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Local Viscoelastic Properties of Live Cells Investigated Using Dynamic and Quasi-Static Atomic Force Microscopy Methods

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ABSTRACT The measurement of viscoelasticity of cells in physiological environments with high spatio-temporal resolution is a key goal in cell mechanobiology. Traditionally only the elastic properties have been measured from quasi-static force-distance curves using the atomic force microscope (AFM). Recently, dynamic AFM-based methods have been proposed to map the local in vitro viscoelastic properties of living cells with nanoscale resolution. However, the differences in viscoelastic properties estimated from such dynamic and traditional quasi-static techniques are poorly understood. In this work we quantitatively reconstruct the local force and dissipation gradients (viscoelasticity) on live fibroblast cells in buffer solutions using Lorentz force excited cantilevers and present a careful comparison between mechanical properties (local stiffness and damping) extracted using dynamic and quasi-static force spectroscopy methods. The results highlight the dependence of measured viscoelastic properties on both the frequency at which the chosen technique operates as well as the interactions with subcellular components beyond certain indentation depth, both of which are responsible for differences between the viscoelasticity property maps acquired using the dynamic AFM method against the quasi-static measurements.

INTRODUCTION

Many recent efforts in cell mechanobiology (1–4) aim to quantitatively measure the mechanical properties of living cells and relate them to cell structure and function. Cells engage in complex processes changing the viscoelastic response (5–9) of the cell membrane, cytoskeleton, and cytoplasm. These changes are often heterogeneous in spatial extent within the cell, and change with time. The measurement of the progressive spatio-temporal variations in viscoelastic properties within living cells in their native physiological liquid environments could shed important insight into cellular processes such as morphogenesis (10,11), mechanotransduction (12–14), migration/locomotion (15–18), metastasis (19–25), apoptosis (26,27), aging (27), focal adhesion (18,28–32), disease progression (33), and drug-cell interactions (34–36).

The atomic-force microscope (AFM) is unique among other cell mechanical measurement techniques (1,6,37,38) in its ability to measure the local force and dissipative gradients as well as map them across the cell surface with sub-10-nm resolution. As a result, the AFM allows researchers to develop quantitative methods to map the local mechanical properties of living cells. The standard force-volume method is the most widely used imaging method to extract, simultaneously, the topography and mechanical properties of a live cell. It is based on the acquisition of slow-speed, quasi-static force-distance curve measurements on a grid of points defined by the user. The mechanical properties in force-volume maps are extracted offline for each recorded force curve by fitting to an analytical tip-sample contact model. Another conventional way to evaluate the viscoelastic properties of live cells is through the acquisition of force-distance curves by applying a rectangular load-relaxation (stress-relaxation) pattern (21,39). Several groups have estimated the local mechanical properties using these methods on live bacteria (40–44), and eukaryotic cells (15,21,34,39,44–50) in their native liquid environment; however, the methods are low-speed and low-resolution, limiting their potential for high throughput biomechanical assay of cells.

To address some of the speed and spatial resolution challenges in conventional AFM techniques for mapping cell mechanical properties, a method called multi-harmonic AFM was recently reported (4) that employed amplitude-modulation atomic force microscopy (AM-AFM), a technique allowing achievement of sub-10-nm resolution high-speed mapping of local nanomechanical properties of live cells in physiological conditions. However, the effective local properties (loss and storage modulus) over the nuclear region were found to be generally 3–5 times larger compared to values acquired from quasi-static force-distance curves. Understanding the basic reasons behind the differences in measured properties using the two methods is key to developing quantitative tools to measure cell viscoelasticity using dynamic AFM.

To address this fundamental issue, in this article we study the local force gradient and damping on live rat fibroblast cells in buffer solution, measured as a function of indentation using a soft AFM microcantilever excited by Lorentz force near its natural frequency (~7–8 kHz). We find that, at small indentations, the dynamically measured force gradient is ~3 times that of the statically measured one, showing the frequency-dependence. However, the ratio of the dynamic to static force gradients begins to increase...
when the AFM tip indents the cell sufficiently to interact with the nuclear region of the cell and its associated cytoskeletal structure, nuclear envelope, and additional subcellular elements. On the peripheral region, the indentation is much smaller and there is less difference between the measured static and dynamic force gradients. On the other hand, the effective contact viscosity is less near the nuclear region than the peripheral regions.

