Acoustic Force Spectroscopy (AFS)

From single molecules to single cells
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From single molecules to single cells
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Chapter 1

Introduction
1.1 Life is a complex system

1.1.1 Complex and Complicated

Complex and complicated are two words that sound and maybe feel very similar, but there is an important difference between them. Perhaps it is easier to understand this difference if you think about the words simple and easy. The famous formula $E = mc^2$ explaining the relationship between energy and mass is simple. I can, however, safely claim that this equation is not an easy concept to develop and understand, but is in fact rather difficult. The same kind of reasoning can be applied to complex and complicated. An airplane is quite complicated. It contains a lot of very specific components that work together making it possible to fly. Because there are so many components with different functions, it is difficult to understand the full working mechanism. However, an airplane is not complex, because it only performs one specialized task. Consequently, by removing just one component, the airplane would most likely be unable to perform its global task. Complexity, on the other hand, can be well exemplified by an ant colony, where millions of ants work together, to build, protect, feed, breed and maintain the colony. Yet, it consists of only ants, each performing a relatively simple task and a single ant is relatively easy to understand. Their interaction, however, gives rise to higher-order, complex behaviour.

A complex system is described as highly composite, built up from very large numbers of mutually interacting subunits, whose repeated interactions result in rich, collective behavior that feeds back into the behavior of the individual parts. Often, the subunits are not completely homogeneous and can be complex themselves, giving rise to an additional layer of complexity.
complicated system can have many parts too. Each part, however, has a very specific functional role and is often not very adaptable to change. One way to discriminate a complex system from a complicated one is to remove one element; a complex system will barely be influenced, while a complicated system will probably stop working.

Apart from simple, complicated and complex systems, there are also chaotic systems. These systems are often unstable, and small changes can have a huge impact on the system, making them extremely hard to predict. An example of a chaotic system is turbulence (Figure 1).

To understand a complicated system, the individual components of the system and their mutual interaction can be studied. For a purely complicated system, the whole system can be understood as a linear superposition of all the components. A complex system, on the other hand, is more difficult to understand. Individual components can be understood, but it is the collective that gives rise to higher-order function, which cannot be directly understood from the sum of all the components. Interdependencies between components and their nonlinear dynamics make it difficult to deduce or predict the higher-order functions from only the individual elements. Therefore, to fully understand a complex system, many approaches are needed. Besides studying the interactions of single components of the system with each other, multiple subunits have to be studied at the same time under various conditions and combined with multiple other subunits, to begin to understand the complete picture.

1.1.2 Life

The amount of different organisms is astonishing: it is estimated that there are ~8.7 million different species on our planet. Only ~1.9 million species of these have been identified and characterized. Because the amount of individuals per species ranges from thousands to quadrillions that have many kind of interaction with each other, life on our planet can be seen as a complex system, with multiple layers of complexity. When you zoom in, you can observe that systems of organisms can be complex, such as the ant colony. If you zoom in further to the level of a single organism, you could find out that they can be complex as well as complicated. At the length scale of an organ, organisms are complicated since each organ has its own specific function and most of all because removing one organ is likely to kill the organism. The opposite is true on the single-cell level, since removing one cell has, most of the time, little effect on the rest of the organism. Therefore, depending on the scale, life can be either complicated or complex.
As biophysicists, we are trying to understand the working mechanisms of life and thereby getting a better understanding of life itself. Often cells are studied, because they are the building blocks of all organisms. The cell can be complicated, if one describes it as a collection of organelles or it can be complex if one zooms in more and looks at the single-molecule level. Because the cell can be complicated as well as complex, it is important to understand what aspects one studies to understand how to tackle the problem of interest. In this thesis, I try to get a better understanding of the cell by studying its components on a molecular level. As this can be seen as a complex system, many approaches are needed to get a full understanding. To this end, we develop novel methodology to approach from a multi-scale perspective the functioning of different components of the cell and of the cell itself.

1.2 The cell

Cells are the smallest units of life that can replicate independently, therefore they are often called “the building block of life”. Their size ranges from 1 \( \mu \)m up to 20 cm and a single organism can consist of one cell, while humans for example contain about \( 10^{12} \) cells\(^4\). Cells are enclosed units, containing organelles that carry out different tasks. At the level of organelles the cell is relatively well understood. For example, the nucleus, which contains the genomic information, can respond to stimuli by synthesizing an RNA molecule (see Figure 1.2). Next, the RNA molecule can be read by the ribosomes that synthesize the proteins, the endoplasmic reticulum regulates the folding of proteins, the Golgi apparatus packages the proteins into vesicles and the cytoskeleton helps transporting these protein cargos (Figure 1.2). Combining
and adding up all the functions and tasks of the cell at the organelle level gives us a good idea of the working mechanisms of the cell, although many details are left out. How misfolding of proteins can lead to Parkinson, how mistakes in DNA repair can lead to cancer or how viruses infiltrate and take over the host cell cannot be understood from this simplified picture. To understand these mechanisms, the cell must be studied at the single-molecule level and, at this level of detail, the cell is a complex system.

In order to get a feeling of the order of complexity of the cell, you could look at the cell as a collection of proteins. Proteins are manufactured on the basis of the genomic code and they are the ‘working horses’ of the cells. A human cell synthesizes about 17 000 different proteins and the number of proteins in a single cell is in the order of millions (3-5 million per cubic micrometer). They complete their tasks alone or in groups. These interdependencies, combined with the huge amount of proteins, make the cell an extremely complex system.

1.3 Methods to study the cell

The cell was first discovered by Robert Hooke in 1665 and the first cell theory, stating that all organisms are composed of one or more cells, was developed by Matthias Jakob Schleiden and Theodor Schwann in 1839. Since then, numerous scientists have studied cell properties and various methods have been developed to study the cell and its components. Light microscopy was first used to visualize the cell and is still one of the most common techniques to study cell behavior. Because of the enormous complexity of the cell, many approaches are needed to understand this system. Consequently, various techniques have been developed and are continuously being developed, for example patch clamping, immunofluorescence, infra-red spectroscopy, mass spectrometry, flow cytometry, computer simulation, gene knockout, force spectroscopy, just to name a few. All these techniques can, in principle, be split up into bottom-up or top-down approaches.

1.3.1 Top-down approach (in vivo)

In essence, a top-down approach studies a system (here the cell) and its components (e.g. single biomolecules or bigger cell structures) in an operational environment (in vivo), in order to gain insight into its working mechanisms. When doing so, microscopy is the preferential tool to monitor the process. However, microscopy itself will always be a passive observation method, therefore it is often used in combination with other techniques allowing for cell manipulation.
Since the first bright-field microscope, lots of different techniques have been introduced to increase imaging capabilities, like phase-contrast, dark-field, interference reflection microscopy and, in particular, fluorescence microscopy, because the use of fluorescence allows for the specific imaging of specific parts of the cell. A big limitation of light microscopy is that the smallest resolvable distance between two objects is determined by the diffraction limit, which is typically in the order of hundreds of nanometers. Recently, super-resolution techniques, like structured illumination microscopy (SIM), stimulated emission depletion microscopy (STED), photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM)\textsuperscript{7}, are able to overcome this diffraction limit and, with these techniques, cell processes can be studied in even more detail. Unfortunately, the resolution achieved with these methods (~10 nm) is still larger than the typical size of a protein (~5 nm), making it impossible to visualize two proteins next to each other, or to observe different conformation states within a proteins.

1.3.2 Bottom-up (\textit{in vitro})

In a bottom up approach, the properties of isolated biomolecules (in vitro) are investigated in order to understand their function and interactions. As expected from a complex system, single-molecule measurements often lead to different outcome than bulk experiments, because, in the latter approach, the stochastic behavior of single biomolecules is averaged out. Some properties of biomolecules can therefore only be obtained by single-molecule experiments. In the bottom-up approach, this can be achieved by isolating individual molecules and observing their behavior using, for example, fluorescence or force probes. It is also possible, and powerful, to combine both methods. Again, fluorescence is passive, but the use of force can actively change the state of biomolecules.

The most common single-molecule force spectroscopy methods are atomic force spectroscopy (AFM), Optical tweezers (OT), Magnetic tweezers (MT) (Figure 5.1). AFM is commonly used as an imaging tool, where a cantilever with a sharp tip scans a surface. Additionally, the tip of the cantilever can be also used as a probe for force-spectroscopy. OT uses a focused laser beam to trap a microsphere and MT applies a magnetic field on magnetic microspheres, to which biomolecules can be attached. By displacing or rotating the microspheres, OT and MT can apply and measure forces on biomolecules of interest.

In order to perform a single-molecule experiment, the molecule of interest
must be isolated and held in place, operations which are often very complicated. Experimental throughput is also often limited, because only one molecule can be studied at a time. Because the single-molecule world is dominated by heterogeneous and stochastic behavior that is very dependent on the environment (e.g. temperature, salt concentration and pH) and the surrounding biomolecules, it will still be a tedious and long process to fully understand all biomolecules and their interactions with each other in every environment. The development of new techniques with high data throughput is therefore essential to move this field forward.

1.4 The outline of this thesis

The key topic of this thesis is the development and application of the novel technique Acoustic Force Spectroscopy (AFS). Using AFS, I study the individual biomolecules present in the cell (e.g. protein and genomic material), as well as properties of individual, whole cells, described in the following chapters:

Chapter 2 introduces the AFS method. Bulk acoustic waves are used to manipulate a surface-tethered microsphere, in a highly controlled fashion. I demonstrate that AFS can apply forces ranging from sub piconewtons to hundreds of piconewtons on thousands of biomolecules in parallel, with sub-millisecond time response and long-time stability. I present a set of proof-of-principle measurements to show the strengths of this technique: performing force-extension curves, constant-force and dynamic force-spectroscopy measurements to obtain the biomechanical properties of DNA, probe DNA-protein interactions and quantify the bond strength of an antigen-anti-body complex.

Chapter 3 describes a set of advances to further improve AFS. I developed a modelling tool to optimize the sample chamber and a calibration method to experimentally validate the modeled force profiles. After optimization, I was able to apply 350 pN on 4.5 μm polystyrene microspheres, without the use of an amplifier, at the coverslip side of the AFS chip. Furthermore, I present the use of a transparent piezo element to generate the acoustic force, making it possible to apply bright-field illumination, without any drawback and I show that AFS can be combined with high-NA oil or water-immersion objectives. These improvements make AFS suitable for an even broader range of applications.

Chapter 4 describes in great detail assays used to perform single-molecule AFS experiments. AFS permits high experimental throughput on many
individual molecules in parallel, a wide range of forces and force loading rates over 6 orders of magnitude. Even though the AFS setup is relatively simple, it can still be challenging to perform high-quality measurements. Therefore, I describe in this chapter how to set up, perform and analyze an AFS measurement to obtain reliable and valuable data.

Chapter 5 presents a study of virus proteins that self-assemble on DNA. Here, AFS is used as a single-molecule method to study the dynamics in real time of two different virus proteins binding and compacting the DNA on a time scale of hours. Combining our results with data obtained from optical tweezers and atomic force microscopy leads to a deeper understanding of the assembly process of these viruses, crucial for the production of viruses as drug delivery systems.

Chapter 6 presents a study of the folding free-energy landscape of a simple and short-handle DNA hairpin using AFS. I resolve folding and unfolding kinetics by observing the state switching of this hairpin from an open to a closed conformation, under the action of both a constant and dynamic force applied on many molecules at the same time. I use the Bell-Evans theory and the Continuous Effective Barrier Approach to map the energy landscape. Our results agree with previous experimental data on this hairpin, validating our approach. Furthermore, I show that the AFS is ideally suited for these kinds of measurements, given its high data throughput and the wide range of loading rates available. Finally, I demonstrate that time and localization accuracy is enhanced by using short construct handles.

Chapter 7 describes how AFS can be applied directly on single cells to measure their adhesion properties. Here, I resolve the adhesion strength and kinetics of individual T-cells binding to a fibronectin-modified surface. I observe that interleukin 7 accelerates the binding kinetics, but adhesion strength remains the same. With these measurements, I demonstrate that AFS can be used to manipulate and track hundreds of cells in parallel in real time, while well-controlled forces can be applied up to 1000 pN. This approach extends the set of applications possible with AFS.

Chapter 8 provides an outlook of how AFS can be further developed and applied. I discuss promising results with the air-filled microspheres, which experience a ~400-fold higher acoustic force than the microspheres currently used. Furthermore, I present a new way of using AFS to measure mechanical properties of cells. Finally, I show that it is possible to measure adhesion strengths and kinetics of cells with AFS at 37°C, approaching a more
physiological environment.
Abstract

Force spectroscopy has become an indispensable tool to unravel the structural and mechanochemical properties of biomolecules. Here we extend the force spectroscopy toolbox with an acoustic manipulation device that can exert forces from subpiconewtons to hundreds of piconewtons on thousands of biomolecules in parallel, with submillisecond response time and inherent stability. This method can be readily integrated in lab-on-a-chip devices, allowing for cost-effective and massively parallel applications.


