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Noncontact microrheology at acoustic frequencies, using frequency-modulated atomic force microscopy

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Abstract

We report an atomic force microscopy (AFM) method for assessing elastic and viscous properties of soft samples at acoustic frequencies under non-contact conditions. The method can be used to measure material properties via frequency modulation and is based on hydrodynamics theory of thin gaps we developed here. A cantilever with an attached microsphere is forced to oscillate tens of nanometers above a sample. The elastic modulus and viscosity of the sample are estimated by measuring the frequency-dependence of the phase lag between the oscillating microsphere and the driving piezo at various heights above the sample. This method features an effective area of pyramidal tips used in contact AFM but with only piconewton applied forces. Using this method, we analyzed polyacrylamide gels of different stiffness and assessed graded mechanical properties of guinea pig tectorial membrane. The technique enables the study of microrheology of biological tissues that produce or detect sound.

Microrheology tools for studying the mechanics of living cells have opened up a new way of thinking about how cells function¹. Rheology is the study and characterization of material properties such as elasticity and viscosity at the macroscopic or bulk length scale. Microrheology characterizes such properties at the micrometer scale. Microrheological methods are described as active or passive². The active methods are typified by (i) magnetic tweezers, in which micrometer-sized spheres are affixed to cell membranes and respond to an applied magnetic field³, (ii) optical tweezers, in which usually a single sphere affixed to a cell membrane responds to changes in light momentum^{4,5}, or (iii) atomic force microscopy (AFM), in which a flexible cantilever driven by a piezo motor applies forces to the cell's surface by indenting it with a micrometer-sized tip⁶. Passive microrheology uses submicrometer-sized spheres that are embedded inside cells and move by Brownian motion².

AFM is the least invasive of the microrheology techniques because it does not require chemical attachment of the probe to the cell. It can also provide topographical images of the sample. However, whereas recent AFM methodologies have been shown to reach megahertz frequencies, they cannot be used for microrheology measurements on soft or viscoelastic samples such as cells and biological tissues⁷. Therefore, the range of frequencies at which

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AUTHOR CONTRIBUTIONS

N.G. conceived, designed and performed the experiments, analyzed the data and wrote the paper. R.S.C. developed the hydrodynamic lubrication theory, conceived and designed the experiments and wrote the paper.

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biological microrheology measurements can be performed remains limited and spans only the lowest part of the acoustic regime^{8,9}. The mechanical behavior of physiological tissues and cells in the acoustic regime is of paramount relevance in the inner ear or the larynx, which are involved in sound detection and production, respectively. In addition, many body tissues experience or produce mechanical stimuli at acoustic frequencies in physiological or pathological conditions, including all sounds detected by a stethoscope.

AFM has been used to generate topographical images of samples in fluid. The initial contact mode with the cantilever's deflection as feedback parameter has been complemented with the invention of the tapping mode, which uses either the amplitude of oscillation or the phase as feedback for surface scanning. Nevertheless, these approaches still rely on the probe being continuously or intermittently in contact with the sample, which is not always desirable.

Frequency-modulated AFM (FM-AFM) has emerged as a powerful tool to study the mechanical properties of single biomolecular interactions and force-extension response of single molecules in liquid^{10,11}. The method is performed at acoustic frequencies and relies on a change in the cantilever stiffness as a result of the tip-sample interaction. This change in stiffness results in a small frequency shift of the resonating cantilever.

Here we describe the use of FM-AFM to perform microrheology at acoustic frequencies. The measured frequency shift results from changes in hydrodynamic mass of a sphere attached to the cantilever rather than changes in cantilever stiffness (Supplementary Note 1). Hydrodynamic mass is the part of the hydrodynamic reaction force proportional to the acceleration of a microsphere attached to an oscillating cantilever. This hydrodynamic reaction force increases as the cantilever approaches a surface. Consequently, the cantilever resonates at a slightly lower frequency when it is closer to the surface¹². This effect is greatest when the surface is rigid and lessens for a surface that is compliant (Fig. 1). We developed a hydrodynamic lubrication theory to determine the elasticity and viscosity of the sample (Supplementary Note 2). We validated the method using polyacrylamide gels of different stiffness. We then used the method to measure the graded mechanical properties of a tissue crucial in hearing, the tectorial membrane, at acoustic frequencies. Our technique is sensitive enough to detect the graded viscoelastic properties of the tectorial membrane along the length of the cochlea, in the 1–15 kPa range.