We conclude that, when using AM-AFM with directly excited probes, the mapped repulsive force gradient appears larger on the nuclear region compared to the peripheral region for two different reasons:

1. Because larger forces need to be applied over the nuclear region to achieve the same oscillation amplitude as on the peripheral regions, this requires the AFM tip to be pressed sufficiently into the cell membrane to interact primarily with the nuclear complex and cytoskeleton in the nuclear region.

2. The inherent viscoelasticity of the cell leads to a different mechanical response at the higher frequency of oscillation in the dynamic AFM method compared to the quasi-static method.

The findings suggest that, at least on live fibroblast cells, maps of material properties that were created using both the force-volume method and the dynamic AFM method are not directly comparable because of the indentation- and frequency-dependence.

See the Supporting Material for additional details on the text.

MATERIALS AND METHODS

Sample preparation

Rat fibroblast cells (ATCC CRL-1213; American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) containing low D-glucose (1000 mg l\(^{-1}\)), 10% Fetal Bovine Serum (Invitrogen), 1% Penicillin-Streptomycin (Invitrogen), and 0.1% Amphotericin B (Sigma-Aldrich, St. Louis, MO). The fibroblast cells were grown to ~75% confluence in 75 cm\(^2\) flasks. The medium was removed every three days, and cells were subcultured every seven days at a rate of 1:8. For the experiments, the cells grown on plastic flasks were trypsinized with 0.5% Trypsin/EDTA solution (Invitrogen) and the cell suspension was deposited on polystyrene-plasma-treated 60 x 15 mm petri dishes (BD Falcon, Franklin Lakes, NJ) precoated with 0.1% gelatin in water (STEMCELL Technologies, Vancouver, British Columbia, Canada). The cells were planted/grown on the dish 1–2 days before experiments and kept in an incubator at 37°C in a 5% CO\(_2\) atmosphere to ensure complete spreading.

Theory of dynamic AFM material property mapping and spectroscopy in amplitude modulation AFM on live cells

It has been shown in a previous work (4) that harmonic signals (multi-harmonic observables) of cantilever vibration can be combined to extract local nanomechanical properties once an analytical tip-sample interaction model is prescribed. In this work, we do not focus on extracting constitutive material properties like storage or loss modulus from the amplitude and phase-shift data of the oscillating cantilever. This would require us to use well-validated elastic contact mechanics models derived from the literature such as that of Hertz (4,15,20,34,46), Sneddon (51), or bottom effect cone correction (BECC) (from Gavara and Chadwick (52)). However, these models have only been validated on live cells using quasi-static indentation curves, and it remains an open question as to which models to choose for dynamic AFM in which the cantilever oscillates in the range of 5–10 kHz. Rheological models for live eukaryotic cells (51) generally do not take into account the heterogeneity of nuclear and peripheral regions. Although three-element models have been used for cell viscoelasticity measurements (9,21,53,54), we use here the two-element Kelvin-Voigt model for the following reasons:

1. The dynamic AFM method we discuss here uses two observables, the cantilever harmonic amplitude and phase to determine the sample viscoelasticity. As a consequence, only two unknown viscoelastic parameters can be determined at each Z location (during approach curves) or at any pixel (during a scan). 2. Thus, the single frequency method presented here can only treat a viscoelastic model with two unknown parameters such as the Kelvin-Voigt element (spring and dashpot in parallel) and the Maxwell element (spring and dashpot in series). 3. Of these two, the Kelvin-Voigt model is physically relevant because the oscillation time of the experiments (8 kHz = 125 μs) is faster than the live fibroblast cell relaxation time (~0.1 s) (55).

In light of this, we convert the amplitude and phase-shift data of the oscillating cantilever into local dynamic repulsive-force gradient (\(k_{\text{dynamic}}\)) and damping (\(c_{\text{sample}}\)) values. Together these are the parameters of an equivalent Kelvin-Voigt element and can be regarded as the local viscoelastic properties of the cell.