*, † These authors contributed equally.
2.1 Introduction

Single-molecule techniques have revolutionized the study of biomolecules, allowing experiments, such as force-induced denaturation of biomolecules, that were hitherto impossible\textsuperscript{8–10}. So far, single-molecule instruments using magnetic forces (magnetic tweezers), mechanical forces (atomic force microscopy) and radiation pressure (optical tweezers) have remained rather complex, requiring specialized expertise for construction and operation and restricting their use mainly to specialized biophysics labs. In addition, most single-molecule techniques allow the user to study only a limited number of biomolecules (often one) simultaneously, thereby lowering experimental throughput and limiting statistics\textsuperscript{9,11}. On the single-molecule level, discriminating heterogeneous behavior and rare events from intrinsic stochasticity requires many independent measurements\textsuperscript{9,12}. Novel methods that are simpler and more cost-effective, have a smaller footprint and allow high throughput are desirable\textsuperscript{13–15}.

Here we present a single-molecule manipulation method, Acoustic Force Spectroscopy (AFS), that uses acoustic forces to stretch multiple molecules individually tethered between a surface and a microsphere. We demonstrate that AFS allows the application of controlled forces of up to 120 pN or higher—strong enough to induce major conformational changes such as DNA overstretching and mechanical unfolding of proteins\textsuperscript{8,16,17}, in a highly parallel fashion.

2.2 Results

2.2.1 Experimental setup

In our AFS instrument (\textbf{Figure 2.1a-d}) we used a piezo element driven by an oscillating voltage to resonantly excite a planar acoustic standing wave over a flow cell. A microsphere (with volume $V$) subjected to this standing wave experiences a force $F$ along the vertical ($z$) direction:

$$ F = -\nabla\nabla \left[ \frac{1}{4} \kappa_m \rho^2 - \frac{3}{4} \frac{(\bar{\rho} - 1)}{2\bar{\rho} + 1} \rho_m \nu^2 \right] $$

in which $\rho$ is the acoustic pressure, $\nu$ the acoustic velocity, and $\bar{\rho} (= \rho_b/\rho_m)$ and $\bar{\kappa} (= \kappa_b/\kappa_m)$ the density and compressibility ratio between the microsphere and the medium, respectively\textsuperscript{18}. In the case of polystyrene or
Results 2.2

Figure 2.1 | Principle of Acoustic Force Spectroscopy

(a) The acoustic force device is integrated in a flow cell, and objects are imaged using an inverted microscope with objective lens (OL), digital camera (complementary metal–oxide semiconductor, CMOS), LED light source (455 nm) and 50/50 beam splitter (BS). (b) The flow cell consists of two glass plates with a fluid chamber in between. An acoustic wave–generating piezo plate is attached to the upper glass slide, which has a sputtered mirroring aluminum layer for illumination. (c) A single DNA molecule, attached at one end to the upper glass plate (black stars) and at the other to a microsphere, is stretched by acoustic forces acting on the microsphere. (d) Digital camera image of a DNA-tethered polystyrene microsphere (4.5-μm diameter; DNA length, 8.4 kbp) and a silica reference microsphere (1.5-μm diameter). Scale bar, 5 μm. (e) Theoretically predicted acoustic energy ($E_{ac}$) of the AFS device driven with a peak-to-peak voltage ($V_{pp}$) of 5 V. (f,g) Predicted acoustic (Ac.) pressure distribution at 6.8-MHz (f) and 9.2-MHz (g) resonance frequencies across the glass and fluid layers. (h) Predicted forces for a 4.5-μm-diameter polystyrene microsphere directed along the $z$ direction (0.5-W input power). Microspheres near the upper surface ($z = 0$) experience a force directed away from the surface at 6.8 MHz resonance and toward the surface at 9.2 MHz.

Silica microspheres in water, the force is dominated by the gradient of the squared acoustic pressure, driving the microspheres towards an acoustic pressure node. We calculated resonance frequencies and force profiles of the flow chamber (originally designed for an instrument combining optical tweezers and microfluidics\textsuperscript{19}) using a one-dimensional model (section 2.4.2)\textsuperscript{20} and compared to the experimentally obtained values. Driving the piezo element at a predicted frequency of 6.8 MHz (measured at 6.7 MHz,
determined from the maximum force obtained with a frequency sweep, Figure 2.1e) resulted in a strong pull away from the surface for microspheres near the upper glass–water interface (Figure 2.1f). We used this force to apply tension to biomolecules tethered between the upper surface and the microsphere. The force was constant within 5% when the microsphere’s axial position was changed by 1 μm, which was the length scale of the force-induced extension of the biomolecules used here. Another predicted resonance at 9.2 MHz (measured at 9.0 MHz) pushed microspheres toward the surface, which was useful because it allowed precise determination of the surface location and in principle could facilitate interactions between functionalized microspheres and surface-attached biomolecules (Figure 2.1e).

We determined microsphere positions by analyzing the images obtained with a 40× air-spaced objective (numerical aperture (NA) = 0.75) and a digital camera (Figure 2.1a). Our tracking software is online available (section 2.4.3) and has a precision of ~2 nm in the x and y directions and ~5 nm in z (at 50 Hz), as determined from the standard deviation (s.d.) of traces of immobilized microspheres (Supplementary figure 2.1). We corrected for mechanical drift by tracking the displacement of an immobilized reference microsphere attached to the surface (Figure 2.1c). Bright-field illumination was provided in epi-configuration, through the objective. The light was reflected by an aluminum layer sputtered on top of the flow cell, below the opaque piezo element. This allowed for a simple, compact design compatible with a standard microscope with epi-illumination (Supplementary figure 2.2).

### 2.2.2 AFS applied to tethered DNA

To validate our approach, we applied acoustic forces to stretch individual dsDNA tethers (8.4 kbp, ~2.8-μm contour length) attached at one end to the flow cell surface and at the other end to a polystyrene microsphere (diameter, 4.5 μm) (Figure 2.2a). Without driving the piezo, we observed diffusive motion of the tethered microsphere comparable to results of a tethered-particle motion experiment. Driving the piezo at 9.0-MHz resonance pushed the microspheres toward the surface, allowing us to determine the zero at the z position. Operating at 6.7-MHz resonance pulled the microsphere away from the surface, quenching the Brownian motion of the microspheres in directions parallel to the surface because of the increased tension on the DNA tether.

To quantify DNA tension, we calculated the power spectrum of the microsphere position fluctuations (in the directions parallel to the glass
Figure 2.2 | Acoustic Force Spectroscopy in action
(a) Time traces showing the x (black) and y (red) position of a DNA-tethered microsphere (polystyrene, diameter, 4.5 μm; DNA length, 8.4 kbp). In the first 35 s, the piezo was driven at 9.0 MHz (peak-to-peak voltage (Vpp) = 0.5 V), pushing the microsphere toward the surface. From 35–80 s, no acoustic force was applied, and after 80 s the piezo was driven at 6.7 MHz (Vpp = 2.4 V), pulling the microsphere away from the surface. (b) Measured and fitted mean power-spectra values of the microsphere’s x and y positions using a Lorentzian function (section 2.4.4). Forces obtained were 0.61 ± 0.07, 3.9 ± 0.1 and 11.8 ± 0.2 pN (fit value ± s.d.) at Vpp = 0.6, 1.6 and 2.9 V piezo-driving voltages, respectively (piezo driven at 6.7 MHz). (c) Forces acting on polystyrene and silica microspheres tethered to the glass surface with DNA (length, 8.4 kbp) as determined from power-spectrum analysis (data are mean ± s.e.m., n = 30). Quadratic fits yielded quadratic constants of 1.54 ± 0.06, 0.19 ± 0.01, 0.089 ± 0.003 pN V⁻² (fit value ± s.d.) for the 4.5-, 1.5- and 1.8-μm microspheres, respectively. (d) Noise characterization of the measured end-to-end distance (Lend). Top, Lend as a function of time (acquired at 50 Hz) for different forces. Bottom, s.d. (σ) of the measured length fluctuations and calculated thermal fluctuations (section 2.4.6).

interface) and fitted it with a model, using the tension as a free fit parameter (Figure 2.2b, section 2.4.4). The planar nature of the acoustic waves allowed for the homogeneous application of forces over an area of several square millimeters (Supplementary figure 2.3). As predicted by equation 2.1, tension scaled with the volume of the particle (Figure 2.2c) and was higher for silica
than for polystyrene microspheres owing to silica’s higher stiffness and density. In further agreement with theory, tension on the DNA tether scaled with the square of the applied voltage (Figure 2.2c), allowing for accurate control of tension with the piezo-driving voltage.

Although acoustic forces acting on the microsphere can be changed rapidly, the speed of the tension response is limited by the effect of solvent drag on microsphere motion. For the microsphere sizes we tested, response time was <1 ms in the enthalpic force regime (i.e., at forces above ~1 pN; section 2.4.5). Theory predicts that AFS precision is ultimately limited by thermal fluctuations, which we verified experimentally by determining the s.d. ($\sigma_L$) of microsphere positions (Figure 2.2d and section 2.4.6). Finally, AFS is very stable and thus allowed forces and positions to be measured over extended periods. Measurements of the length of a DNA molecule subjected to a 9-pN force showed no substantial drift over 16 h (Supplementary figure 2.4).

### 2.2.3 Force-extension, constant-force and dynamic force spectroscopy measurements

To demonstrate the use of AFS with a range of forces relevant to biomolecules, we performed experiments on individual dsDNA molecules (Figure 2.3a). AFS allowed us to accurately determine the force-extension behavior of both torsionally unconstrained (8.4 kbp, 2.8-μm contour length) and torsionally constrained DNA (10.1 kbp, 3.4-μm contour length) over the full range of forces for which the double helix remains intact (0–65 pN and 0–110 pN for torsionally unconstrained and constrained DNA, respectively16,17). These experimental curves were well described by the extensible worm-like chain model22.

Next, we used AFS to investigate how protein binding affects DNA mechanical properties by probing RecA filament assembly on torsionally unconstrained dsDNA (8.4 kbp) in the presence of ATP. A single RecA protein binds to 3 bp and increases their contour length by 0.51 nm23. A dsDNA molecule completely coated with RecA thus has a contour length 1.5-fold longer than that of naked DNA. Such an increase could be clearly resolved in force-extension measurements using AFS (Figure 2.3b).

To obtain insight into the dynamics and stochasticity of cooperative RecA binding to DNA, we used AFS to perform constant-force measurements of the extension of multiple DNA molecules simultaneously in the presence of RecA
2.2

Figure 2.3 | Force-extension and constant-force measurements
(a) Stretching curve of 8.4-kbp torsionally (Tors.) unconstrained DNA and 10.1-kbp torsionally constrained DNA and their corresponding extensible worm-like chain fits (8.4-kbp DNA: persistence length, 52 nm; contour length, 2.87 μm; stretch modulus, 2,000 pN; 10.1-kbp DNA: persistence length, 50 nm; contour length, 3.46 μm; stretch modulus, 2,100 pN). (b) Experimental force-extension curves of an 8.4-kbp DNA molecule measured in the absence or presence of 1 μM RecA (pH 6.4). Fits yielded contour lengths of 2.82 ± 0.02 and 4.26 ± 0.04 μm (fit value ± s.d.) for bare DNA and RecA-coated DNA, respectively. (c) Normalized length-time traces of two DNA molecules (8.4 kbp) in the presence of 0.5 μM RecA (pH 7.4): DNA length increased at 40 pN and decreased at 2.5 pN. At 30 pN, stochastic length variations were observed.

and ATP. It is well documented that RecA binding to dsDNA depends strongly on DNA tension: the binding rate increases with tension, whereas the unbinding rate decreases24. Monitoring of DNA extension showed that, indeed, RecA-filament formation was enhanced at a tension of 40 pN, leading to an effective increase in DNA length Figure 2.3c). RecA-filament disassembly was dominant at 2.5 pN, resulting in shortening of the DNA molecule. At an intermediate tension of 30 pN, competition between filament formation and disassembly led to heterogeneity between two DNA tethers: one increasing length owing to filament growth after a rare nucleation event, followed by a period of decrease due to disassembly, the other showing no change.
Figure 2.4 | Dynamic force spectroscopy measurements
(a) Typical field of view (scale bar, 200 μm) of the dynamic force spectroscopy measurements, showing thousands of microspheres (diameter, 2.8 μm). The majority of these were tracked in real time. Zoom shows an area with several regions of interest (black squares) around tracked microspheres (black points) and the identification number given by the tracking software. (b) Rupture-force distribution of the Dig::anti-Dig bond at different loading rates. Each histogram of rupture counts was obtained from a single field of view. Inset, most probable rupture force (F*), obtained from the Gaussian fits, as a function of loading rate. Error bars, s.e.m.; n = 145.

