Tuning the Music: Acoustic Force Spectroscopy (AFS) 2.0

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ABSTRACT

AFS is a recently introduced high-throughput single-molecule technique that allows studying structural and mecanochemical 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 modelled 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.

1. Introduction

The ability to mechanically manipulate single biomolecules is leading to insights in fundamental cell processes \cite{1-4}. Single-molecule force-spectroscopy techniques are commonly used to study, for example denaturation of biomolecules \cite{5}, the binding, unfolding and folding of proteins \cite{6} and the replication and repair of nucleic acids \cite{7}. However, single-molecule instruments can be rather complex and often have a low experimental throughput \cite{2,8}. Moreover, the dynamics of single molecules are intrinsically stochastic, meaning that, to probe the heterogeneous behaviour, many independent measurements should be performed \cite{2,9}.

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 \cite{10} (Fig. 1A). 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 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 beads. We optimize the layer thicknesses of the system to generate a strong force at the bottom surface and quantify the modelled 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 is it possible to turn AFS into a distance clamp.

2. Material and methods

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 μm for the matching, fluid and capping layers, respectively (Fig. 1). 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.
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 Fig. 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 (Fig. 1B, III).

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 10× microscope objective (Nikon, CFI Plan Fluor 40× and CFI Plan Fluor 10×) 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.

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 [10] and to run a MATLAB script to determine the acoustic properties of different dimensions of the system.

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 [11], 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 [12]. 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 Fig. 2). Tracking software is freely available (http://figshare.com/articles/AFS_software/1195874).

2.6. DNA Tethering

Both torsional unconstrained pKYBI (8.4 kbp) [13] and lambda (45.5 kbp) DNA tethers were used [10]. 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. 11333089001) 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 into 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 -20°C, 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 streptavidin (0.0187 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. One-dimensional acoustical model

The analysis of the forces on suspended particles resulting from acoustic pressure dates back to the 1930s [14] and 1950s [15].
Here, we will make use of the approach developed by Gor‘kov [16], who derived the acoustic radiation force \( F_{\text{rad}} \) as the gradient of a potential \( (U_{\text{rad}}) \), 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.

### 3.1. KLM model

We use for our model an equivalent circuit model, KLM [17]. 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. [18,19], using the equations listed by Kinsliers et al. [20]. 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:

\[
F_{\text{rad}}(x) = -\nabla U_{\text{rad}}(x)
\]

(1)

Where \( U_{\text{rad}} \) is given by:

\[
U_{\text{rad}} = V_p \left( \frac{1 - \kappa_p/\kappa_l}{4} \kappa_l p(x) \right)^2 - \frac{3}{4} \left( \frac{\rho_p/\rho_t - 1}{2\rho_p/\rho_t + 1} \right) \rho_l v(x)^2
\]

(2)

Here \( V_p \) is the volume of the particle, \( \kappa_p \) and \( \kappa_l \) the compressibility of the particle and the fluid, respectively and \( \rho_p \) and \( \rho_t \) the density of the particle and the fluid, respectively [21]. 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 beads (Supplementary Fig. 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, \( Q \) [22]. 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 [15], 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 [10]. Note that our model is 1-dimensional and can only be used for a system that is constant over the measured surface. From Supplementary Fig. 8 of Sitters et al. [10], 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 a tilt causes the force to deviate at most 10\% over the measured area.