The theory behind this is described below. Assuming that the Lorentz force cantilever driving frequency is near the first resonance frequency, the single degree-of-freedom equation of motion governing the tip motion \(q(t)\) when it oscillates far from the sample surface is:

\[
\ddot{q} + \frac{1}{\omega_{\text{far}} Q_{\text{far}}} \dot{q} + q = \frac{F_{\text{mag}} \sin(\omega_{\text{res}} t)}{k_{\text{cant}}} ,
\]

where \(\omega_{\text{far}}\) is the cantilever frequency (rad/s), \(Q_{\text{far}}\) is the quality factor far from the sample surface, \(\omega_{\text{res}}\) is the cantilever drive frequency (rad/s), \(F_{\text{mag}}\) is the magnitude of the magnetic excitation force, \(k_{\text{cant}}\) is the calibrated cantilever spring constant, and \(q\) is the tip velocity. Solving the steady-state vibration response \(q(t) = A_1 \sin(\omega_{\text{res}} t - \phi_{1})\), it can be easily shown that when \(\omega_{\text{res}}\) is tuned to the peak amplitude of the resonance curve far from the surface, the following expressions hold:

\[
\omega_{\text{res}} / \omega_{\text{far}} = \sqrt{1 - 1/2Q_{\text{far}}^2} ,
\]

\[
A_{1\text{far}} = F_{\text{mag}} k_{\text{cant}} \frac{Q_{\text{far}}}{\sqrt{1 - 1/4Q_{\text{far}}^2}} ,
\]

\[
\tan \phi_{1\text{far}} = 4Q_{\text{far}}^2 - 2 .
\]

Here, \(A_{1\text{far}}\) is the oscillation amplitude and \(\phi_{1\text{far}}\) is the phase lag far from the sample surface. Note that by Eq. 2, when tuning the cantilever far from the sample, the phase lag at the frequency of peak amplitude with low \(Q\), say 2, is not to be set to 90°; instead, it should be set to \(\phi_{1\text{far}} = 75°\). Thus, when the drive is tuned at the peak amplitude of resonance far from the sample we must have

\[
F_{\text{mag}} = k_{\text{cant}} A_{1\text{far}} Q_{\text{far}} \sqrt{1 - 1/4Q_{\text{far}}^2} .
\]
Viscoelasticity of Live Cells Investigated Using AFM

For soft cantilevers in liquids, their resonance frequencies (measured in rad/s) and $Q$ factors near the sample (i.e., $\omega_{\text{near}}$ and $Q_{\text{near}}$) are different from the values measured far from the surface (i.e., $\omega_{\text{far}}$ and $Q_{\text{far}}$) because of the hydrodynamic coupling between the cantilever and the sample (56). As a result, the oscillation amplitude and the phase lag near the surface (i.e., $A_{\text{near}}$ and $\phi_{\text{near}}$) are different from their values far from the sample (i.e., $A_{\text{far}}$ and $\phi_{\text{far}}$). As shown by Raman et al. (4), the quantities near and far from the sample are related by

$$1 - \left( \frac{\omega_{\text{far}}}{\omega_{\text{near}}} \right)^2 = \frac{A_{\text{far}}^2}{A_{\text{near}}^2} \frac{1 - 1/4Q_{\text{far}}^2}{Q_{\text{near}}^2} \cos(\phi_{\text{near}}),$$

$$\frac{\omega_{\text{far}}}{Q_{\text{near}}^2} = \frac{A_{\text{far}}^2}{A_{\text{near}}^2} \frac{1 - 1/4Q_{\text{far}}^2}{Q_{\text{near}}^2} \sin(\phi_{\text{near}}).$$

(3)

It is important to note that the hydrodynamic coupling to the surface is different on live fibroblast cells that are tall (~2–4 μm in height) and soft (1–1000 kPa), as compared to when the cantilever is located at the same height above a rigid surface. For example, when the cantilever is excited at resonance far from the sample so that $\phi_{\text{far}} = 74^\circ$ and with a free amplitude $A_{\text{far}} = 9$ nm, we find that when brought close to the sample, just before the tip-sample interactions begin, $A_{\text{near}}\phi_{\text{near}}$ values on the gelatin-coated dish and live cell are typically 2.5 nm/95° and 3.5 nm/89°, respectively. This clearly demonstrates that the squeeze-film hydrodynamic effect is much stronger on the substrate than on the live cell. This hydrodynamic correction is essential to account for while measuring the viscoelastic properties of live cells. This correction is relatively straightforward to perform for the case when the cantilever is excited at very low frequencies (57), where the cantilever inertia is not important. Here we have presented the correction for the case when the cantilever is excited near its resonance frequency, and accounts for both the added damping and the added mass of the fluid in the near vicinity of the sample surface.