Finally, to demonstrate the massive multiplexing capability of AFS, we used dynamic force spectroscopy to unravel the energy landscape of the digoxigenin (Dig)::anti-Dig antigen-antibody interaction. We tracked over 2,000 microspheres in real time in x and y within one field of view (Figure 2.4a). We applied very strict selection criteria to discriminate singly tethered from doubly tethered and surface-attached microspheres, yielding approximately 8% (~150) singly Dig::anti-Dig–tethered spheres (using a 1.1-kbp DNA linker) in a typical field of view (section 2.4.7, Supplementary figure 2.5). Force calibration was performed independently for each of these spheres (section 2.4.4, Supplementary figure 2.6). When we used the tracking software, a single run of the machine yielded a complete bond-ruptures force
histogram (analysis software is available online, section 2.4.3).

We performed measurements at seven loading rates spanning six orders of magnitude (from $6.3 \times 10^{-4}$ pN s$^{-1}$ to $5.4 \times 10^{2}$ pN s$^{-1}$; Figure 2.4b). Note that even higher loading rates are attainable when a camera with a higher frame rate is used. A plot of the rupture force against the logarithm of the loading rate showed two distinct linear regimes (inset, Figure 2.4b) that correspond to two energy barriers along the unbinding pathway$^{25}$. Linear fits yielded a dissociation rate at zero force of $k_{\text{off}} = (7 \pm 1) \times 10^{-5}$ s$^{-1}$ (fit value ± s.d. given throughout) and a distance from the ground state to the energy barrier of $\Delta x_1 = 1.54 \pm 0.07$ nm at low loading rates; for high loading rates these values were $k_{\text{off}} = (2.7 \pm 0.7) \times 10^{-2}$ s$^{-1}$ and $\Delta x_2 = 0.57 \pm 0.05$ nm (section 2.4.7). Previous single-molecule studies have yielded higher off-rates for Dig::anti-Dig bonds, indicating weaker binding$^{14,26}$, which is most likely because of the different antibodies used$^{27}$ (section 2.4.7). Most dynamic force spectroscopy experiments to date have been performed using atomic force microscopy (AFM), which requires that individual bonds be broken one at a time. When we used the slowest loading ramp ($6.3 \times 10^{-4}$ pN s$^{-1}$), it took on average several hours for a single bond to rupture. Consequently, measuring a histogram consisting of ~150 rupture events with this loading rate using AFM would take several months, which makes this regime difficult to access for AFM measurements.

2.3 Conclusions

AFS is an accurate single-molecule technique that provides insight into protein-DNA and protein-protein interactions by force-extension, constant-force and dynamic force spectroscopy measurements and can readily be extended to studies of other molecules and molecular interactions. Moreover, AFS is a unique single-molecule tool that combines a high–dynamic force range with massive multiplexing capabilities. Also taking into account its relative simplicity, low cost and compactness, which allow straightforward implementation in lab-on-a-chip devices, we anticipate that AFS will help to spread single-molecule methods from the realm of fundamental research in specialized laboratories toward more widespread application in areas such as molecular biology and medical diagnostics.

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2.4 Supplementary methods

2.4.1 Experimental setup

For the force-extension and constant-force measurements (Figure 2.1 and 2.2a–c), a 40× microscope objective was used to image and illuminate the tethered microspheres, together with automatic focus-drift correction (Nikon, Perfect Focus, CFI Plan Fluor 40×, NA 0.75). Images were taken with a CMOS camera (Thorlabs, DCC1545M). For the dynamic force spectroscopy measurements (Figure 2.4) a 10× microscope objective (Nikon, CFI Plan Fluor 10×, NA 0.30) was used in combination with a 0.45× c-mount adaptor (Nikon, MQD42040). Images were obtained with a CMOS camera (Thorlabs, DCC3240M, with a full-frame frame rate of 60 Hz. For illumination, a fiber-coupled blue LED (Thorlabs, M455F1) was coupled into the imaging path with a 50:50 beam-splitter plate (Thorlabs, EBS2). Reference images required for particle tracking in z were obtained by mounting the AFS device consisting of a flow chamber and a piezo plate. The borosilicate-glass flow chamber (Micronit Microfluidics B.V.) consisted of bottom and top glass plates of 170 μm and 1 mm thickness, respectively, the fluid channel width was 2 mm and thickness was 100 μm. On top of the flow cell, a thin (~200 nm) aluminum layer was applied, serving as a mirror allowing for bright-field microscopy in epiconfiguration. The disk-shaped piezo element (Meggitt Sensing Systems, material PZ26) had a diameter of 7 mm and a thickness of 0.22 mm and was equipped with wrap-around gold-nickel (1.5–2 μm nickel and a few nanometers of gold) electrodes allowing connection of both electrodes from the same side. The piezo element was glued to the chamber with a cyanoacrylate adhesive (Permatex, 40150A) (Supplementary figure 2.2). The piezo element was driven with a function generator (Agilent, 33250A) in combination with an RF-amplifier (SCD, ARS 2_30_30, 50-Ω impedance, 10-W max. output power). A maximum driving voltage of 20 V (peak to peak, \(V_{pp}\)) was applied, which did not result in ultrasonic cavitation. Powers used in our measurements did not lead to sample heating of more than a few degrees (Supplementary figure 2.7). When the piezo element was directly connected to the function generator, the maximum voltage that could be applied was \(V_{pp} = 4.0\) V (at 6.7 MHz). The thickness of the individual layers was not critical for the construction of an AFS device because tolerances were quite large (Supplementary figure 2.8). One important feature was the use of a thin layer of glue to attach the piezo element to the flow chamber\(^{20}\), which we achieved by applying force to the piezo plate while gluing.
2.4.2 One-dimensional acoustical model

To provide insight into the operation of the AFS device and for identifying acoustic resonances with the desired properties, we used a one-dimensional model, as previously described. The model considers the different layers (piezo, glass and fluid) as analogous to a set of electrical transmission lines in which the voltage represents the acoustic pressure. The impedance of every layer is determined by the density and the speed of sound of the different layers. The piezo layer is driven by an applied voltage, which is coupled to mechanical quantities via a transformation ratio that is defined by the piezo material. When the thickness, impedance and damping of all layers are known, resonance frequencies, acoustic pressures and velocities along the different layers can be calculated. Our layered resonator was modeled with a transducer of PZT4D material (thickness $T = 220 \, \mu m$, speed of sound $c = 4530 \, m \, s^{-1}$, density $\rho = 7700 \, kg \, m^{-3}$, piezo-constant $h_{33} = 2.37 \times 10^9 \, V \, m^{-1}$, permittivity $\varepsilon_r = 6.195 \times 10^9$), and carrier ($T = 1000 \, \mu m$) and reflector ($T = 170 \, \mu m$) layers of glass ($c = 6000 \, m \, s^{-1}$, $\rho = 2200 \, kg \, m^{-3}$) and water as the fluid ($T = 100 \, \mu m$, $c = 1480 \, m \, s^{-1}$, $\rho = 1000 \, kg \, m^{-3}$). Forces were calculated for polystyrene particles ($c = 1960 \, m \, s^{-1}$, $\rho = 1000 \, kg \, m^{-3}$). The Quality (Q)-factor (ratio between the stored and dissipated acoustic energy) accounts for the damping and all other losses throughout the resonant device. These values are generally much lower than material Q-factors and are commonly determined experimentally. We assumed $Q = 230$ in every layer to match the calculated with the measured forces. The electrodes of the transducer and the glue layer were neglected in the acoustical model because of their relatively small thickness.

2.4.3 Microsphere tracking

Acquired images were processed in real time to extract the microsphere positions in three dimensions. To determine the $x$ and $y$ position, we applied a cross-correlation algorithm, whereas for the $z$ position, a look-up table (LUT) was used, which contained a library of radial profiles previously acquired as a function of microsphere $z$ position. The precision of $x$- and $y$-position determination was about 3 nm, and for $z$-position determination, it was about 5 nm, at an acquisition rate of 50 Hz (Supplementary figure 2.1). Tracking and analysis software is freely available:

http://figshare.com/articles/AFS_software/1195874

2.4.4 Force calibration

Experimental power spectra were obtained for each microsphere and binned
using equal-sized blocks on the frequency axis (having n points per block). These binned power spectra were fitted by a Lorentzian using importance-weighted least-squares:\(^31,\!32\):

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

(2.2)

with \(D = k_B T/\gamma_{fax}\) as the microspheres’ diffusion constant, \(k_B\) Boltzmann’s constant, \(T\) the temperature, \(f\) the frequency and \(k\) the stiffness of a Hookean spring acting on the microsphere in the \(x\) and \(y\) dimension, \(k = F/(L_{ext} + R)\). In this expression of \(k\), \(F\) is the force, \(L_{ext}\) is the measured extension of the DNA and \(R\) is the radius of the microsphere. Because microsphere and surface were in close proximity, their hydrodynamic interaction was taken into account using Faxén’s law to calculate the effective drag on the microsphere:\(^33\). Both \(D\) and \(F\) were used as free fit parameters. The values for \(D\) obtained from the fits were bias corrected by \(D_{cor} = D \times (n/(n – 2))\) to account for the non-Gaussian–distributed data in each block:\(^34\). We plotted the bias-corrected power spectra together with the log-binned experimental spectra (Figure 2.2b). We did not correct for aliasing or motion blurring as these effects did not contribute substantially to the calibrations performed here (Supplementary figure 2.9).

### 2.4.5 Response time

When the acoustic force acting on a tethered microsphere is changed by altering the driving voltage, the tension acting on the molecule does not change immediately but is delayed owing to viscous drag. To estimate the system response time, we used Newton’s second law, neglecting inertia because of the low Reynolds number of the system:

\[
F(t) - \gamma \dot{z}(t) - k_z z(t) = 0
\]  

(2.3)

with \(\dot{z}\) as the time derivative of the position \(z\), \(\gamma\) the drag coefficient and \(k_z\) the stiffness of the Hookean spring acting on the microsphere when it is displaced by a distance \(z\) from its equilibrium position at \(z = 0\). When the acoustic force \(F(t)\) is suddenly switched from \(F_1\) to \(F_2\) at \(t = 0\), then the microsphere approaches its equilibrium position following:

\[
z(t) = \frac{F_2}{k_z} \left(1 - \frac{F_2 - F_1}{k_z} e^{-\frac{k_z}{\gamma} t}\right)
\]  

(2.4)

The response time of the system is thus of the order \(\gamma/k_z\), which is ~0.4 ms for
a 4.5-μm diameter microsphere, assuming a 8.4-kbp DNA stretched within the enthalpic regime \( (k_{\text{DNA}} \approx 100 \text{ pN } \mu\text{m}^{-1}) \). This is much longer than the switching time of the driving voltage (which is of the order of 100 ns) and the time it takes until the acoustic standing wave reaches its final amplitude \( (2Q/\omega_0 \approx 10 \mu\text{s}) \). The response time can thus be reduced by increasing system stiffness (using shorter DNA, as in Chapter 6) or decreasing microsphere drag (using smaller microspheres section 9.1).

### 2.4.6 Thermal fluctuations and instrumental uncertainty

The tethered microspheres undergo Brownian motion, leading to fluctuations in DNA end-to-end length. The s.d. of these fluctuations are estimated to be\(^{12}\):

\[
\sigma_{\text{therm}} = \sqrt{\frac{2k_BT}{\pi k_{\text{DNA}}} \arctan \left( \frac{2\gamma_{\text{fax}}fs}{k_{\text{DNA}}} \right)}
\]

(2.5)

with \( fs \) as the sampling frequency. For a 4.5-μm microsphere tethered by a DNA molecule with length 8.4 kbp and stretched with a force of 8.0 pN, position fluctuations with an s.d. \( \sigma_{\text{therm}} \approx 3 \text{ nm} \) are expected (sampling frequency 50 Hz). We measured larger fluctuations \( \sigma_L \approx 10 \text{ nm} \), indicating that the instrumental uncertainty lay around 10 nm. A major contribution to this uncertainty was the tracking uncertainty, which was 5 nm for immobilized microspheres (Supplementary figure 2.1) and which was most likely larger for tethered spheres owing to motion blurring.

### 2.4.7 Dynamic force spectroscopy measurement

Bond-rupture measurements were performed to characterize the force-dependent unbinding kinetics of digoxigenin (Dig) and its antibody (polyclonal anti-digoxigenin (anti-Dig) from sheep, Roche, Cat. No. 11 333 089 001). In the assay, DNA molecules (1,091 bp) labeled with a single Dig antigen at one end and a streptavidin at the other end were used, which were tethered between an anti-Dig–coated surface and to a 2.85-μm streptavidin–coated microsphere. Since the Dig::anti-Dig bond is by far the weakest link, all rupture events could be safely assigned to these bonds (Supplementary figure 2.6).