Our technique overcomes the limitations of contact and tapping-mode AFM because the probe is never in contact with the sample and the amount of force applied onto the sample is reduced to piconewtons.

RESULTS

FM-AFM design to measure material properties

We developed a new theory to measure material properties through frequency modulation based on the hydrodynamics of thin gaps. The key feature of our FM-AFM design is its ability to oscillate a cantilever tip at kilohertz frequencies and nanometer-scale amplitudes while the distance between the probe and the sample's surface is controlled in the range of tens of nanometers (Fig. 1). We used the 'cantilever tune' mode of the Bioscope II AFM instrument, which vertically oscillates the cantilever over a selectable range of frequencies and then measures its phase lag with respect to the oscillation of the driving piezo. Moreover, this instrument allows the distance of the cantilever to the sample's surface to be adjusted with nanometer resolution.

FM-AFM is typically performed near resonance peaks, using the frequency at which the cantilever motion lags the piezo motion by a phase of $\pi/2$. Although in liquid AFM cantilevers display multiple resonance peaks, only a small number of them display a $\pi/2$ phase lag between the cantilever and the piezo motion (Supplementary Note 3). The cantilevers we used in our experiments displayed a $\pi/2$ phase lag close to the largest resonance peak, which was around 30 kHz. To fulfill the thin gap assumptions of our theory, the surface of the tip probing the sample must have rotational symmetry. Therefore, we used tipless cantilevers, and we attached to them a 10 μm latex bead. Also, we used bead-sample gaps (~ 100 nm) much smaller than the bead size and oscillation amplitudes (~ 5 nm) much smaller than the bead-sample gap (Fig. 1).

Frequency shift caused by a compliant surface

First, we tested the prediction that a frequency shift will occur when the cantilever oscillates close to a compliant surface, using polyacrylamide gels with a reported Young's modulus (E) of ~ 50 kPa¹³. We recorded frequency sweeps around the frequency $f_{\pi/2}$, where the phase lag was $\pi/2$ (Fig. 2a). As the bead was moved closer to the sample, the phase-frequency curves shifted up, and thus $f_{\pi/2}$ shifted to higher frequencies. Phase-frequency curves theoretically should have a positive slope¹⁴, but the Bioscope II software inverts the relationship so the $f_{\pi/2}$ shift is actually to lower frequencies. The amplitude of oscillation displayed a small reduction as the bead approached the sample (Supplementary Fig. 1). Notably, the shifts observed for $f_{\pi/2}$ followed a $1/3$ exponent with increasing bead-sample distance (Fig. 2b), as predicted by our theoretical results.

Calculation of mechanical properties

As a validation of the use of FM-AFM to estimate mechanical properties of soft samples, we repeated our measurements with gels of different stiffness. We tested gels with known E values spanning two orders of magnitude (1–220 kPa) to assess the applicability of the technique to the wide range of mechanical properties observed under physiological conditions¹⁵ (Online Methods). Using our model, we calculated E values of the gels from the measured frequency shifts ($\Delta f_{\pi/2}$). We also compared the mechanical properties of polyacrylamide gels at acoustic and quasistatic regimes by acquiring standard force-displacement curves on the same gel location immediately after FM-AFM measurements and analyzing them with the Hertz contact model¹⁶.

We observed frequency shifts for all gels when we moved the bead closer to the surface, with larger $\Delta f_{\pi/2}$ for stiffer gels. The relationship between bead-sample distance and $\Delta f_{\pi/2}$ displayed a $1/3$ exponent for all gels tested (Fig. 2b). The E values measured via FM-AFM and contact AFM (C-AFM) methods were on the same order of magnitude for all gels. The behavior at acoustic frequencies depended on the composition of the gel (Fig. 2c). For the softest and stiffest gels, E value at acoustic frequencies was larger than at quasistatic frequencies. Indeed, for the intermediate regime of stiffness, the gels displayed softening at acoustic frequencies.