When the cantilever is brought closer to the sample so that it interacts with the soft cell surface, the equation of motion becomes:

$$\ddot{q} + \frac{1}{\omega_{\text{near}}^2} q + \frac{1}{\omega_{\text{near}}^2 Q_{\text{near}}^2} \ddot{q} = \frac{F_{\text{mag}}}{k_{\text{cant}}} \sin(\omega_{\text{dr}} t) + F_{\text{ts}}(Z + q, \dot{q}),$$

(4)

where $F_{\text{ts}}$ is the tip-sample interaction force, and $Z$ is the distance between the tip and sample assuming that the cantilever is rigid or un bent. As observed in Raman et al. (4) and also demonstrated in Results and Discussion, the cantilever motion while interacting with live cells is dominated by the zeroth and first harmonics, leading to the following form of the tip motion:

$$q(t) = A_0 + A_1 \sin(\omega_{\text{dr}} t - \phi_1).$$

(5)

To convert zeroth and first harmonics observables into local viscoelastic properties, we first annotate the tip indentation $\delta(t)$ into the sample as

$$\delta(t) = -(Z + q) = -Z - A_0 - A_1 \sin(\omega_{\text{dr}} t - \phi_1).$$

(6)

As described in Results and Discussion and in Raman et al. (4), the tip oscillation amplitude $A_1$ is much smaller compared to the net average indentation $\delta_0 = -(Z + A_0)$ while imaging live cells in AM-AFM. Next, we describe the interaction forces as a Taylor series expansion in $(\delta - \delta_0)$ and discard higher-order terms,

$$F_{\text{ts}} = F_{\text{ts}}(\delta_0) + c_{\text{sample}}^{\text{dynamic}} (\delta - \delta_0) + c_{\text{sample}}^{\text{dynamic}} \delta,$$

(7)

where $c_{\text{sample}}^{\text{dynamic}}$ and $c_{\text{sample}}^{\text{dynamic}}$ represent the parameters of a Kelvin-Voigt element, specifically the conservative local force gradient (stiffness) and damping (viscoelastic properties) at that particular indentation value. Moreover, $k_{\text{sample}}^{\text{dynamic}}$ and $c_{\text{sample}}^{\text{dynamic}}$ used here are equivalent to $K$ and $K'$ of Mahaffy et al. (58). Substituting Eq. 6 into Eq. 7, substituting the resulting expression for $F_{\text{ts}}$ into Eq. 4, and taking into account Eqs. 2, 3, and 5, one can match the harmonic terms of each side of the equation of motion (Eq. 4) to yield the following:

$$j_{\text{sample}}^{\text{dynamic}} = \frac{k_{\text{sample}}^{\text{dynamic}} A_{\text{far}}^2}{Q_{\text{far}} A_1} \cos(\phi_1) - \frac{k_{\text{sample}}^{\text{dynamic}} A_{\text{far}}^2}{A_{\text{near}} Q_{\text{far}}} \cos(\phi_{\text{near}})$$

$$\times \sqrt{1 - 1/4Q_{\text{far}}^2},$$

$$\epsilon_{\text{sample}}^{\text{dynamic}} = \frac{k_{\text{sample}}^{\text{dynamic}} A_{\text{far}}^2}{Q_{\text{far}} A_1 \omega_{\text{dr}}} \sin(\phi_1) - \frac{k_{\text{sample}}^{\text{dynamic}} A_{\text{far}}^2}{A_{\text{near}} Q_{\text{far}} \omega_{\text{dr}}} \sin(\phi_{\text{near}})$$

$$\times \sqrt{1 - 1/4Q_{\text{far}}^2}.$$  

(8)

These equations allow the conversion of the harmonic observables ($A_0, A_1, \phi_1$) into the local viscoelastic properties, i.e., the dynamic repulsive force gradient ($k_{\text{sample}}^{\text{dynamic}}$) and damping ($\epsilon_{\text{sample}}^{\text{dynamic}}$), while taking into account the near-surface hydrodynamic corrections.