Note that our experimental approach allowed us to perform such experiments for a few thousand microspheres in parallel. Evaluation of this large amount of measured position data to extract rupture forces was done in parallel as well. It was therefore not more time consuming to analyze thousands of traces than to analyze a single trace, apart from the computation time (which was well below 1 min). Analysis software is freely available (http://figshare.com/articles/AFS_software/1195874 and Supplementary...
Supplementary methods 2.4

Tracking the positions of all microspheres individually makes it possible to discriminate between single-tethered microspheres and stuck or multiple-tethered microspheres (Supplementary figure 2.5). By selecting on the total amount of motion both microspheres that were stuck to the glass or hindered in some way in their motion (low RMS) and microspheres that were tracked poorly (high RMS due to tracking errors) were discarded. The amount of motion was quantified using root mean square motion of the microsphere in x and y over a period of more than 15 minutes:

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

with x and y are the average positions over time t. In our experiments, microspheres with RMS values below 350 nm and above 550 nm were discarded. Determination of the symmetry of motion allows discrimination between microspheres tethered with a single and multiple DNA molecules. Only single-tethered particles exhibit symmetrical motion around their anchor points. Motion symmetry was calculated using the covariance matrix:

$$\Sigma = \begin{pmatrix} \sigma_{xx} & \sigma_{xy} \\ \sigma_{yx} & \sigma_{yy} \end{pmatrix}$$

With

$$\sigma_{xy} = \langle xy - \bar{x}\bar{y} \rangle_t$$

The eigenvalues (1, 2) of $\Sigma$ are equal for a perfectly symmetric motion. We used $s = \sqrt{\lambda_{\text{max}}/\lambda_{\text{min}}} < 1.2$ as a threshold. Forces on single-tethered microspheres were then calibrated by applying a low acoustic force for a few minutes and measuring the microsphere positions. Power spectra of the x and y-motion were calculated and fitted for every individual microsphere. Finally a linear force ramp was applied (because of the quadratic dependence of force on applied voltage this corresponds to a voltage that increases like a square root function). The voltage and corresponding force at which a bond rupture takes place was determined from the frame at which a given microsphere lefted the region of interest. To determine this frame, the measured region of interest was compared to a template image made when the microsphere was present at the beginning of the measurement. This was done by setting the following threshold:

$$\Delta \text{Template} = \sum_{n=1}^{144} \sqrt{(I_{\text{measured}} - I_{\text{template}})^2} > 200$$
with \( I_n \) the intensity of the \( n \)-th closest pixel to the sub-pixel tracked center of the microsphere (Supplementary figure 2.6).

Energy landscape of the antibody-antigen bond. The lifetime due to thermal activation of a weak non-covalent bond decreases when subjected to a force. Applying a force tilts the energy landscape, effectively lowering energy barriers, decreasing the likelihood that the bond stays intact. The faster the force is increased, the less time the system has to overcome the energy barriers via thermal fluctuations. Therefore, the most probable rupture force of a population of bonds will increase as a function of loading rate. Linear regimes that appear over multiple orders of magnitude of loading rates can be interpreted as distinct energy barriers along the unbinding pathway. For the DIG:anti-DIG bond two regimes are observed, which we associate with two barriers. Parameters of these barriers are obtained by fitting the linear regimes with

\[
F^* = \frac{k_B T}{\Delta x} \ln \left( \frac{r\Delta x}{k_B T \cdot k_{off}} \right)
\]

where \( \Delta x \) is the distance between the bound and the transition state, \( k_{off} \) is the dissociation constant at zero force and \( r \) is the loading rate.

### 2.4.8 DNA tethers

Both torsionally unconstrained (8.4 kbp) and constrained (10.1 kbp) DNA tethers were used. The 8.4 kbp DNA was obtained from a pKYBI vector that was restricted with KpnI and EcoRI (Fermentas), resulting in a 8364 bp fragment. To the 3’-end overhang, a 29-mer (5’-C(T-DIG)CTC(T-DIG)CTC(T-DIG)TC TC(T-DIG)CTC TT GTAC -3’) was ligated. The 5’-end overhang was filled in using Klenow exo- polymerase (Fermentas) using biotin-14-dATP (Invitrogen) and dTTP (Fermentas), resulting in a DNA construct labeled with biotin on one end and digoxigenin on the other, on the same strand. The 10kbp torsionally constrained DNA was a gift by David Dunlap. This plasmid is based on pWM530 and was generated by doubling the length of an earlier construct to give an 11564 bp sequence. Double digesting with Spel and ApaLI produced a fragment of 10160 bp, which subsequently was ligated to ~1.4 kbp PCR fragments as tethers, containing either biotin or digoxigenin. For the bond rupture measurements of DIG (digoxigenin) and antiDIG DNA (1091 bp) with a single DIG label was used as described previously. The DNA tethers were attached to the surface using Anti-Digoxigenin (Roche, Cat. No. 11 333 089 001). To this end the flow cell was incubated for several minutes with an antibody-containing solution (20 μg/ml) for several minutes. Streptavidin-
coated polystyrene (Spherotech) or silica microspheres (BaseClear) were attached to the other end of the DNA molecules.

### 2.4.9 Buffers and RecA solutions

Experiments on naked DNA were conducted in PBS (138 mM NaCl, 2.7 mM KCl and 10 mM phosphate (pH 7.4); Sigma) supplemented with 5 mM NaN3 (sodium azide; Sigma), 0.5 mM EDTA (ethylenediaminetetraacetic acid; Sigma) and 20 mg ml⁻¹ Casein (Sigma). To fully coat DNA with RecA (Figure 2.3b), a 70 mM MES (pH 6.4) buffer (2-(N-morholino)ethanesulfonic acid; Sigma) was used, with 1 μM RecA (New England Biolabs), 50 mM KCl, 10 mM MgCl₂, 1 mM DTT (dithiothreitol; Sigma) and 1mM ATP (adenosine triphosphate; Sigma). To analyze the binding and unbinding of RecA to DNA (Figure 2.3c) a 70 mM Tris-HCl (pH 7.5) buffer was used, containing 0.5 μM RecA, 10 mM MgCl₂, 5mM DTT and 1 mM ATP.
2.5 Supplementary figures

Supplementary figure 2.1 | Accuracy of tracking 4.5 μm diameter polystyrene microsphere

Measurements are performed at 50 Hz in x, y, and z of a (a) and a 1.5 μm diameter silica microsphere (b). These microspheres were stuck nonspecifically to the flow cell surface. To correct for mechanical drifts a reference microsphere (1.5 μm diameter silica) was tracked simultaneously and its position was subtracted. The standard deviations of the position distributions for the 4.5 μm diameter polystyrene microsphere and 1.5 μm diameter silica microsphere are \( \sigma_{x,4.5} = 3 \text{ nm} \), \( \sigma_{y,4.5} = 3 \text{ nm} \), \( \sigma_{z,4.5} = 4 \text{ nm} \) and \( \sigma_{x,1.5} = 2 \text{ nm} \), \( \sigma_{y,1.5} = 1 \text{ nm} \), \( \sigma_{z,1.5} = 5 \text{ nm} \) respectively.

Supplementary figure 2.2 | Picture of the AFS device

The piezo element, with wires connected to the electrodes, is glued onto the aluminum layer. A US quarter dollar coin is placed at the side for size perspective.
**Supplementary figure 2.3 | Histogram of the measured force distribution**
The measured force on DNA (8.4 kbp) tethered microspheres (silica, diameter 1.5 μm) distributed over a distance of more than a millimeter. The grey curve represents the force distribution expected from the size variation from microsphere to microsphere ($\sigma_{\text{microsphere}} \approx 6\%$). This variation is probably the limiting factor of the force homogeneity in our setup.

**Supplementary figure 2.4 | The stability of the AFS setup**
The measured end-to-end distance of a DNA molecule (10.1 kbp) to illustrate the stability of the AFS apparatus. The DNA molecule was tethered to a 4.5 μm polystyrene microsphere that was subjected to an acoustic force of 9 pN. No significant drifts were observed over a timescale of 16 hours.

**Supplementary figure 2.5 | Selection criteria for single tethers**
XY scatter plots for a microsphere that was stuck (left panel), double tethered (middle panel) and single tethered (right panel). Stuck microspheres were discarded by their low RMS value and microspheres tethered to multiple molecules were discarded by their asymmetric motion (high symmetry value).
Supplementary figure 2.6 | Typical bond-rupture measurement
A typical bond-rupture measurement of a DIG::anti-DIG bond. This measurement was performed for thousands of microspheres in parallel. At the beginning of the measurement no force was applied to determine the RMS and symmetry value of each tether in order to discriminate between single tethered microspheres and microspheres that were stuck or tethered to multiple molecules (Supplementary figure 2.5). To calibrate forces acting on individual microspheres, a constant voltage was applied for several minutes and the power spectrum of the quenched x, y motion was analyzed for each microsphere independently. Finally, a linear force ramp (~0.02 pN/s) was applied. After the Dig::anti-Dig bond breaks, the microsphere leaves the selected tracking region of interest, leading to an increased value of Δ Template (equation 7.1) yielding a random tracked positions. Note that, in these experiments, the positions were not corrected for drift.
Supplementary figure 2.7 | Sample heating

Temperature measurement of the sample using a Platinum thermocouple that is embedded in the upper glass layer close to the piezo. The left panel shows that the heating is approximately $3^\circ C$ if $4 \text{ Vpp}$ is applied for several minutes. Using a heatsink, a block of aluminum that is connected to the flow cell next to the piezo, decreases heating of the sample by a third. A more efficient heatsink will probably reduce the heating further. The right panel shows that we measure almost no heating for fast force ramps to high voltages (20 Vpp). Note that heating can be decreased if silica microspheres are used instead of polystyrene spheres since less than half the power is needed to achieve the same forces. Another option to decrease the amount of input power is simply using larger microspheres.
Supplementary figure 2.8 | Influence of layer thicknesses

Simulated frequencies of three acoustic resonances of the probe chamber and the corresponding forces at the upper glass/water interface per electrical input power of the transducer when varying the thickness of an individual layer. A positive force is directed towards the fluid layer and is calculated for 4.5 μm polystyrene microspheres. The dashed lines indicate the actual thicknesses for our device, which are also the values used in the simulation for the layers that are not varied. The results show that varying the fluid layer thickness has the largest influence on the force.
Supplementary figure 2.9 | Effect of blurring and aliasing
Theoretical power spectra calculated for a typical, tethered microsphere (diameter 4.5 μm; DNA length 8.4 kbp) that is subjected to a force of 5 pN. For this frequency range motion blurring due to the finite integration time (15 ms) effectively suppresses the extra power due to aliasing. Therefore there is no significant difference between the power spectrum without blurring or aliasing effects (black line) and the power spectrum that takes both effects into account (blue line).
Chapter 3

Tuning the music

Acoustic Force Spectroscopy 2.0

Abstract

AFS is a recently introduced high-throughput single-molecule technique that allows studying structural and mechanochemical properties of many biomolecules in parallel. To further improve the method, we developed a modelling tool to optimize the layer thicknesses, and a calibration method to experimentally validate the modeled force profiles. After optimization, we are able to apply 350 pN on 4.5 μm polystyrene beads, without the use of an amplifier, at the coverslip side of the AFS chip. Furthermore, we present the use of a transparent piezo to generate the acoustic force and we show that AFS can be combined with high-NA oil or water-immersion objectives. With this set of developments AFS will be applicable to a broad range of single-molecule experiments.

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3.1 Introduction

The ability to mechanically manipulate single biomolecules is leading to insights in fundamental cell processes\textsuperscript{8,9,37,38}. Single-molecule force-spectroscopy techniques are commonly used to study, for example denaturation of biomolecules\textsuperscript{36}, the binding, unbinding and folding of proteins\textsuperscript{39} and the replication and repair of nucleic acids\textsuperscript{10}. However, single-molecule instruments can be rather complex and often have a low experimental throughput\textsuperscript{9,11}. Moreover, the dynamics of single molecules are intrinsically stochastic, meaning that, to probe the heterogeneous behavior, many independent measurements should be performed\textsuperscript{9,12}.

Acoustic Force Spectroscopy (AFS) is a recently introduced single-molecule technique that distinguishes itself by a high experimental throughput, a wide range of forces that can be applied and an unmatched range of force loading rates (Chapter 2). In its original implementation the method has several drawbacks: it makes use of an opaque piezo-element to generate the acoustic force, the force profile perpendicular to the surface is not constant over the fluid layer and it is not possible to apply significant forces close to the coverslip side.

Here we present innovative solutions to address these limitations. The use of a transparent piezo-element allows trans-illumination of the sample, which increases the tracking accuracy of microspheres. We optimize the layer thicknesses of the system to generate a strong force at the bottom surface and quantify the modeled forces by direct measurement of the force profile. Furthermore, we show that AFS is also compatible with high numerical aperture (NA) water or oil-immersion objectives. In addition, we made the force gradient that is present in the fluidic cell flatter by using multiple frequencies at the same time. A flatter force profile improves the accuracy of the force application because of the diminished force variations when a measured construct changes length. Finally, we demonstrate that it is possible to turn AFS into a distance clamp.

