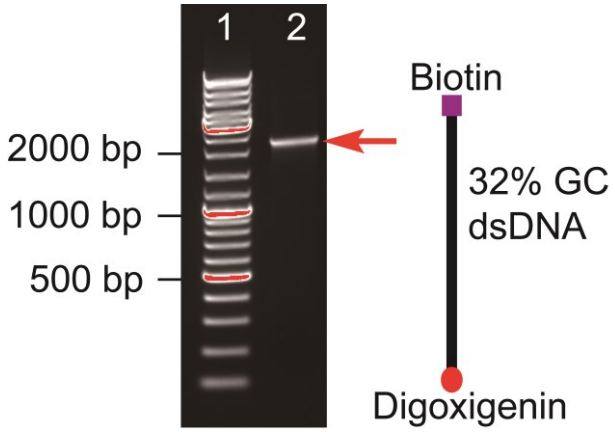

Figure 6.4: **Visualization of PCR product size by agarose gel electrophoresis.** (1) 2 µL of the GeneRuler DNA marker. (2) 2 µL of the purified PCR product, and it is ready for use in Acoustic Force Spectroscopy experiments. The schematic representation of PCR-generated 5' digoxigenin and 3' biotin modified-DNA.

3.2 Preparation of reference beads

1. Mix 8 µl of 0.5% (w/v) 1.9 µm polystyrene beads into 1 ml of buffer A.
2. To coat polystyrene beads with DIGs, incubate the bead solution with DIG-NHS at RT for 3 hours with tumbling.
3. Centrifuge the bead solution at 2000 x g for 1 minute, and discard the supernatant.
4. Remove the free DIG-NHS by washing the bead solution with 1 ml buffer C. Centrifuge at 2000 x g for 1 minute 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 µl of the bead solution in 500 µl PBS.

2. Vortex the sample thoroughly.
3. Centrifuge at 2000 x g for 2 minutes and discard the supernatant, but avoid discarding beads. Resuspend the beads in 500 µl PBS.
4. Repeat steps 2 and 3, carefully discard ~450 µL of supernatant, leaving ~50 µl in tube.
5. Add 4 µl of the reference beads to the 50 µl solution from step 4 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 before each experiment.

1. Install the flow cell chip into its holder. The holder is connected to tubing (see Fig 6.3).
2. Introduce 0.2 ml of bleach into the flow cell through the tubing and incubate for 10 minutes.
3. Rinse the tubing and the flow cell with MilliQ.
4. Introduce 0.2 ml $Na_2O_2N_3$ into the flow cell via the tubing and incubate for 10 minutes.
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, 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 µl of anti-digoxigenin solution into the flow chamber and incubate for 20 minutes.
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. [8]
3. Inject 0.1 ml of buffer D and incubate for 30 minutes for the first time 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 minutes for the second time 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 minutes. 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 minutes.
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. 6.2)
2. Start the bead tracking program in LabView.
3. Select 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 [11].
4. Remove untethered beads by flushing the flow cell with the measurement buffer at a flow rate of 0.2 µl/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” botton 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 in 16.6 ms, each stage 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 x-y motions of the tethered beads in the absence of force, the x-y 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 minute recording is sufficient.
8. To calibrate the force-voltage relationship of each tethered beads, apply a series of different forces on the tethered particles. To collect sufficient data for power spectra fitting (see section 3.7.1), perform 2 minutes of z-position recording for each forces.

9. Apply low force (~10 pN) and slow flowing rate (0.2 µl/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 steps) 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 x-y motions of each tethers.

   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:

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

   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.

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

   In which $\boldsymbol{\lambda_{major}}$ and $\boldsymbol{\lambda_{minor}}$ represent the maximum and minimum value along the axes of the x-y-scatter plot 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.
3. Remove the drift from the measured data by calculating the average drift of the stuck beads. Stuck beads are selected based on their X and Y motions. In our studies we use RMS < 200 / s ≈ 1 (see **Note 4**].

4. Determine the anchor points of the tethers by selecting the x-y motion trace without force applied during the measurement. Use “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.
5. Calibrate the force-voltage relationship by selecting the voltage-time plot in the program where constant voltages were applied. Generate and fit power spectrum. [Note 5]
6. 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 3.7.2)

3.7.2 Extensible worm-like chain model (eWLC) model Fitting

The extensible worm-like-chain model in formula (4) describes the behavior of elastic polymers such as DNA and protein-DNA complexes [8].