It is important to reiterate the different assumptions under which the above equations are accurate:

**Assumption 1**

The cantilever is driven directly, not acoustically or via sample excitation, so that the cantilever has a well-defined transfer function (59,60) with the Lorentz force being the only source of excitation without interference from fluid-borne excitation (61) that arises when the either the cantilever or the sample are excited using piezoelectric transducers.

**Assumption 2**

The tip is in continuous contact with the sample and that the oscillation amplitude is small relative to the net indentation of the tip into the sample.

**Assumption 3**

The eigenmode of the cantilever oscillating away from the sample remains unchanged when compared to being pressed against the sample. This assumption in fact enables us to state that the $k_{\text{cant}}$ does not change when coupled to the sample. This assumption is known to be correct only when the contact stiffness/repulsive gradient is much smaller than the cantilever stiffness (namely 0.09 N/m) (62).

**Assumption 4**

We need to recognize, as stated before, that the hydrodynamic correction is different on the live cell compared to that on the gelatin-coated glass surface.

Typically, $A_{\text{near}}$ and $\phi_{\text{near}}$ on a gelatin-coated dish are ~1.5–4 nm/95–98°, whereas on live cells the values are ~2–5 nm/85–89° on nucleus and ~1.8–3.5 nm/92–95° on periphery. However, Eq. 8 uses a single value of $A_{\text{near}}$ and $\phi_{\text{near}}$. Because we are more interested in the properties of the cell than the gelatin surface, we choose to process all the data using $A_{\text{near}}$ and $\phi_{\text{near}}$ on top of the cell. This implies that the predicted repulsive gradients on a gelatin-coated dish are systematically larger than the actual value due to the hydrodynamic effect. However, we expect the correct values to be mapped on the live cell. All computations and data processing were performed using the software MATLAB (The MathWorks, Natick, MA). For each set of multi-harmonic observable ($A_0, A_1, \phi_1$) curves the viscoelastic properties maps ($k_{\text{sample}}^{\text{dynamic}}$ and $\epsilon_{\text{sample}}^{\text{dynamic}}$) were extracted by using Eq. 8.
Quasi-static force spectroscopy

In quasi-static force spectroscopy, an unexcited AFM probe approaches and is retracted from the sample surface and the deflection of the microcantilever tip $q$ is recorded as a function of $Z$-piezo extension. The resulting force-distance ($F$-$Z$) curve is converted to a force-indentation ($F$-$d$) curve by $d = Z + q$. The slope of the $F$-$d$ curve is the local repulsive force gradient $f_{\text{rep}}$ and can be calculated as a function of the indentation $d$ at each point.

AFM experimental protocol

The experimental setup is as follows: Before beginning the AFM imaging, cells were rinsed thoroughly with 2 mL PBS twice and then 2 mL of fresh sterile PBS was added to simulate near-physiological conditions when imaging. All live cell AFM imaging and viscoelastic measurements were performed with a model No. MFP-3D-Bio AFM (Asylum Research, Santa Barbara, CA) mounted on a model No. IX-71 inverted optical microscope (Olympus, Melville, NY) which was itself placed on a vibration table inside an acoustic isolation enclosure. This allows easy positioning and monitoring of cells. We used soft microcantilevers (model No. BL-TR400PB microcantilever; Olympus) with a nominal spring constant of 0.09 N/m, and nominal tip radius of 42 nm ($\pm$ 12 nm). The iDrive (Asylum Research) AC mode was used for the experiment that uses Lorentz force excitation to apply an oscillating driving force directly to the microcantilever. The importance of using such directly excited probes for quantitative measurements in liquids has been discussed before (61,63). During each experimental measurement, we first localized the cells using the inverted optical microscope, and checked for viability by means of cell morphology and extracellular matrix spreading/anchorage.

Before doing experiments on live fibroblast cells to measure quantitative local viscoelastic properties, certain calibrations need to be performed. The AFM probe must be engaged on a stiff substrate (mica) and a force-distance curve obtained to calculate the optical photodiode deflection sensitivity. Then, the probe is withdrawn from the stiff sample surface and the cantilever spring constant was calibrated by the thermal noise method (64,65). Typical estimated values for effective cantilever spring constants were in the range of 0.03–0.1 N/m and $Q$-factors were in the range of 1.5–2.