3.2 Material and methods

3.2.1 AFS-chip properties

An AFS chip consists of two glass layers with a fluid channel in between and a piezo element on top. The flow cells are custom fabricated by LUMICKS B.V.. Flow cells of 2 different dimensions are used in this report. Flow cell 1 has layer thicknesses of 1000, 100 and 175 μm and flow cell 2 has 616, 84 and 175
Figure 3.1 | Illustration of the AFS setup

(A, I) The AFS chip is imaged using an inverted microscope with objective lens (OL), a digital CMOS camera and LED light source (455 nm). (a II) The flow cell consists of two glass plates with a fluid chamber in between. A transparent piezo element is attached to the upper glass slide. Using an overhang the piezo is electronically connected. (a III) Diagram hardware configuration, the function generator is controlled by the computer via USB, while it is connected in series with an amplifier, a transformer and to the piezo element. An oscilloscope is connected to measure the voltage over the piezo. (b) A picture of the AFS chip (I,III) and a digital camera image (b II and IV) are shown for the non-transparent and transparent piezo chip, respectively. (c) Modeled force profiles for transparent and opaque piezo element, in both cases two resonance frequencies are displayed with a force directed toward and away from the top surface (indicated with the arrows acting on microspheres). Forces are calculated for a 4.5-μm-diameter polystyrene microsphere with an input power of 0.1-W.

μm for the matching, fluid and capping layers, respectively (Figure 3.1a II). The total tilt of the flow cells is less than 1 milliradian and the surface roughness is less than 1 nm (LUMICKS specifications). Both flow cells are glued (using Permatex, 40150A) either to a 500 μm piezo or to a 200 μm piezo resulting in 4 different types of AFS-chips.

3.2.2 Electrical connection of the chip

The piezo is driven by a function generator (Siglent, SDG830) at frequencies ranging from 1 to 30 MHz via a power RF-amplifier (SCD, ARS 2_30_30, 50-Ω impedance, 10-W max. output power). A transformer is used to match the output electrical impedance of the amplifier and the function generator to the electrical impedance of the layered resonator. The electrical impedance of each layered resonator configuration used is measured and a custom transformer is made to match the impedance (Supplementary figure 3.1). The peak-to-peak voltage over the piezo was recorded with an oscilloscope (Tektronix, TDS1002). The function generator is connected to the computer via USB and controlled with a LabVIEW interface (Figure 3.1b III).
3.2.3 Imaging the sample
An inverted bright-field microscope (Nikon eclipse Ti-E) was equipped with a 1 megapixel 60 Hz frame-rate CMOS camera (Thorlabs, DCC3240M) read out via USB by a computer. A collimated LED (Thorlabs, M455L3-C5) was coupled into a condenser lens (Nikon, LWD 0.52) to illuminate the sample. The sample was then imaged with a 40× or a 10x microscope objective (Nikon, CFI Plan Fluor 40X and CFI Plan Fluor 10X) in combination with a 0.45× c-mount adaptor (Nikon, MQD42040). A nanometer piezo translation stage (PI, P-517.2CL) driven by a digital piezo controller (PI, E-710.4CL) was used, providing the option to generate a look-up table (LUT) to determine the z position of the microspheres.

3.2.4 Computer
We use a computer with two Xeon E5 2643v2 processors, both containing 6 cores for parallel processing. This computer was used to run a LabVIEW program dedicated for controlling the experimental sequence (section 3.2.5) and to run a MATLAB script to determine the acoustic properties of different dimensions of the system.

3.2.5 Microsphere tracking.
Acquired images were processed in real time to extract the microsphere positions in three dimensions. To determine the x- and y-position, we applied a quadrant-interpolation algorithm\(^40\), whereas for the z position, a look-up table (LUT) was used, which contains a library of radial profiles previously acquired as a function of microsphere z position\(^30\). The precision of x- and y-position determination was about 1.3 nm, and for z-position determination, it was about 3.8 nm, at an acquisition rate of 60 Hz (Supplementary figure 3.2). Tracking software is freely available (http://figshare.com/articles/AFS_software/1195874).

3.2.6 DNA Tethering
Both torsional unconstrained pKYBI (8.4 kbp)\(^41\) and lambda (45.5 kbp) DNA tethers were used (Figure 2.3). Experiments on DNA tethers were all conducted in PBS (138 mM NaCl, 2.7 mM KCl and 10 mM phosphate (pH 7.4); Sigma). Before experiments are conducted, flow cells were treated with polystyrene (3% w/v, Sigma-Aldrich, 331651) in toluene solution to make the surface hydrophobic. This solution was rinsed out with PBS.

For attachment of the pKYBI DNA, the flow cell was incubated with the anti-Dig antibody-containing solution (20 µg/ml, Roche, Cat. No. 11 333 089 001) in
PBS for 20 min. A two-step passivation was used, incubating Bovine Serum Albumin (BSA) (0.2% w/v, Sigma–Aldrich, A7906) and then pluronic (0.5% w/v, BASF, pluronic® F 108NF Prill) both 30 min in PBS, reducing nonspecific sticking of the DNA and microspheres. Thereafter, buffer containing the DNA was incubated for 20 min. In the last step, 4.5 μm streptavidin coated polystyrene microspheres (0.15% w/v, SVP-40-5, Spherotech, Inc) were flown in to the chamber to let them incubate for 20 min.

For attachment of Lambda DNA, biotin-modified casein was produced by reacting casein solution (2% w/v, Sigma–Aldrich, C8654) with an equimolar amount of EZ-Link™ Sulfo-NHS-LC-LC-Biotin (Thermoscientific, 21338) in a borate buffer (pH 8.3) for several hours. The reacted solution was stored at -20C, and could after thawing be used for surface coating. A mixture of biotin-modified casein (0.02% w/v) and casein (1% w/v) in PBS was incubated for 20 min., then streptavindin (0.0167 μg/ml, Thermo Fisher, 43-4301) in PBS was incubated for 20 min. Thereafter, the DNA was incubated for 20 min. In the last step, the 4.5 μm streptavidin coated polystyrene microspheres were incubated for 20 min.

### 3.3 One-dimensional acoustical model

The analysis of the forces on suspended particles resulting from acoustic pressure dates back to the 1930's and 1950's. Here, we will make use of the approach developed by Gor'kov, who derived the acoustic radiation force \( F^{rad} \) as the gradient of a potential \( U^{rad} \), which depends on the acoustic kinetic and potential energy densities and on the density and compressibility of both the fluid and the suspended particles. This approach allows us to predict the resonance frequencies and the corresponding forces in the fluid layer of our layered resonator.

#### 3.3.1 KLM model

We use for our model an equivalent circuit model, KLM. The concept of this approach is to use an electric circuit as an analogue for the propagation of acoustic field. The first step is to calculate the cumulative impedance \( Z \) of all layers together, as described in refs, using the equations listed by Kinslers et al. From the impedance of the system follows the transducer response, allowing computation of the propagation of the acoustic energy through the layers. An acoustic force is applied on each layer boundary and from this force the pressure \( p(x) \) and velocity \( v(x) \) fields can be calculated. Knowing this, the radiation force can be computed:
\[ F^{rad}(x) = -\nabla U^{rad}(x) \]  

(3.1)

Where \( U^{rad} \) is given by:

\[
U^{rad} = V_p \left( \frac{1 - \kappa_p \kappa_f}{4} - \kappa_f \rho_f |p(x)|^2 - \frac{3}{4} \left( \frac{\rho_p}{\rho_f} - \frac{1}{\rho_f} \right) \rho_f |v(x)|^2 \right) 
\]

(3.2)

Here \( V_p \) is the volume of the particle, \( \kappa_p \) and \( \kappa_f \) the compressibility of the particle and the fluid, respectively and \( \rho_p \) and \( \rho_f \) the density of the particle and the fluid, respectively. With these equations it is possible to calculate the direction and the strength of the acoustic force that can be applied on a particle. We have developed a MATLAB code that, given a set of system configurations and properties, can calculate the resonance frequencies and the corresponding force profiles within the fluid layer. Typically there are several strong force peaks that are potentially useful for force application on the microspheres (Supplementary figure 3.3). This software is freely available (https://figshare.com/articles/AFS_1D_model/3166753).

In our model losses in each layer are incorporated by the quality factor, \( Q \). A value for \( Q \) is hard to predict because it includes effects of viscous dissipation in the bulk, viscous friction at the walls, sound waves emitted into the chip holder, fluidic connectors, surrounding air, electronic coupling and roughness of the materials used. In our model \( Q \) is considered to be constant over all frequencies and for all layers as used in our original implementation (section 2.4.2). Note that our model is 1-dimensional and can only be used for a system that is constant over the measured surface. From Supplementary figure 2.8, it can be concluded that small changes of the fluid-layer thickness have the largest effect. While the resonance frequencies are relatively insensitive, a change in fluid-layer thickness of less than one micrometer will result in a relative force change of less than 10\% (for the strong resonance peaks). The surface roughness of our glass materials is less than 1 nm, therefore the roughness will have a negligible effect on the generated forces. The tilt of the glass layers in our chips is at most 1 milliradian, causing a fluid-layer thickness change of at most 1 \( \mu \)m over a distance of 1 mm (a typical field of view). The result of such tilt causes the force to deviate at most 10\% over the measured area.
3.3.2 Parameters used in the model

Our layered resonator was modeled with a PZ26 (thickness $T = 220 \, \mu m$, speed of sound $c = 4530 \, m \, s^{-1}$, density $\rho = 7700 \, kg \, m^{-3}$, piezo-constant $h_{33} = 2.37 \times 10^9 \, V \, m^{-1}$, permittivity $\varepsilon_r = 6.195 \times 10^9$) and transparent transducer (thickness $T = 500$ and $200 \, \mu m$, speed of sound $c = 7340 \, m \, s^{-1}$, density $\rho = 4628 \, kg \, m^{-3}$, piezo-constant $h_{33} = 5.10 \times 10^9 \, V \, m^{-1}$, permittivity $\varepsilon_r = 7.437 \times 10^{10}$), a matching layer (typically $T = 1000 \, \mu m$) and reflector (typically $T = 175 \, \mu m$) layers of glass ($c = 6000 \, m \, s^{-1}$, $\rho = 2240 \, kg \, m^{-3}$) and water as the fluid (typically $T = 100 \, \mu m$, $c = 1480 \, m \, s^{-1}$, $\rho = 1000 \, kg \, m^{-3}$). Forces were calculated for polystyrene particles ($c = 1960 \, m \, s^{-1}$, $\rho = 1000 \, kg \, m^{-3}$). The piezo size is 5 by 5 mm, but set to 5 by 2.5 since this is the area under the fluid. The Quality (Q)-factor is set on 230 for all layers. The electrodes of the transducer and the glue layer were neglected in the acoustical model, because of their relatively small thickness.

3.3.3 The quality number (QN)

Grölsch et al.\textsuperscript{43} introduced the dimension-less performance number ($\eta_{eff}$) as a measure of the efficiency of a certain resonance frequency. This is essentially the efficiency of the acoustic wave over a full period. For our purpose we are interested in the strength and the direction of the force at the boundaries in the flow chamber, therefore we introduce a new quantity, the acoustic Quality Number (QN) with the dimension N/W:

$$QN(z) = \frac{F_{rad}(z)}{P_{ei}} = \frac{8|Z|^2 F_{rad}(z)}{U_{pp}^2}$$

(3.3)

Here $P_{ei}$ is the electrical input power, $U_{pp}$ is the peak-to-peak voltage, and $|Z|$ is the absolute of the cumulative impedance. QN is dependent on $z$, where $z$ is here the height within the fluid channel. This means that we can use QN to optimize the acoustic force for every height location within the fluid channel. Furthermore, QN depends on microsphere size and material. We calculate the QN for 4.5 $\mu m$ diameter polystyrene microspheres, the ones we typically use in our experiments.

3.4 Results

3.4.1 Transparent piezo

In the original AFS setup, a PZ26 opaque piezo element was used to generate
the acoustic wave (Chapter 2). This made the implementation of bright-field microscopy impossible and a more complicated epi-illumination method had to be used. Here we introduce the use of transparent piezo elements as developed by Brodie et al. These transparent piezos are made of lithium niobate crystals with indium tin oxide electrodes (LUMICKS B.V., AFS_trP). We measured the transparency of these piezos by making an image of the fluid channel with and without the piezo element (Supplementary figure 3.4), showing that the piezo transmits 55% of the light with a wavelength of 455 nm, in agreement with previous measurements. With these piezos, bright-field illumination (Figure 3.1a I) can be implemented resulting in a more homogenous illumination. Therefore a higher tracking accuracy could be reached (Supplementary figure 3.2) and a larger field of view in which tethers can be tracked (Figure 3.1b II and IV). We modeled the force profiles for a 500 μm thick transparent piezo and compared them to the PZ26 piezos (Figure 3.1c), demonstrating that using the transparent piezos similar forces can be applied in two directions, albeit with a slightly steeper force gradient. These results show that the transparent piezos perform acoustically very similar to the opaque ones, while they allow substantially improved imaging conditions.