### 3.2. Parameters used in the model

Our layered resonator was modelled with a PZ26 (thickness \( T = 220 \mu \text{m} \), speed of sound \( c = 4530 \text{ m s}^{-1} \), density \( \rho = 7700 \text{ kg m}^{-3} \), piezo-constant \( h_{33} = 2.37 \times 10^9 \text{ V m}^{-1} \), relative permittivity \( \varepsilon_r = 700 \) ) and transparent transducer (thickness \( T = 500 \mu \text{m} \), speed of sound \( c = 7340 \text{ m s}^{-1} \), density \( \rho = 4628 \text{ kg m}^{-3} \), piezo-constant \( h_{33} = 5.10 \times 10^9 \text{ V m}^{-1} \), relative permittivity \( \varepsilon_r = 6.195 \times 10^{10} \) ), a matching layer (typically \( T = 1000 \mu \text{m} \)) and reflector (typically \( T = 175 \mu \text{m} \)) layers of glass (\( c = 6000 \text{ m s}^{-1} \), \( \rho = 2200 \text{ kg m}^{-3} \)) and water as the fluid (typically \( T = 100 \mu \text{m} \), \( c = 1480 \text{ m s}^{-1} \), \( \rho = 1000 \text{ kg m}^{-3} \)). Forces were calculated for polystyrene particles (\( c = 1960 \text{ m s}^{-1} \), \( \rho = 1000 \text{ kg m}^{-3} \)). 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. The quality number (QN)

Grölsch et al. [15] introduced the dimension-less performance number \( (\eta_{Qg}) \) 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 \( N/W \):

\[
QN(z) = \frac{F_{\text{rad}}(z)}{P_{et}} = \frac{8|Z|F_{\text{rad}}(z)}{U_{pp}^2}
\]

(3)

Here \( P_{et} \) 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 bead size and material. We calculate the \( QN \) for 4.5 \( \mu \text{m} \) diameter polystyrene microspheres, the ones we typically use in our experiments.

### 4. Results

#### 4.1. Transparent piezo

In the original AFS setup, a PZ26 opaque piezo element was used to generate the acoustic wave [10]. 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. [23]. 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 Fig. 4), showing that the piezo transmits 55\% of the light with a wave length of 455 nm, in agreement with previous measurements [23]. With these piezos, bright-field illumination (Fig. 1A, I) can be implemented resulting in a more homogenous illumination. Therefore a higher tracking accuracy could be reached (Supplementary Fig. 2) and a larger field of view in which tethers can be tracked (Fig. 1B, II and IV). We modelled the force profiles for a 500 \( \mu \text{m} \) thick transparent piezo and compared them to the PZ26 piezos (Fig. 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.
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 [15]:

$$ R = \frac{Z_1 - Z_2}{Z_1 + Z_2} \tag{4} $$

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 $\sim$80%, resulting in acoustic energy loss, affecting the quality factor of the acoustic resonator, Fig. 2A. Our model is not able to describe such more complex systems. To test whether liquid-immersion objectives are compatible with AFS, we measured directly the forces applied to DNA-tethered particles using different objectives (Fig. 2B). From this data it is evident that AFS still works with water and oil-immersion objectives, but that acoustic forces are reduced ($\sim$70% and $\sim$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 the current implementation of AFS, but at the cost of a substantial reduction of the forces that can be applied.

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 (Fig. 1C). 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, the QN together with the force gradient is used to quantify the performance of each resonance. We set the limits of the system thickness between 100 and 5000 m for the piezo and the matching layers, and 10–300 m for the fluid and the capping layers. All the different combinations of these four parameters were modelled, within the given limits. The step size of this parameter space was 20 m for the piezo and matching layers and 10 m for the fluid and the capping layers, resulting in total fifty million configurations.

This four-dimensional parameter space is visualized in different ways in Fig. 3. From the contour plot Fig. 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 m. 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 m thick piezo, while it is maximal for 100–200 m thick piezos. In Fig. 3B the piezo-layer thickness is fixed to 200 m and fluid and matching-layer thicknesses are varied, indicating that a fluid-layer thickness of around 45 m or a multiple of this is optimal and that a thicker fluid layer deteriorates the system. In Fig. 3C the fluid layer is fixed to a thickness of 84 m and the piezo to 200 m. Optical microscopes are often optimized for 175 m 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 m away from the surface over a range of 2 m. The gradient was extracted from the slope and normalized with the intercept. In Supplementary Fig. 5 the QN values are shown where the gradient is less than 2% per m. In contrast to flow cell 1 with a piezo of 200 m, flow cell 2 with a piezo of 200 m did show a flat force profile. The most optimal force profiles of these flow cells are shown in Fig. 3D and E. For bottom force directed towards the fluid layer QN reaches 9.99 $\times$ 10$^{-9}$ and 9.10 $\times$ 10$^{-9}$ N/W for flow cell 1 and 2 with 200 m 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 obtained. Our original geometry [10] yields a QN of 4.3 $\times$ 10$^{-9}$ N/W measured at the top surface, meaning that with the optimized, 200 m thick piezo, according to our model the maximum force increases by a factor of 2.3 and it is applied at the bottom surface. Fig. 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 bead diameter and compressibility using Eq. (2). For DNA-stretching experiments, we typically use 4.5 m polystyrene beads, with a QN of 0.88 $\times$ 10$^{-9}$ N/W. With these beads, 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 overstretched DNA, without substantial heating of the sample (less than 2 degrees over a time scale of 2 min).