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

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, Lp is the persistence length and Lc is contour length. A typical value for the stretch modulus of double-stranded DNA is about 1000 pN [9].

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 (Lp), contour length (Lc), 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 protein concentration dependent manner. In our experimental system, DNA was compacted at the concentration below 400 nM. Above 400 nM, DNA was extended by the filament of HU proteins (See Fig. 6.5).

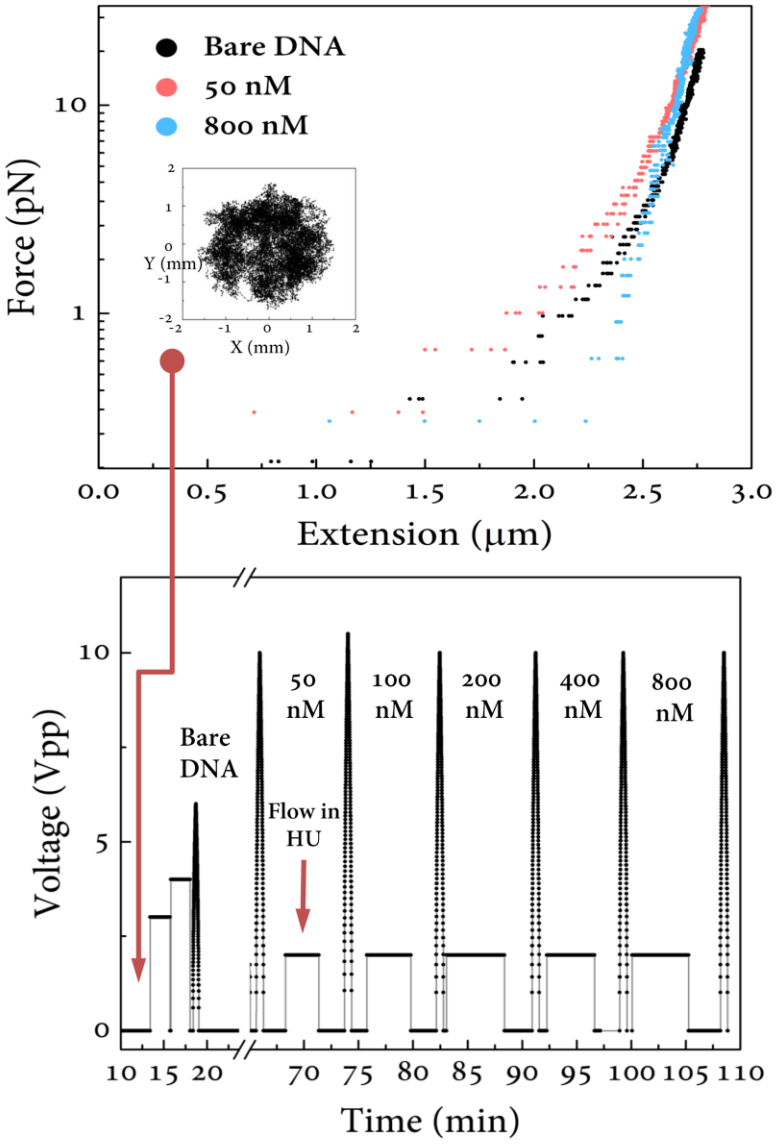

Figure 6.5. Stretched HU-DNA complex and the flow chart of force application. The top figure shows three force-extension curves at HU absent (black), low concentration (red), and high concentration (blue). Top inside is the freely movement of a tether bead (symmetry in 1.2). Bottom plot shows the force v.s. time. Low force ~2 pN was applied while introducing proteins.

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 was studied by using AFS system (see Fig. 6.6). It shows that DNA was stiffened by H-NS at 2000 nM, which is in agreement with previous study [10,11].