3.4.2 Use of liquid-immersion objectives
For many microscopy experiments, the use of high-NA oil or water-immersion objectives is required. So far, we have only demonstrated AFS for use with air objectives, resulting in a negligible loss of acoustic energy due to minimal coupling of the resonator with the objective and the rest of the setup, as can be understood by considering the reflectivity $R$, of an acoustic wave passing through the interface of two media:

\[ R = \frac{Z_1 - Z_2}{Z_1 + Z_2} \]  

(3.4)

Here, $Z_i$ is the specific acoustic impedance, which is equal to the speed of sound times the density of the medium i. An acoustic wave traveling from a glass to an air layer is reflected by >99.99%. From glass to water the reflectivity is ~80%, resulting in acoustic energy loss, affecting the quality factor of the acoustic resonator, Figure 3.2a. Our model is not able to describe this more complicated system. To test whether liquid-immersion objectives are compatible with AFS, we measured directly the forces applied to DNA-tethered particles using different objectives (Figure 3.2b). From this data it is evident that that AFS still works with water and oil-immersion objectives, but that acoustic forces are reduced (~70% and ~84% lower forces, respectively).
and that resonance frequency are slightly shifted (less than 0.3%). Hence, in case the high NA of a liquid-immersion objective is absolutely required, this is compatible with our current implementation of AFS, but at the cost of a substantial reduction of the forces that can be applied.

### 3.4.3 Optimizing layer thicknesses

An AFS sample chamber can be brought in acoustic resonance using only a specific set of frequencies, depending on chamber geometry and materials used. With the system previously reported it is not possible to apply a significant force at the bottom of the fluid layer i.e. the side of the cover glass. In addition, the previous configuration had a force profile with a relatively steep gradient (Figure 3.1c, close to the top surface (at 100 μm)). To optimize the sample chamber, we developed a MATLAB program that can calculate the resonance frequencies of a chamber geometry and the corresponding force profiles in the fluid layer. One configuration can be calculated within 5 ms, therefore a large set of configuration can be computed to find the optimal dimensions for our system. Specifically, we used the model to optimize layer thicknesses, in order to apply a force at the bottom fluid layer with a minimized force gradient. As explained in section 3.3.3, the QN together with
Figure 3.3 | Layer thickness optimization for AFS chip

(a–e) Contour plot of the QN (N/W) 3 μm above the capping layer with the force directed towards the fluid layer for a 4.5 μm polystyrene microsphere. The scale bar is equal for all plots. Flow cell 1 (section 2.1) with a 200 and 500 μm thick piezo is marked in the contour plots with a green and red dot, respectively. Flow cell 2 (also with 200 and 500 μm thick piezo) is marked with a black and yellow dot, respectively. (a) Combination of piezo and matching layer thickness, the fluid and capping thickness ranging between 10-300 and 50-300 μm, respectively, with 10 μm steps. The maximal QN is shown within this range. (b) Combination of matching and fluid layer thickness, the piezo thickness is set to 200 μm and a variable capping layer 50-300 μm with 10 μm steps, the maximal QN is shown within this range. (c) Combination of matching and capping layer thickness, piezo and fluid layer thickness are set to 200 and 84 μm, respectively. (d–e) The modeled acoustic radiation force over the fluid channel for the strongest resonance frequencies away and towards the bottom surface of the flow cell 1 (d) and 2 (e) both with the 200 nm piezo, when applying 0.1 W. Note that flow cell 2 has a flatter force profile close to the bottom surface. Arrows point towards the direction the force is acting for microspheres close to the bottom surface.

the force gradient is used to quantify the performance of each resonance. We set the limits of the system thickness between 100–5000 μm for the piezo and the matching layers, and 10–300 μm for the fluid and the capping layers. All the different combinations of these four parameters were modeled, within the given limits. The step size of this parameter space was 20 μm for the piezo and matching layers and 10 μm for the fluid and the capping layers, resulting in in total fifty million configurations.

This four-dimensional parameter space is visualized in different ways in
Figure 3.3. From the contour plot Figure 3.3a, it can be concluded that a thicker matching layer results in a lower QN, although the acoustic power is almost constant between 100 and 1000 μm. Furthermore, the contour plot indicates that optimizing the thickness of the piezo layer is crucial to obtaining a significant force at the bottom surface: this force is almost zero for a 500 μm thick piezo, while it is maximal for 100 to 200 μm thick piezos. In Figure 3.3b the piezo-layer thickness is fixed to 200 μm and fluid and matching-layer thicknesses are varied, indicating that a fluid-layer thickness of around 45 μm or a multiple of this is optimal and that a thicker fluid layer deteriorates the system. In Figure 3.3c the fluid layer is fixed to a thickness of 84 μm and the piezo to 200 μm. Optical microscopes are often optimized for 175 μm cover-glass thicknesses, equivalent to the capping layer in our model. The figure shows that such a thickness does not present a negative trade-off in acoustic power.

QN does not contain information on the steepness of the force gradient. For actual experiments, this is an important parameter to keep the force relatively constant over the size of the measured molecule. In order to determine the gradient, we fitted the force profile for a given chamber geometry with a straight line, 3 μm away from the surface over a range of 2 μm. The gradient was extracted from the slope and normalized with the intercept. In Supplementary figure 3.5 the QN values are shown where the gradient is less than 2% per μm. In contrast to flow cell 1 with a piezo of 200 μm, flow cell 2 with a piezo of 200 μm did show a flat force profile. The most optimal force profiles of these flow cells are shown in Figure 3.3d and e. For bottom force directed towards the fluid layer QN reaches 9.99 × 10^{-6} and 9.10 × 10^{-9} N/W for flow cell 1 and 2 with 200 μm piezo, respectively. Also, with flow cell 2 the force gradient is less than 2% per micrometer and an efficient force directed towards the surface can be found. Our original geometry (Chapter 2) yields a QN of 4.3 × 10^{-9} N/W measured at the top surface, meaning that with the optimized, 200 μm thick piezo, according to our model the maximum force increases by a factor of ~2.3 and it is applied at the bottom surface. Figure 3.3 shows that, in principle, a QN of a factor of 3 higher can be obtained. The optimal configuration results in the highest attainable forces, but has two severe drawbacks: the force varies more than other configurations over the flow and it requires glass of non-standard thicknesses. QN can be calculated for any microsphere diameter and compressibility using equation 3.2. For DNA-stretching experiments, we typically use 4.5 μm polystyrene microspheres, with a QN of 0.88 × 10^{-9} N/W. With these microspheres, a maximum force of 350 pN can be reached using the maximum output power.
of the function generator (0.25 W), more than enough to overstretch DNA, without substantial heating of the sample (less than 2 degrees over a time scale of 2 minutes).

### 3.4.4 Direct measurement of the force profile

Our model allows us to predict the force profile along the fluid channel of the flow cell. So far, forces have been experimentally determined on tethered particles, a time-consuming process, providing only information on forces at one specific height. Here we show another method to verify the force profiles by direct measurement of the free movement of particles (6.84 μm diameter silica, Bangs Lab. SS06N, experiencing ~1.3 pN of gravitational force), driven by a combination of acoustic force and gravity (Figure 3.4a). These measurements were performed in the presence of 1% w/v casein to prevent sticking of the particles to the surface. For measurements of the force profile on the matching-layer side of the flow cell, the cell is used upside down, imaging through the piezo with a long-working distance objective (section 3.2.3). After switching on the acoustic force, particles are pushed away from the surface in the direction of a node of the acoustic standing wave. After switching off the force, particles sink to the bottom with a constant speed until they reach the surface (Supplementary figure 3.6). The forces acting on a suspended particle in solution are the gravity force, the buoyancy force, the Stokes drag force and the acoustic radiation force, and for a particle moving at constant velocity these forces cancel out:

\[
F_{\text{Grav}} - F_{\text{Buoyancy}} + F_{\text{drag}} - F_{\text{rad}} = 0 \tag{3.5}
\]

\[
F_{\text{Grav}} = ρ_p g \tag{3.6}
\]

\[
F_{\text{Buoyancy}} = -ρ_m g \tag{3.7}
\]

\[
F_{\text{Stokes}} = v_p υ_{\text{faxon}} \tag{3.8}
\]

Here \( g \) is the gravitation acceleration. For a silica microsphere of a diameter of 6.84 μm with a density of 2000 kg/m³ this means that \( F_{\text{Grav}} = 3.29 \text{ pN} \) and the \( F_{\text{Buoyancy}} = -1.64 \text{ pN} \), so the total constant force on the particle is 1.64 pN. \( υ_{\text{faxon}} \) is the effective drag coefficient in the low-Reynold's number regime, corrected for hydrodynamic surface effects. Gravity and buoyancy force are constant, while the drag force is directly related to the particle's velocity, meaning that from the particle velocity the drag force can directly be
Figure 3.4 | Mapping the acoustic force within the fluid layer
Free suspended 6.84 μm silica microspheres in solution are used in this experiment. (a) Schematic image, showing the concept of this experiment (corresponding data is shown in Supplementary figure 3.6). This illustration is not to scale. (b) Measured (dots) and modeled shape (line) of the force profile of the 6.0 and 7.3 MHz resonance frequencies at to top surface of flow cell 1 with 500 μm piezo. The orange dots represent the measured force profile when the two resonance frequencies applied at the same time. Arrow indicates the direction of the force. (c) Measured (dots) and modeled shape (line) of the force profile of two different resonance frequencies at the bottom surface of flow cell 1 and 2 (section 2.1) with 200 μm piezo. Arrow indicates the direction of the force. (d) Force profiles in B and C are fitted with a sin function showing a linear force power behavior. The slope of the fits yielded linear constants of 18.07 ± 0.17, 13.34 ± 0.12, 5.83 ± 0.06, 2.014 ± 0.015 nN/W (fit value ± s.e. of the sin fit Supplementary figure 3.7) for flow cell 1 at the bottom surface with 200 μm piezo at 14.3 MHz, flow cell 1 at the top surface with 500 μm piezo at 7.28 MHz, flow cell 2 at the bottom surface with 200 μm piezo at 14.8 MHz, flow cell 1 at the top surface with 500 μm piezo at 6.0 MHz, respectively.
calculated.

<table>
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<tr>
<th>System</th>
<th>Max QN in flow cell measured for 6.8 μm silica</th>
<th>Max QN in flow cell modeled 6.8 μm silica</th>
<th>Difference (%)</th>
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<td>Flow cell 1, 200 μm, 14.3 MHz</td>
<td>18.1 ± 0.2</td>
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<td>33</td>
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<tr>
<td>Flow cell 1, 500 μm, 7.2 MHz</td>
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<td>Flow cell 1, 500 μm, 6.0 MHz</td>
<td>2.01 ± 0.02</td>
<td>.935</td>
<td>115</td>
</tr>
</tbody>
</table>

**Table 3.1** Modeled and calibrated QN values

The measured force profiles at the top and the bottom surfaces are displayed in Figure 3.4b and c, respectively and show a high degree of (quantitative) agreement with the modeled force profiles. One resonance frequency predicted by our model (16.2 MHz; Figure 3.3d) could experimentally not be found, possibly because it is close to the resonance frequency of the piezo element itself. Acoustic forces scale linear with applied electric power. To demonstrate this dependency, force profiles of different applied powers were measured and fitted with a sin function (Supplementary figure 3.7). The force amplitude plotted against the power proves this a linear behavior (Figure 3.4d). Note that QN, of a 6.84 μm silica microsphere, is equal to the slopes of these force–power curves. In Table 3.1, the modeled and measured QN values are displayed, showing a clear correlation between measured and modeled QN. These experiments show that our method to quantify the acoustic force profile is straightforward and quick, and can be used to calibrate any layered acoustic resonator comprising a fluid layer.
3.4.5 Optimizing the acoustic-force profile

The acoustic force profiles typically used in AFS (Figure 3.1c), have a sine shape with a wavelength of ~100 $\mu$m. For tethered constructs with a length of micrometers, the force profile can be assumed to be constant, however for longer constructs the force gradients can have a significant effect. One way to flatten the force profiles is by driving two different resonances simultaneously (Figure 3.4b), resulting in a superposition (in this case the sum) of the profiles of the two resonances, which can be used to create a flatter force profile for longer constructs.

For some experiments, for example those involving a substantial lengthening of the molecule at a more or less constant force (e.g. the overstretching of double-stranded DNA), it is advantageous to not use a constant force profile but to employ a distance clamp instead. In those experiments, a strong force gradient, resulting effectively in a distance clamp, is beneficial. An AFS experiment demonstrating this distance-clamp mode of AFS is shown in Figure 3.5. Here, a 45.5 kilo base pairs phage lambda DNA is overstretched by ramping the voltage applied to the piezo (resonance frequency 6.0 MHz). In order to overstretch the DNA from 16 $\mu$m to 25 $\mu$m the voltage needs to be increased from 39 to 54 V_pp, allowing gradual and controlled stretching of the molecule. At each voltage setting, a stable equilibrium is created due to the force gradient: when a thermal fluctuation moves the tethered particle away from the surface, it will experience a lower acoustic force and the DNA will pull back the microsphere. When the particle moves towards the surface, it will experience a higher acoustic force and it will extend the DNA. In a force clamp, this distance change would occur almost instantaneously. The trap stiffness follows from the gradient of the force profile and is of the order 6.6 pN/$\mu$m. These results show that in AFS, the user can control the shape of the force profile, which allows easy switching between force-clamp and distance-clamp experiments.