Another important mechanical property that can be measured via our FM-AFM method is the effective viscosity (μ_{eff}) of the sample. Values of μ_{eff} for all gels were larger than those for water (Supplementary Table 1) and increased as the density of cross-links was increased, reflecting a reduction in the gel's ability to flow as cross-links were added to the meshwork. Together, the calculated μ_{eff} , E and complex shear modulus ($G = G' + iG''$, where G' is the storage modulus, G'' is the loss modulus, and i is the imaginary number; Supplementary Table 1) showed that our FM-AFM method can be used to measure mechanical properties of samples at acoustic frequencies.

Estimation of the effective area and applied force

In FM-AFM, the cantilever tip is not in contact with the surface, which makes the estimation of the contact area not as straightforward as for flat-ended contact. Specifically, the contact area will not only be determined by the radius of the bead but also by the bead-sample gap (Supplementary Note 2 equation 6). With the parameters used here, our FM-AFM technique can be used to measure the averaged mechanical properties of a $\sim 1.6 \mu\text{m}^2$ area on the sample's surface. This value is in the range of the $\sim 1.4 \mu\text{m}^2$ effective area for a pyramidal tip used in C-AFM. A similar estimation for the depth of sample being probed can be made by computing the penetration depth (Supplementary Note 2 equation 5), which is $\sim 200 \text{ nm}$. Therefore, we estimated that the volume being probed in our experiments was $\sim 0.32 \mu\text{m}^3$. We can also compute the amount of force that is being applied to the sample through fluid coupling between the sample's surface and the oscillating bead; the force can be estimated as the product of the hydrodynamic mass ΔM and the acceleration of the bead $\omega^2 A$, in which A is the amplitude of oscillation and ω is the frequency in radians per second. The force (F), $2kA\Delta\omega/\omega$, in which k is the cantilever's stiffness was about 1 pN in our experiments. The force applied in C-AFM is orders of magnitude larger, typically in the range of nanonewtons.

To verify that the effective area of FM-AFM is similar to that of a pyramidal tip but with greatly reduced force application, we performed measurements on a $10 \mu\text{m}$ latex bead ($E > 100 \text{ kPa}$) partially embedded in a soft polyacrylamide gel ($E = \sim 10 \text{ kPa}$). This arrangement produced a controlled local heterogeneity on the micrometer scale. The E value on multiple points along the crossing line was measured using C-AFM with a pyramidal tip and FM-AFM. To compare these results with those arising from a larger effective area, a $10 \mu\text{m}$ spherical probe was also used to measure sample height and E on the same locations using C-AFM. As expected, both pyramidal-probe C-AFM and FM-AFM detected the presence of the bead (as measured by changes in E) at similar locations (Fig. 3a), starting at $\sim 3.5 \mu\text{m}$ away from the center of the bead. In contrast, we observed changes in E values at distances $5 \mu\text{m}$ from the center of the bead with spherical-probe C-AFM (Supplementary Fig. 2a). For both C-AFM measurements, E values over the bead were smaller than over the gel (Fig. 3b and Supplementary Fig. 2b). This artifactual observation arises from the microsphere being pushed down inside the soft gel owing to the large forces applied by the probe in C-AFM. As a result, the measured E value reflects the increased series compliance of the gel and bead arrangement. In contrast, when FM-AFM is used, the force applied to the sample is much smaller, reducing the pushing effect and yielding a less artifactual value for E (Fig. 3b). Together, those results suggest that FM-AFM probes a similar effective area as a pyramidal tip without the need for large force application and subsequent sample alteration.

The variability of E values measured with FM-AFM was larger than that measured with C-AFM (Fig. 3). Although this indicates that the consistency of FM-AFM technique is now surpassed by C-AFM, we envisage that its variability could be reduced using a setup specifically designed for frequency modulation with enhanced electronics to provide better signal-to-noise ratio values on phase measurements¹¹. We describe some possible modifications to our current setup to increase the accuracy of the technique in Supplementary Note 3.