The AFM cantilever was directly driven (Lorentz force excitation) at the peak of the resonance curve of the fundamental flexural mode (typically 7.5–9 kHz) and then engaged to the live fibroblast cell in its buffer solution. Phase contrast optical imaging was used to identify a viable cell on which to position the AFM cantilever. The AFM beam deflection was then recorded to calculate the $Z$-piezo extension. The $Z$-piezo extension was used to calculate the changes in amplitude and phase that are closely related to the cell viscoelastic properties. Therefore, an equivalent spring-dashpot model for the cantilever in contact with the cell can be used to extract the viscoelastic properties. To see this figure in color, go online.

multi-harmonic theory described above to reconstruct frequency- and indentation-dependent local force and dissipation gradients on live fibroblast cells.

Here we present data acquired on three fibroblast cells from different cell cultures on different days using different AFM probes (10 repeats). The results are consistent and repeatable. Many additional cells have been studied, but we present those for which comprehensive quasi-static and dynamic data sets were acquired.

RESULTS AND DISCUSSION

Cantilever response while interacting with a live cell

Fig. 1 $b$ shows the cantilever’s frequency response to Lorentz force excitation off and in contact with the live cell nucleus and periphery as well as the gelatin-coated dish. This clearly shows that the cell adds stiffness and damping to the
cantilever oscillation, confirming the validity of modeling the interaction as a Kelvin-Voigt viscoelastic element consisting of a repulsive force gradient and viscous dashpot model.

Before describing the detailed results, it is worth highlighting repeatable key features one can observe from dynamic approach curves on the cell:

Our first important finding is that the AFM tip requires large net sample indentation to reduce the cantilever amplitude to a setpoint value $A_1$ not only on the cell nuclear region but also on the periphery. To demonstrate this we measured dynamic approach curves at different locations in the image and observe the mean indentation, oscillation amplitude reduction, and phase-lag shift (Fig. 2). In Fig. 2 it can be clearly seen that as the cantilever tip is pressed down against the top of the cell, the first harmonic amplitude $A_1$ decreases very slowly whereas the zeroth harmonic amplitude $A_0$ increases, rapidly becoming $>A_1$. From Fig. 2 it can be deduced that on top of the cell nucleus it takes ~400 nm of Z-piezo extension beyond initial interaction with the cell surface to reduce the AFM probe oscillation amplitude to $A_1 \sim 0.75 A_{1\text{near}}$. Also, from Fig. 2, c and d, on the cell periphery region and gelatin-coated dish it takes ~50 and 25 nm of Z-piezo extension, respectively, to reduce the amplitude to a value of $A_1 \sim 0.75 A_{1\text{near}}$. Thus, at the imaging setpoint $A_1 \sim 0.75 A_{1\text{near}}$, indentation/amplitude ratio ($\delta/A_1$) is $>100$ times over that of the nucleus, to $5-10$ times over the peripheral region and $2-5$ times over the gelatin-coated dish. In other words, for the microcantilever to reach a specific amplitude reduction, it requires a large net indentation into the living cell. This implies that when imaging, the AFM tip is in permanent contact with a large net indentation allowing the use of linear theory to extract $k_{\text{sample}}^{\text{dynamic}}$ and $c_{\text{sample}}^{\text{dynamic}}$, as described in Materials and Methods.

The second important finding is that the resonance frequency of the cantilever shifts significantly due to hydrodynamic squeezing effects from initial measurements taken while tuning to when it begins to interact with the sample. This can be observed in Fig. 2, b–d, by noting that the phase lag before interaction begins is $<85-100^\circ$ even though while tuning (at ~4 μm far from the cell surface) the phase lag was set to 75° whereas the drive frequency was chosen to match the resonance peak. As mentioned, we use Lorentz force excitation to drive the microcantilever near resonance; therefore, this phase shift is due to near-surface hydrodynamic effects that have been considered in the theory presented before.