3.5 Discussion and conclusion

We have shown that AFS can be combined with a transparent piezo element without any disadvantage. This gives the option for trans-illumination, resulting in a larger field of view and higher tracking accuracy of tethered particles. Furthermore, we demonstrate that AFS can be combined with high-NA water and oil-immersion objectives, yet with reduced acoustic power. High-NA water and oil-immersion objectives and trans-illumination permit new measurement opportunities with AFS, such as low-light fluorescence.
Figure 3.5 | Overstretching curve of 45.5 kbp lambda DNA
Flow cell 1 and 500 μm piezo was used at 6.0 MHz resonance frequency (Figure 3.4b). During overstretching with this frequency the DNA experiences a distance clamp, illustrated by the graph inset. Here the force profile is visualized for four points in the overstretching curve where 30, 39, 46, and 54 V_{pp} is applied on point A-B, respectively. In this experiment the force is corrected this the force profile measured in Supplementary figure 3.7b.

detection, including confocal, super-resolution or total internal reflection fluorescence microscopy. Together, these improvements make integration of AFS in most existing microscopes substantially easier.

We presented a 1D MATLAB model that calculates the resonance frequencies and the corresponding force profiles of any layered resonator. We have used this model to successfully optimize the layer thicknesses of the system and showed that we can generate high forces at the bottom of the AFS chamber (i.e. the side of the cover slide). In fact, the developed model can also be used to improve the design of any layered resonator in, for example, sorting applications, cell mechanic manipulation devices, particle agglutination diagnostics and for functional biosensors.

At the same time it should be noted that the energy losses in the system are not captured by the model (section 3.3.1). Moreover, the predictions are made using a 1-dimensional model while the flow chamber is a 3-dimensional object. Such 3D object has (weaker) additional acoustic reflections, which are not
Discussion and conclusion

For these reasons the strength of a predicted resonance frequency always needs to be validated experimentally. We made experimental validation simpler by our new method to directly measure the force profile in the longitudinal direction. Quantifying the force profiles for different system configurations showed that our model can, in most cases, accurately predict the resonance frequencies and the shape of the force profile in the fluid layer. In Table 3.1 the measured and modeled QN are compared, showing a strong correlation. It seems, however, that the less efficient resonance frequencies are underestimated in our model. Also, one resonance frequency (Figure 3.3 e) could not be observed experimentally, likely because it is close to the resonance of the piezo itself.

Finally, we showed with our force-profile calibration method that two resonance frequencies can be applied at the same time to change the shape of the force profile within the device (Figure 3.4b). Shaping force profiles can be used to create a more homogeneous force field which is useful when studying biomolecules that undergo large length changes. By doing the opposite, and measure at a location in the force profile where the force gradient is very high, AFS could be transformed into a distance clamp (Figure 3.5). A distance clamp has the advantage that it is better in probing multiple rupture events on the same construct. This can for example be used to study the overstretching of DNA as well as protein unfolding, DNA hairpin disruption or the denaturalization of any other bio-construct in a multiplexed fashion. To conclude, AFS is a young technique with much room for improvements. Each of these new AFS developments presented here can open the doors for increasingly diverse measurement opportunities on massively parallel single-molecule systems.

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3.6 Supplementary figures

**Supplementary figure 3.1 | Picture of the custom-made transformer**

A ferrite core (Ferroxcube, TN14/9/5-4C65) and isolated copper wire are used. The winding ratio is 5 to 25 to match the electric impedance of the layered resonator (here 1250 Ω) to the impedance of the function generator and amplifier (50 Ω).

![](image)

**Supplementary figure 3.2 | Accuracy of tracking at 60 Hz in x, y, and z of a 4.5 μm diameter polystyrene microsphere**

The microspheres were stuck nonspecifically to the flow cell surface with 1 Molar MgCl. To correct for mechanical drifts a reference microsphere was tracked simultaneously and its position was subtracted. The standard deviations of the position distributions for the 4.5 μm diameter polystyrene microsphere is $\sigma_x = 1.3$ nm, $\sigma_y = 1.3$ nm and $\sigma_z = 3.8$ nm.
Supplementary figure 3.3 | Theoretically predicted resonance frequencies and corresponding force profiles of flow cell 1 with a 200 μm piezo
(a) The acoustic energy density ranging from 0 to 30 MHz, driven with 1 Vpp. (b) The predicted force profiles of the 4 strongest resonance peaks for a 4.5-μm-diameter polystyrene microsphere directed along the z direction for 0.1-W input power.

Supplementary figure 3.4 | 2 images showing the transparency of the piezo element
On the left an image is made where the light goes through the flow cell and on the right the same image is made only the light travels through the flow cell and the transparent piezo element. The average pixel intensity is 104 left and 57 right meaning that the piezo transmits 55% of the light.
Supplementary figure 3.5 | Combination of matching and fluid layer thickness, the piezo and capping layer thickness are set to 200 μm and 175 μm, respectively

All QN values that have a higher force gradient than 2% per μm are neglected. Flow cell 1 and flow cell 2 with a 200 μm thick transparent piezo are marked with a green and black dot, respectively. The corresponding force profiles are shown in Figure 3.3d and e.

Supplementary figure 3.6 | Time trace showing the z position of the microspheres together with the applied voltage on the piezo

Here, flow cell 1 is used with the 500 μm transparent piezo resonated at 7.3 MHz. To determine the force profile the velocity is measured between the moment the force is turned on till the moment it reaches the acoustic node (~40 μm). Using Formula 5, the acoustic force profile can be calculated.
Supplementary figure 3.7 | Underlying data of Figure 3.4b and c

Here, the microspheres position in the lateral direction are displayed, while the piezo is driven with different powers. The camera image was cropped to get a higher frame rate ~300 Hz and is afterwards averaged with a bin of 10. A sin function is fitted to this data, where only the amplitude is used as fit value. (a) Flow cell 1 with 500 μm piezo with 7.3 MHz resonance frequency is driven with a power of 0.5, 0.7, 0.9, 1.2, 1.5, 1.9, 2.3 and 2.7 mW, corresponding fitted amplitudes values are 6.67 ± 0.13, 9.0 ± 0.2, 12.4 ± 0.2, 16.6 ± 0.2, 21.1 ± 0.2, 26.1 ± 0.5, 31.2 ± 0.3 and 35.6 ± 0.4 pN, respectively. (b) Flow cell 1 with 500 μm piezo with 6.0 MHz resonance frequency is driven with a power of 1.5, 1.8, 2.3, 2.7, 3.3, 3.8, 4.4, 5.1, 5.8, 6.5 and 7.3 mW, corresponding fitted amplitudes values are 3.07 ± 0.04, 3.73 ± 0.10, 4.52 ± 0.18, 5.39 ± 0.06, 6.70 ± 0.05, 7.72 ± 0.10, 8.67 ± 0.08, 10.06 ± 0.10, 11.8 ± 0.2, 13.2 ± 0.2, 15.0 ± 0.2 pN, respectively. (c) Flow cell 1 with 200 μm piezo with 14.3 MHz resonance frequency is driven with a power of 0.5, 0.7, 0.9, 1.2, 1.5, 1.9, 2.3 and 2.7 mW, corresponding fitted amplitudes values are 4.05 ± 0.07, 5.45 ± 0.09, 7.09 ± 0.14, 9.03 ± 0.13, 29.8 ± 0.4, respectively. (d) Flow cell 2 with 200 μm piezo with 14.8 MHz resonance frequency is driven with a power of 0.9, 1.6, 2.5, 3.6, 4.9, 6.4, 8.1 and 10 mW, corresponding fitted amplitudes values are 5.04 ± 0.12, 7.9 ± 0.3, 13.3 ± 0.4, 20.2 ± 0.3, 28.9 ± 0.2, 37.6 ± 0.2, 47.6 ± 0.6, and 59.4 ± 1.8 respectively. (fit value ± s.d.).
Chapter 4 | Single-Molecule measurements using Acoustic Force Spectroscopy

Abstract

Single-molecule force spectroscopy is a powerful tool to investigate the forces and motions related to interactions of biological molecules. Acoustic Force Spectroscopy (AFS) is a recently developed measurement tool to study single molecules making use of acoustic standing waves. AFS permits high experimental throughput, because many individual molecules can be manipulated and tracked in parallel. Moreover, a wide range of forces can be applied, as well as a force loading rate with range of six orders of magnitude. At the same time, AFS stands out because of its simplicity and the compactness of the experimental setup. Even though the AFS setup is simple, it can still be challenging to perform high-quality measurements. Here we describe, in detail, how to setup, perform and analyze an AFS measurement.

4.1 Introduction

Using single-molecule experiments, properties of individual biomolecules are investigated in order to distinguish inhomogeneity and stochasticity, which are difficult to measure in ensemble measurements. Force spectroscopy explores biomechanical properties and can thus provide insights in the structure, binding properties and interactions of biomolecules. Techniques that can be used for this purpose are, for example, atomic force microscopy, optical tweezers and magnetic tweezers. Here we discuss AFS, a recently developed technique that uses acoustic standing waves to apply forces on a field of single tethered biomolecules. The main advantage of AFS is that it is relatively simple and compact, and that it works in a highly multiplexed fashion. Forces in AFS can be applied in the range of sub-pN to hundreds of pNs, with force loading rates ranging between $10^{-4}$ pN s$^{-1}$ and $10^{2}$ pN s$^{-1}$ on thousands of constructs in parallel. It has been shown that AFS can be used to study the mechanical properties of DNA molecules, the strength of DNA-protein interactions and to probe the energy landscape of antigen-antibody bonds.

The original implementation AFS (Chapter 2) had several drawbacks and the technique was greatly improved as described in Chapter 3. A newly developed transparent piezo element was combined with AFS to allow for transillumination, improving the tracking accuracy and the measurable field of view. It was also shown that AFS can be used with high NA water- and oil-immersion objectives. The acoustic properties of AFS were improved by optimizing the layer thickness of the chips in order to generate a more efficient force at the cover slip side. These improvements resulted in better optical and acoustic performances making the AFS more compatible for integration in existing microscopes, giving rise to many new measurement possibilities.

Even though the AFS employs a relatively simple experimental setup and is even commercially available (LUMICKS B.V.), it can still be challenging to perform a good single-molecule measurement. Therefore we describe here in detail how to perform an AFS experiment on DNA molecules. We will list all hardware, software and biomaterials necessary to perform an AFS measurement. Furthermore, we describe in detail the biological protocols used to make a DNA tethered surface. Lastly, we describe step-by-step an AFS measurement, where we select single DNA tethers, calibrate the force per construct and make an overstretching curve of DNA.
4.2 Material

4.2.1 AFS experimental setup
Most AFS experiments are done with the AFS module or the AFS stand-alone from LUMICKS B.V. The AFS stand-alone is a complete system including microscope, while the AFS module only includes the chip, the holder and the function generator. Here, measurements are performed with the LUMICKS AFS module together with the following additional equipment.

1. Inverted microscope: Nikon eclipse Ti equipped with CFI Plan Fluor DLL 40x, 0.75 NA objective (Nikon) and a tube 0.45 x c-mount adaptor (Nikon, MQD42040).
2. Illumination light: a collimated LED (Thorlabs, M660L4).
3. Digital camera: 1 Megapixel CMOS (Thorlabs, DCC3240M), recording 60 frames per second at full resolution.
4. Translation stage: multi-axis piezo translation stage (PI, P-517.2CL), driven by a digital piezo controller (PI, E-710.4CL).
5. Computer: desktop computer, with two Xeon E5 2643v2 processors to run the live tracking software.
6. Oscilloscope: 2 channels signal input and data readout by the computer.

4.2.2 DNA labeling
1. pKYBI plasmid (8,393 bp; see Note 1).
2. Cutting enzymes: EcoRI-HF (R3101S) and KpnI-HF (R3142S), used in CutSmart Buffer (New England Biolabs).
3. Nucleotides: 2 mM dTTP and 0.4 mM Biotin-14-dATP.
4. Klenow Fragment exo-.
5. Custom KpnI_Dig primer: 5'-C(T-Dig)CTC(T-Dig)CT CTC(T-Dig)TC(T-Dig) CTT CTC TT GTAC-3'.
6. T4 DNA Ligase and T4 DNA Ligase buffer (Thermo Scientific).
7. Nuclease-free sterile water.
8. PCR Purification kit: we use QIAquick (QIAGEN).
9. Centrifugal filters: Amicon Ultra 0.5 mL (Sigma-Aldrich).
11. dH2O: ultrapure water.

4.2.3 Surface chemistry buffers
1. Bleach: <5% (w/v) sodium hypochlorite.
2. 1 M Na₂S₂O₃ in ultrapure water.
3. PBS buffer: 138 mM NaCl, 2.7 mM KCl and 10 mM phosphate pH 7.4;
supplemented with 5 mM sodium azide and 0.5 mM EDTA.

4. 20 μg/ml anti-digoxigenin antibody from sheep (Sigma-Aldrich) in PBS. Store aliquoted at -20 °C.