FM-AFM on sloped surfaces

We chose the minimum tip-sample distance used in our experiments (50 nm) to avoid steric and van der Waals interactions. The polystyrene bead used as the tip in our experiments contains a small amount of surfactant on its surface, which has been shown to induce tip-sample repulsive forces at distances less than 50 nm in water¹⁷. However, physiological samples are mostly probed in salty solutions, which greatly reduces the characteristic length

of repulsive forces^{17,18}. This allows physiological samples in ionic solutions to be measured at smaller minimum tip-sample distances, which can then yield even larger frequency shifts. Repulsive forces owing to the presence of glycocalyx on cellular membranes act on tip-membrane distances of up to 10 nm¹⁹. Therefore, this constitutes the minimum tip-sample distance for FM-AFM measurements on cellular samples.

Adhered cells can be taller around the nucleus and thus exhibit sloped surfaces. To test the performance of FM-AFM on sloped surfaces, we used a tilting device, which causes the oscillation of the bead to have a 10° angle with respect to the vector perpendicular to the sample's surface²⁰. For the tilted conditions, the frequency shifts still followed a 1/3 exponent with increasing bead-sample distance, and E values showed no significant difference ($P = 0.23$) with those collected under the nontilted condition (Supplementary Fig. 3). Therefore, in theory, our FM-AFM method could be used to probe moderately sloped cell surfaces.

Graded mechanical properties of the tectorial membrane

We used our FM-AFM technique to measure the mechanical properties of the tectorial membrane in the inner ear at acoustic frequencies. We wanted to assess the previously reported graded viscoelastic properties of the tectorial membrane along the length of the cochlea, in areas sorted as apical, middle or basal. We performed measurements on tectorial membranes obtained from guinea pigs, using the same protocol and driving frequency described for polyacrylamide gels (Online Methods). The tectorial membrane E values we obtained at acoustic frequencies were in the kilopascal range (Table 1) and slightly larger than those previously reported in quasistatic conditions^{20–23}. Accordingly, other physiological samples have been shown to display stiffening with increasing probing frequencies²⁴. Furthermore, our results provide evidence that the previously reported graded mechanical properties of the tectorial membrane are maintained at acoustic frequencies, with the basal part of the tectorial membrane being significantly ($P = 0.006$) stiffer than the apical part. Our measurements also show that μ_{eff} of the tectorial membrane (Table 1) is significantly ($P = 0.007$) graded from base to apex. This gradient in viscosity may arise from the gradient in the density of collagen fibers composing the tectorial membrane, as previously reported²⁰. These results show that our FM-AFM microrheology technique can be applied to characterize the viscoelastic properties of biological tissues under physiological conditions.

DISCUSSION

Here we showed that the phase lag between the probe and the piezo changes as the probe is moved away from the sample. Using this principle, we envision that a new method of scanning AFM could be developed in which a bead is forced to oscillate close to a sample. By using a constant probing frequency and the $\pi/2$ phase as the setpoint for the feedback system, the cantilever could be kept at a constant height with respect to the sample, enabling scanning of the surface without contacting it. This will prevent application of large forces to the sample, which may be undesirable in soft tissues or mechanosensitive cells²⁵. FM-AFM microrheology to measure mechanical properties of soft samples should be particularly useful to the field of hearing, where multiple cells and tissues are mechanically excited at acoustic frequencies.

ONLINE METHODS

Preparation of polyacrylamide gels

Polyacrylamide gel disks were prepared as described previously²⁶. To prepare gels of different stiffness, initial solutions of 40% acrylamide (A) and 2% bis-acrylamide (B) were diluted in water to provide the following concentrations: 5% A and 0.025% B; 5% A and 0.1% B; 8% A and 0.1% B; 8% A and 0.25% B; 12% A and 0.25% B; and 15% A and 1.3% B. Acrylamide and bis-acrylamide were obtained from Bio-Rad. Gel polymerization was initiated by addition of ammonium persulfate (1:200 volume; Bio-Rad) and tetramethylethylenediamine (TEMED, 1:2,000 volume; Sigma Chemical). A 20 μ l drop of the gel mixture was deposited on the chemically activated glass bottom of a petri dish (MatTek Corporation) and covered with a 10-mm-diameter circular coverslip. After gel polymerization and coverslip detachment, gel disks remained firmly attached to the glass bottom. The dimensions of the gel disk were $\sim$ 150 μ m thick and 8 mm in diameter. Gels were kept at 4 °C in water until measurement. Gels were brought to room temperature (19–22 °C) before beginning measurements.