**Depth-dependent viscoelastic properties**

We then measured the viscoelastic properties of live fibroblasts cells using the dynamic and quasi-static AFM

**FIGURE 2** Dynamic approach curves performed on different locations across the sample. (a) Three-dimensionally rendered topography image of a live fibroblasts. (Crosses) Measurement locations. (b–d) Dynamic-approach curves for multi-harmonic observables ($A_0$, $A_1$, and $f_1$) acquired in the nucleus and periphery of a live fibroblast cell and the stiffer gelatin-coated petri dish, respectively. (Green vertical lines) Typical imaging setpoint ratios of 85 and 75%. (Curves) Behavior of the multi-harmonic amplitudes and phase ($A_0$, $A_1$, and $f_1$) as the microcantilever moves toward and interacts with different regions of a live fibroblast cell in a soft repulsive regime. It is clear that the strongest and most easily accessible harmonic signals that reflect local viscoelastic properties are those from the 0th and 1st harmonics for live cells. Topography image was acquired using a Lorentz force excited Olympus (Melville, NY) microcantilever (BL-TR400PB: $k_{\text{cant}} = 0.083$ N/m, $Q = 1.9$, $f_{\text{drive}} = 7.79$ kHz, and $A_{1\text{far}} = 8.5$ nm). To see this figure in color, go online.

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spectroscopy methods as described in Materials and Methods, and obtained the following key results:

When comparing the static and dynamic force gradients (at \(0^\circ\) \(\omega_d\) and \(\omega_d\), respectively) on the cell nucleus as a function of indentation (Fig. 3a), the dynamic values are three times larger than those measured statically until a critical depth of ~100 nm, beyond which the ratio of dynamic force gradient \(k_{\text{dynamic}}^{\text{sample}}\) to \(k_{\text{static}}^{\text{sample}}\) becomes larger. However, in the case of the periphery shown in Fig. 3b, \(k_{\text{dynamic}}^{\text{sample}}\) and \(k_{\text{static}}^{\text{sample}}\) are comparable and vary in a similar manner with average indentation. These two plots are the averages of 10 repeats at the same location on the cell. The data are highly repeatable with a typical variance of 10% over the 10 repeats for all the cells discussed in this article. From Fig. 3a and b, we note the ratio of \(k_{\text{dynamic}}^{\text{sample}}\) to \(k_{\text{static}}^{\text{sample}}\) strongly depends on indentation depth on the nuclear region but not significantly on the cell periphery.

Sample dynamic damping \(c_{\text{dynamic}}^{\text{sample}}\) also strongly depends on indentation depth becoming larger as the tip is pressed down on the cell. Dynamic damping was also extracted for the two regions (Fig. 3c) showing that there is a variation in damping across the cell. The plots in Fig. 3c are the averages of 10 repeats at the same location on the cell. The data are highly repeatable with a typical variance of 10% over the 10 repeats for all the cells discussed in this article. The damping behavior for the two regions shows an intrinsic critical indentation depth where it deviates from zero value and increases monotonically. Finally, we note that the damping is larger on the periphery than the nucleus.

The viscoelastic properties of live fibroblast cells exhibited a weak dependence on the operating oscillation amplitude. Dynamic force-indentation curves at different cantilever free oscillation amplitudes from 5 to 25 nm for a maximum loading force of ~2.5 nN were performed and no significant difference in dynamic viscoelastic properties was observed. This suggests that dynamic viscoelastic properties measurements are independent of the free oscillation amplitude.

Fig. 4 shows the extracted viscoelasticity properties of three different fibroblasts cells from different cell cultures. In the nuclear region of all cells the indentation depth dependency is observed, where the ratio of \(k_{\text{dynamic}}^{\text{sample}}\) to \(k_{\text{static}}^{\text{sample}}\) increases beyond a certain indentation while on the periphery \(k_{\text{dynamic}}^{\text{sample}}\) and \(k_{\text{static}}^{\text{sample}}\) follow a similar depth dependence. Also, \(c_{\text{dynamic}}^{\text{sample}}\) on the nucleus and periphery of all cells demonstrates the indentation-depth dependency.