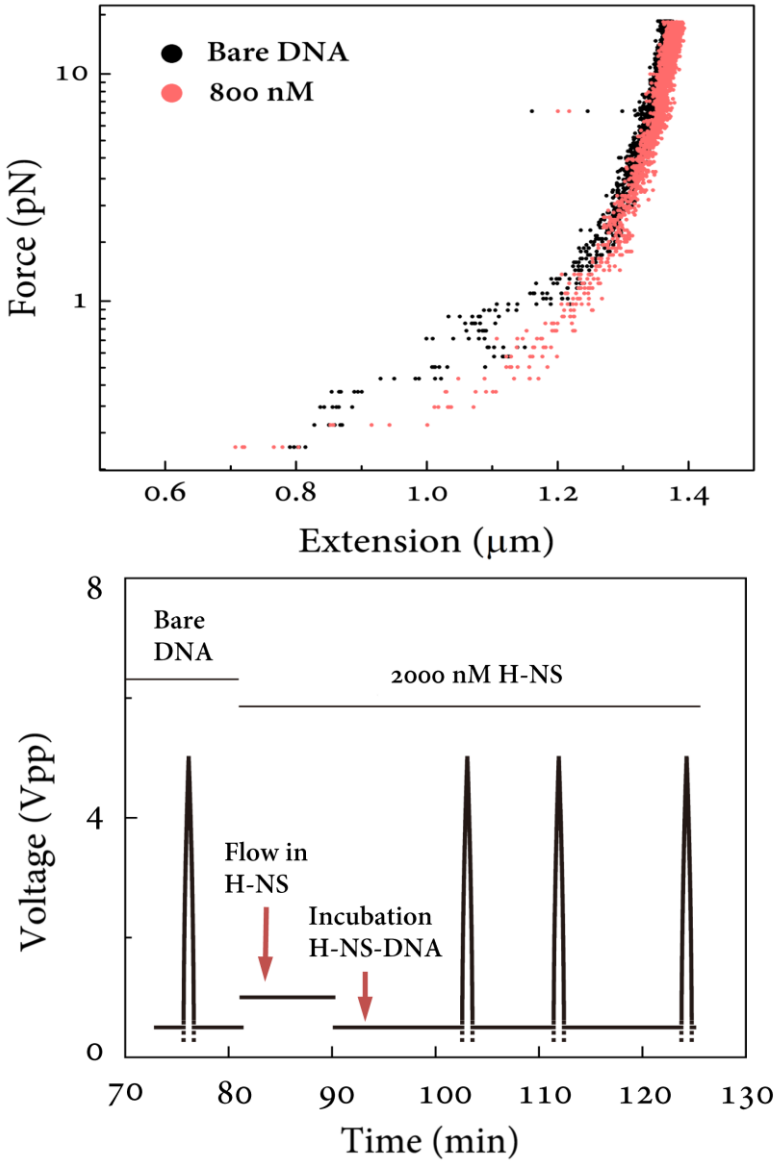

Figure 6.6. Stretched H-NS-DNA complex and the flow chart of force application. Top plot shows force-extension curves of 4000 bp bare DNA (red) and H-NS-DNA complex (blue). Bottom plot shows the applied voltage v.s. time. Low force 0.8 pN was applied while introducing proteins and 0.2 pN was applied while incubating H-NS-DNA complex.

Notes

1. DNA substrates can also be prepared by other approaches, e.g. by filling in Digoxigenin/Biotin at two ends of 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.
4. To correct drift signal, the program subtracts the displacements from x, y, z on the average traces of the selected stuck beads. The average displacement of the stuck beads is as a starting point, 0.
5. The force power spectrum fitting is described in Norrelykke and Flyvbjerg [12]. Two important parameters 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 [13]. Nevertheless, the microsphere size varies in the same batch. Checking whether the theoretical value is overlaps with the fitted value gives a reference of the error. This function is already included in the AFS data analysis program. Lorentzian formula and importance-weighted least squares fitting generate the force power spectra fitting [14,15]:

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

where $\mathbf{D} = \mathbf{k_B T} / \boldsymbol{\gamma_{fax}}$ is the microsphere diffusion constant, $\boldsymbol{k_B}$ *is* the Boltzmann constant, $\boldsymbol{T}$ *is* the temperature, $\boldsymbol{\gamma_{fax}}$ *is* the effective drag coefficient, $\boldsymbol{f}$ *is* the frequency and *k is* the stiffness of a Hooke spring acting on the microsphere. $\boldsymbol{k} = F / (L_{ext} + R)$*;* in the expression

of k, 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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