5. Streptavidin coated polystyrene microspheres: 4.5 μm diameter, 0.5% (w/v). Store at 4 °C.

6. Casein buffer: stock solution 1% (w/v) in PBS.
7. BSA buffer: stock solution 1% (w/v) in PBS.
8. Pluronic (F-127) buffer: stock solution 5% (w/v) in PBS.
9. DNA measuring buffer: PBS buffer supplemented with 0.02% (w/v) Casein and 0.02% (w/v) Pluronic.
10. 0.5 M Borate, pH 8.3; supplemented with 5 mM sodium azide and 0.5 mM EDTA.
11. 0.5 M HEPES, pH 7.5.

4.3 Methods

4.3.1 Measuring the impedance of the AFS chip

The acoustic and electric properties of the AFS chip can be modeled (Chapter 3), however, we have noticed that significant deviations from the ideal behavior exist from chip to chip. To account for that, we calibrate each AFS chip. The resonance frequencies and the impedance (Z) of the chip can be determined electronically in the following way.

1. Connect the AFS chip to the function generator and add a resistance (R) in series to the chip. Measure with the oscilloscope the electrical potential (V) over the piezo and electrical potential over the piezo plus the resistance (see Figure 4.1a).

2. The impedance of the AFS chip can be calculated using Ohm’s law:

\[ V_R = V_{all} - V_{piezo} \]  \hspace{1cm} (4.1)

\[ I = \frac{V_R}{R} = \frac{V_{all} - V_{piezo}}{R} \]  \hspace{1cm} (4.2)

\[ Z = \frac{V_{piezo}}{I} = \frac{V_{piezo} \cdot R}{V_{all} - V_{piezo}} \]  \hspace{1cm} (4.3)
Methods 4.3

Figure 4.1 | Measuring the impedance of the AFS chip
(a) Electric diagram used to measure the impedance of the AFS chip. (b) A graph showing the impedance of the AFS chip measured between 13.5 and 15.5 MHz filled with air and water channel. The arrow shows the used resonance for this chip configuration (14.3 MHz).

3. Connect the oscilloscope to the computer and sweep the frequency while keeping the voltage constant. Using the equations above, the impedance can be calculated. An example is shown in Figure 4.1b, here a resonance frequency is found at 14.3 and 15.0 MHz. The impedance is around 80 Ω for both these resonance frequencies.

4.3.2 DNA labeling protocol for surface tethering

DNA labeling is performed in three steps (Figure 4.2), first a DNA plasmid is linearized by cutting it with two digesting enzymes and directly labeled at the 5’ side with Biotin using polymerase and biotin labeled nucleotides. In the second step, the 3’ is labeled with digoxigenin using a primer. Finally, the DNA is ligated to repair the backbone of the DNA. See Note 2 for other constructs. The detailed procedure is as follows.

1. Mix 27 μL dH2O, 5 μL 10x Cut Smart buffer, 15 μL pKYB1 (216 ng/μL), 1.5 μL EcoRI-HF and 1.5 μL KpnI-HF. Volume of pKYB1 and dH2O can be adjusted according to the pKYB1 concentration.
2. Incubate for 45 min at 37°C (a to b in Figure 4.2).
3. Use PCR Purification Kit to remove the remaining nucleotides and elute in 60 μL (see Note 3).
4. Mix 23.5 μL dH2O, 5 μL 10x Klenow buffer, 60 μL pKYB1 cut and purified (step 3), 8 μL of 0.4 mM Biotin-14-dATP, 1.5 μL of 2 mM dTTP and 2 μL Klenow polymerase exo-.
Figure 4.2 | A schematic drawing of the DNA labeling protocol

In the first step (a) the pKYBI DNA plasmid is cut with EcoRI and KpnI. This results in a DNA strand with an overhang at the 5' end at one side and an overhang on the 3'end at the other side (b). First the 5' side is labeled via polymerase with Biotin-14-dATP (c). After the primer can be attached to the 3'end side (d). In the last step the DNA is ligated (e).

5. Incubate at 37°C for 1 hour (b to c in Figure 4.2).
6. Use PCR Purification Kit to remove the remaining nucleotides and elute in 30 μL (see Note 3).
7. Mix 5 μL 10x T4 DNA Ligase buffer, 5 μL dH2O, 30 μL pKYBI cut and purified vector (step 6), 8 μL KpnI-DIG primer, 2 μL T4 DNA Ligase.
8. Incubate at 23°C for 2 hours (c to e in Figure 4.2).
9. Use Amicon Ultra 0.5 mL centrifugal filters to purify the DNA construct.

4.3.3 Labeling microspheres with digoxigenin

Microspheres labeled with digoxigenin are used to stick beads on the surface in order to correct for drift during the measurement.

1. Mix 8 μL streptavidin-coated polystyrene, microspheres with 30 μM
digoxigenin-NHS, 100 mM borate and 0.05% (w/v) Pluronic in a total volume of 400 μL.
2. Incubate for 3 hour at room temperature, while tumbling the sample constantly.
3. Wash the microspheres are by spinning down with 2000 g, removing the residue and add 1 mL of 10 mM HEPES plus 0.1% Pluronic. This washing step is reaped 2 times.
4. Finally, store these microspheres in 1 mL of 10 mM HEPES plus 0.1% Pluronic at 4 °C.

4.3.4 Preparation of DNA tethered to the surface and microspheres
DNA tethered surfaces are prepared fresh each day to prevent interference between measurements and degradation of the surface. All steps are performed at room temperature and liquids are flushed in at room temperature.

1. To clean the surface, incubate bleach for at least 10 min. After, flush the channel dry and flush bleach through again, repeat this step multiple times. End by flushing it dry and incubated Sodium Thiosulfate for 10 min, this will inactivate the residual bleach. Flush with MQ and flush dry. The chamber is now cleaned and ready for surface preparation.
2. Flush in with PBS and make sure that there are no bubbles in the chamber. Flush 30 μL of 20 μg/mL Anti-Digoxigenin and incubate for 20 min.
3. For passivation, use a combination of BSA and pluronic. First flush with 400 μL of PBS with 0.2% (w/v) BSA and let this incubate for 30 min. Then flush with 400 μL of PBS with 0.5% (w/v) pluronic and incubate for 30 min.
4. To attach the DNA to the surface, flush first 400 μL of the DNA buffer. Then Flush in the 30 μL of 11.4 pg/μL DNA and incubate 20 min.
5. Clean the microspheres before measuring. Take 10 μL of microsphere solution, add 1 mL DNA measuring buffer and spin down for 2 min at 2000 g and remove the residue. Add 1 mL DNA measuring buffer again, spin down for 2 min at 2000 g and remove residue. Now fill up to 30 μL with DNA measuring buffer and add 1 μL of digoxigenin microspheres (see section 4.3.3).
6. Flush with DNA measuring buffer. Now flush in the microsphere and let them incubate for 20 min. After, flush out all the microspheres that have not attached to the surface. Be careful not to flush too fast, otherwise your tethers will detach from the surface (see Note 4). The tethers are now ready for measuring.
4.3.5 Measurements on tethered particles

The Tracking software we used is provided with the stand-alone LUMICKS system, a previous academic generation of this software is available online https://figshare.com/articles/AFS_software/1195874. The tracking software analyzes the images from the camera in real time and also controls the function generator and the piezo stage or step motor. A detailed manual is also provided with the software.

1. The microspheres can be selected by double-clicking on the screen. Their x and y position are directly tracked and displayed in the preview tap. Algorithms of determining the position of the microsphere are based on cross correlation (XCOR) or Quadrant interpolation (QI). Where QI is more precise; however, XCOR costs less computation power.

2. Before measuring the z dimension a look up table (LUT) has to be made, where the range of the LUT has to be bigger than the maximal extension of the molecule. Here a 2.8 μm DNA molecule is used, when overstretched the DNA molecule it reaches a length of ~4.8 μm. There the range of the LUT should be bigger than 5 μm.

3. Turn on the Perfect Focus System (PFS), this keeps the objective at a constant distance from the bottom of the flow cell.

4. The measurement can now be started. A typical measurement is shown in Figure 4.3.

4.3.6 Data analysis

We use a LabVIEW-based program to analyze our data. This program is available online at https://figshare.com/articles/AFS_software/1195874. Here we explain how single tethered constructs can be selected, forces can be calibrated and force-distance curves can be extracted. The steps required to make a force-distance curve of DNA are shown in Figure 4.3.

1. To select single tethers we use a histogram of the xy location of the tethered particle. A typical xy histogram of a particle attached with a single tether is round and has specific RMS value (Figure 4.3a). To remove double tethers or other none specific interaction we quantify the xy motion with the RMS and symmetry number35. A higher symmetry value can suggest a double tether (Figure 4.3b). However, some xy pattern cannot be explained with single or double tethers only (see Note...
**Figure 4.3 | AFS data analyses tethered particles**

**a** and **b** showing xy plot of tethered particle with an RMS of respectively 1.3 and 0.8 μm a symmetry values of 1.1 and 2.5. **c** A histogram of the RMS values of one measurement, in black all RMS values are plotted and in gray the RMS values with a symmetry value below 1.4. **d** a plot of the x, y and z values measured. **e** A plot of the end-to-end length of the DNA molecule and the applied voltage on the piezo. **f** Measured and fitted power-spectra values of the microsphere's x and y positions using a Lorentzian function (piezo driven at 14.3 MHz). **g** Forces acting on the tethered microsphere as determined from power-spectrum analysis (panel f), in gray a quadratic fit yielded a quadratic constants of 1.6 pN V⁻². **h** Overstretching curve of 8.4-kbp torsionally unconstrained pKYBI DNA, using the force voltage⁻² ratio calculated in panel g.
5. Here, we excluded all tether with a symmetry values lower than 1.4 (Figure 4.3c).

2. Remove system drift by subtracting the displacements of at least two surface-attached microsphere. To determine the end-to-end length of the DNA molecule, the anchoring point to the surface should be found. This is done in a region where no force is applied to the tether (0–20 min Figure 4.3d and e). The x and y movement is subtracted with their average location and the minimum of the Z location is used as the zero position where the microsphere touches the surface. Using Pythagoras’ theorem, the end-to-end of the DNA molecule can be calculated.

3. To calibrate the force voltage$^2$ ratio, a constant force is applied on the molecule (22–20 min Figure 4.3d and e). Taking the power spectrum of the x and y position, the force acting on the tethered microsphere can be derived from the corner frequency$^{31,34}$ (see Figure 4.3f). To verify power spectrum fit see Note 6.

4. From theoretical considerations it is known that the force scales quadratically with the voltage$^{18}$. By making a quadratic fit through the measured forces the set-force can be calibrated for different voltages (see Figure 4.3g).

5. In the final part of the measurement, the voltage is ramped with a square-root function, resulting in a linear force ramp. Using the force–voltage ratio a force–distance curve can be extracted (see note 7, for fitting the force–distance curve).

4.4 Notes

1. The pKYBI plasmid is not commercially available anymore; however, it can be extracted from an E. coli strain that contains the plasmid. This can be obtained using miniprep kit (27104, Qiagen). The concentration of a stock can be measured with a Nanodrop.

2. In this book chapter it is explained how to make a DNA-tethered construct. After making the construct it is relatively straightforward to flush in other buffers or proteins and measure the mechanical effect on the DNA molecules. We use a Dig::anti-Dig bond to attach the DNA to the surface and Biotin::streptavidin to attach the DNA to the microspheres. The DNA molecule could also be replaced with any other biomolecule to measure the mechanical properties, as long as it has the digoxigenin and biotin handles. At last, rupture forces could be measured as seen in Figure 2.4 or even more elegantly by using DNA nanoswitches$^{58}$.

3. For PCR Purification, the protocol of QIAGEN can be used. We modified
the elution volume for optimal reaction concentration of the DNA in the next step. Also, we decreased the centrifugation force to 12,000 g to reduce nicking of the DNA.

4. Flushing liquid through the flow cell can be tricky: flow drag acting on a microsphere attached to the surface can result in breaking the construct. To prevent this, flow speeds can be controlled with a syringe pump. Another way to control drag forces is to keep an air column in the syringe, since air is compressible this will slow down the flow in the chamber.

5. xy location histograms of tethered particles can vary substantially in shape due to multiple tethers, non-specific interactions, inhomogeneous microspheres or irregularities in the surface\textsuperscript{39}. By analyzing the xy motion the DNA-tethering protocol can be optimized.

6. The way we fit our power spectrum is described by Norrelykke and Flyvbjerg\textsuperscript{34}. It fits the corner frequency and the diffusion coefficient. The corner frequency depends on the force applied; however, the diffusion coefficient dependence could be theoretically predicted from the microsphere size and the distance from the surface\textsuperscript{33}. It is always a good check to see if the theoretically predicted diffusion coefficient overlaps with the fitted one.

7. The force-distance curve of DNA can be fitted with the Worm-Like Chain (WLC) model (equation 6.16). Since this model uses three non-independent variables it can be quite tricky to get reliable fit values. In Broekmans, O. D.\textsuperscript{60} a method is described to do this fitting in a reproducible way.

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