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 m diameter
silica, Bangs Lab. SS06N, experiencing ~1.3 pN of gravitational force), driven by a combination of acoustic force and gravity (Fig. 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 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 surface (Supplementary Fig. 7). 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 speed until they reach the surface (Supplementary Fig. 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:

\[ F_{\text{Grav}} - F_{\text{Buoyancy}} + F_{\text{drag}} - F_{\text{rad}} = 0 \tag{5.1} \]

\[ F_{\text{Grav}} = V\rho_p g \tag{5.2} \]

\[ F_{\text{Buoyancy}} = -V\rho_m g \tag{5.3} \]

\[ F_{\text{Stokes}} = \frac{6}{C_2} \frac{F_V}{\text{fixed}} \tag{5.4} \]

Here \( g \) is the gravitation constant. For a silica bead of a diameter of 6.84 \( \mu \text{m} \) with a density of 2000 kg/m\(^3\) this means that \( F_{\text{Grav}} = 3.29 \) pN and the \( F_{\text{Buoyancy}} = -1.64 \) pN, so the total constant force on the particle is 1.64 pN. \( C_2 \) is the effective drag coefficient in the low-Reynold's number regime, corrected for hydrodynamic surface effects [24]. 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 calculated.

The measured force profiles at the top and the bottom surfaces are displayed in Fig. 4B and C, respectively and show a high degree of (quantitative) agreement with the modelled force profiles. One resonance frequency predicted by our model (16.2 MHz; Fig. 3E) 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 Fig. 7). The force amplitude plotted against the power proves this a linear behaviour (Fig. 4D). Note that \( Q_N \), of a 6.84 \( \mu \text{m} \) silica bead, is equal to the slopes of these force-power curves. In Table 1, the modelled and measured \( Q_N \) values are displayed, showing a clear correlation between measured and modelled \( Q_N \). These experiments show that our method to quantify the acoustic force profile is straightfor-
ward and quick, and can be used to calibrate any layered acoustic resonator comprising a fluid layer.

4.5. Optimizing the acoustic-force profile

The acoustic force profiles typically used in AFS (Fig. 1C), have a sine shape with a wavelength of ~100 μm. 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 (see Fig. 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 Fig. 5. Here, a 45.5 kilobasepair 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 μm to 25 μm [25] the voltage needs to be increased from 39 to 54 Vpp, 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 higher acoustic force and the DNA will pull back the microsphere. When the particle moves towards the surface, it will experience a lower 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/μm. 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.

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 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 [26,27], cell mechanic manipulation devices [28,29], particle agglutination diagnostics [30,31] and for functional biosensors [32].

At the same time it should be noted that the energy losses in the system are not captured by the model (Section 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 captured in the 1D model (Fig. 1B, III). 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 1 the measured and modelled 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 (Fig. 3E) 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 (Fig. 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 measuring at a location in the force profile where the force gradient is very high, AFS could be transformed into a distance clamp (Fig. 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 denaturation 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.

Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2016.05.002.

References

Competing financial interests
D.K., G.S., G.J.L.W. and E.J.G.P. have filed a patent application (PCT/NL2014/050377) covering the acoustic force spectroscopy method. G.S., G.J.L.W. and E.J.G.P. are co-founders and co-owners of LUMICKS B.V.

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