Tectorial membrane isolation

Tectorial membranes of juvenile female pigmented guinea pigs were isolated as previously outlined²⁰. Several samples were used for each guinea pig. All animal procedures were conducted according to approved US National Institutes of Health animal protocol number 1186-07.

AFM setup

Measurements were performed using a Bioscope II AFM (Veeco Metrology Inc) instrument mounted on the stage of an Axiovert 200 inverted microscope (Zeiss) placed on a vibration-isolation table (Isostation). A latex bead (10 μ m diameter) glued to a tipless V-shaped gold-coated silicon nitride cantilever (NP-020, Veeco Metrology Inc) was used as a probe. The spring constant of the cantilever was 0.158 ± 0.008 N m⁻¹ as calibrated using the thermal fluctuations method²⁷. For FM-AFM, we used the ‘cantilever tune’ feature of the tapping mode to sweep the cantilever drive frequency over a selectable frequency range. A crucial feature of the cantilever tune mode is the possibility to position the tip of the cantilever at a controlled height over the sample’s surface with nanometer resolution. For C-AFM, we recorded force-displacement curves using ‘contact mode’. To test the performance of FM-AFM on sloped surfaces, a custom-made PVC wedge was positioned between the stage and the AFM, with a resulting AFM tilting of 10°. As a result, the oscillation of the bead had a 10° angle with respect to the vector perpendicular to the sample’s surface. There was no relative displacement between the cantilever, the laser source and the photodiode.

Protocol

Measurements were performed in liquid, using ultrapure water as bathing solution. Once the coverslip containing the gel was placed on the stage of the microscope, the cantilever was positioned far above the glass surface and allowed to thermally equilibrate. Then, the relationship between photodiode signal and cantilever deflection was calibrated by taking a force-displacement curve at a bare region of the glass coverslip and measuring its slope. For FM-AFM experiments, the cantilever was moved over the gel and placed in contact with the surface of the gel. Then, the cantilever tune mode was launched, positioning the tip of the cantilever 200 nm above the sample. An initial frequency sweep was performed to locate $f_{\pi/2}$ of interest. Also, the drive amplitude of the piezo was adjusted to ensure that the amplitude of oscillation of the cantilever at $f_{\pi/2}$ was below 5 nm. Frequency sweeps were recorded with a 3 kHz frequency range around $f_{\pi/2}$. Sweeps were performed for multiple

distances between the bead and the sample, from 50 nm to 150 nm using 10-nm increases. For each bead-sample distance, three sweeps were recorded. Finally, the cantilever was moved 4 μm over the sample and three final sweeps were recorded to provide f_{∞} . Immediately after FM-AFM measurements, the contact mode was engaged and the tip was brought in contact with the gel's surface. Ten force-displacement curves were recorded using 3- μm ramps with ~500 nm indentation at 1 Hz.

Data processing

All computations were performed using Matlab (The Mathworks). For FM-AFM, each recorded frequency sweep, phase-frequency curves were fitted with a second-order polynomial on the vicinity of the $\pi/2$ to determine $f_{\pi/2}$. Values of $f_{\pi/2}$ for the three frequency sweeps recorded at each height were averaged together. Values of f_{∞} were estimated similarly using the frequency sweeps recorded far away from the sample's surface. $\Delta f_{\pi/2}$ -height curves were fitted with a 1/3 exponent and only those yielding r^2 values larger than 0.9 were used. $d\Phi/df$ was computed using the frequency sweeps recorded 100 nm above the sample and fitting the whole phase-frequency curves with a third-order polynomial. Estimates of $d\Phi/df$ changed by less than 2% between the highest and lowest points measured over the sample (Supplementary Fig. 4). Young's modulus (E) value and effective viscosity (μ_{eff}) of the sample were computed with Supplementary Note 1 equations 3 and 4 for the measured $\Delta f_{\pi/2}$, f_{∞} , $d\Phi/df$ and other parameters of the setup. For C-AFM, E value was computed by fitting force-displacement curves with the Hertz contact model for an indenting sphere¹⁶:

$$F = \frac{4E}{3(1-\nu^2)} a_0^{1/2} \delta^{3/2}$$

or Sneddon's formula for an indenting cone²⁸:

$$F = \frac{2}{\pi \tan \alpha} \frac{E}{(1-\nu^2)} \delta^2$$

in which F is the applied force, ν is Poisson's ratio and was assumed to be 0.5, δ is indentation, and α is the opening angle of the cone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Concept of FM-AFM to measure mechanical properties of soft samples. **(a)** A hydrodynamic reaction force will emerge when a sphere is forced to oscillate close to a surface (left). This effect will lessen when the sphere is far from the surface (middle) or when the surface is compliant (right). **(b)** Sketch of a typical AFM setup; a cantilever with a bead attached to its end is used to probe the sample. A laser beam is focused onto the tip of the cantilever, and the reflected light is measured by a photodetector. Vertical oscillations of the cantilever and its height with respect to the sample are controlled with a piezo. **(c)** The thin gap assumption of our theory requires bead-sample gaps (h_m) much smaller than the bead size (a_0) and oscillation amplitudes much smaller than the bead-sample gap.

Figure 2.

Estimation of mechanical properties through measurement of frequency shifts. **(a)** Shifts observed on the phase-frequency curves recorded around $f_{\pi/2}$ when the oscillating sphere is moved closer to the sample's surface. Dotted line shows $\pi/2$ phase intercept. Estimated E value of the gel was 50 kPa. Inset, $f_{\pi/2}$ was computed by fitting a subset of points around the $\pi/2$ intercept with a second-order polynomial. **(b)** Representative examples of single measurements performed at one gel location for gels of different stiffness. Fitted lines correspond to the frequency shifts predicted by our theoretical results, with a $1/3$ exponent with increasing bead-sample distance. **(c)** Acoustic values of E computed using the

measured frequency shifts versus quasistatic values of E computed using force-displacement curves and the Hertz contact model. Plotted are mean E values for five different gels. Error bars, s.d. ($n = 5$). Dotted line is the identity line. Data points above the dotted line display stiffening at acoustic frequencies, and data points below the dotted line exhibit softening.

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Figure 3.

Effective probe area of pyramidal-probe C-AFM versus FM-AFM. **(a)** Topography along a line crossing the highest part of a 10- μm latex bead, obtained with a pyramidal tip in C-AFM (top). The gel surface was fitted with a solid line, whereas the bead surface was fitted with a 10 μm diameter circle. E value obtained with a pyramidal tip in C-AFM along the crossing line (middle) and with FM-AFM at the same locations as above (bottom). The shaded area corresponds to the region of the bead emerging from the gel, as determined by the topographic measurements. **(b)** Averaged E values over the gel or the bead obtained with C-AFM and FM-AFM. Values are mean $\pm$ s.d. ($n = 22$ over gel, $n = 7$ over bead).

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Table 1

Viscoelastic properties of the tectorial membrane

Location	E (kPa)	μ_{eff} (Pa $\times$ s)	G' (kPa)	G'' (kPa)	Loss tangent (G''/G')
Apex	3.3 ± 2.3	0.007 ± 0.006	1.1 ± 0.8	1.3 ± 1.1	1.2 ± 1.9
Middle	5.7 ± 3.3	0.01 ± 0.008	1.9 ± 1.1	1.8 ± 1.5	0.9 ± 1.3
Base	14 ± 16	0.03 ± 0.04	4.7 ± 5.3	5.6 ± 7.4	1.2 ± 2.9

Data are mean $\pm$ s.e.m. ($n = 4$). One-way ANOVA reported significant differences in E ($P = 0.006$) and μ_{eff} ($P = 0.007$) along the length of the cochlea.