Our observation that the ratio of dynamic and static stiffness increases beyond a certain indentation level on the nuclear region, but not on the periphery, could arise from the following possibilities:

As the AFM probe approaches and is being pressed down into the cell, the AFM tip initially interacts with brushes and the brush-type structures layer (combination of glycocalyx, microvilli, and microridges) on the cell membrane surface (67,68), and it is possible that the viscoelastic properties of these extramembrane components are very different from that of the underlying subcellular components leading to the observed depth-dependent divergence of the static and dynamic stiffness. However, if this were the reason for the observations, the depth-dependent divergence of static and dynamic stiffness should be detected not only in the nuclear region but across the entire cell, which is not the case.

Another explanation for the observed divergence between the dynamic and static force gradients on the nuclear region could be that the increase in dynamic and static force gradient ratio begins when the tip indents sufficiently to interact with the nuclear complex. The nuclear complex is expected to have quite different viscoelastic properties (69) compared to the cytoskeleton that lays just beneath the cell membrane. This hypothesis is consistent with the observation that the indentation-depth-dependent
divergence of static and dynamic stiffness is observed only over the nuclear region.

Implications on mapping in vitro local viscoelastic properties

Now that we have studied the viscoelastic properties as a function of indentation depth at high excitation frequency on different cell locations, we turn our attention to viscoelastic property maps that can be easily extracted in AM-AFM scan over live cells. In Fig. 5, we show a series of AM-AFM images acquired over the live fibroblast cell in physiological conditions. The multi-harmonic observables ($A_0$ and $\phi_i$) are converted to detailed local material property maps of effective dynamic repulsive force gradient $k_{\text{dynamic sample}}$ and damping $c_{\text{dynamic sample}}$ as described earlier. We also convert these maps into loss tangent $\tan\delta$ (Fig. 5 h).

The loss tangent is the ratio between the energy dissipated and the energy stored in one cycle of oscillation in contact with the sample (70). In this case, the loss tangent is defined as

$$\tan\delta = \left(\frac{c_{\text{dynamic sample}} + \omega_0^2}{k_{\text{dynamic sample}}}\right).$$

The map in Fig. 5 f suggests that $k_{\text{dynamic sample}}$ over the nuclear region is greater than in the peripheral part of the cell and the gelatin-coated dish. Moreover, from Fig. 5 g, the $c_{\text{dynamic sample}}$ over the nuclear region is lower in magnitude than the periphery and dish. This result was also observed in Raman et al. (4). On the other hand, many prior works have shown that the gelatin-coated dish has the largest elastic modulus, followed by the peripheral region, and lastly the nucleus (45,47,48). Also, it was expected that the cell nucleus should be the surface with higher damping compared to the peripheral region and the dish. This apparent contradiction between the maps in Fig. 5 and known properties can be explained in terms of the force spectroscopy results presented earlier. Fig. 6 shows a repulsive gradient with respect to applied force plot on the nucleus of a live fibroblast cell. It can be clearly seen that, at forces $>200$ pN, $k_{\text{dynamic sample}}$ increases rapidly. We actually apply forces $>750$ pN when imaging in AM-AFM over the nuclear region, and as a result we observe large $k_{\text{dynamic sample}}$. However, on the peripheral region (Fig. 6), we apply very small forces $<150$ pN while imaging in AM-AFM yielding relatively small $k_{\text{dynamic sample}}$, lesser than the nucleus. At such low forces, the force gradient on the gelatin is also very small in
Comparison with microrheological models of live cells

It is interesting to compare the results in this work to prior works on the rheology and viscoelasticity of live cells. Previous work by Alcaraz et al. (51) has used a power-law frequency-dependent structural damping model to estimate the complex shear modulus $G^*(\omega)$ of a live cell. This model has been validated systematically at low frequencies (~0.1–100 Hz), although not at the high frequencies (5–10 kHz) as presented in this work. A benchmark calculation using values reported by Alcaraz et al. (51) for A549 human lung epithelial cells, suggests $G_{\text{storage}}$ (8 kHz)/$G_{\text{storage}}$ (0.25 Hz) ~ 9.8. However, the ratio of $k_{\text{dynamic}}^\text{static}(\omega_{dr})$ to $k_{\text{static}}$ is smaller than the one predicted by the rheological power-law model ~3.5–6.5. There could be several reasons for this:

1. The rheological models do not take into account the indentation depth dependence;
2. Cell microrheological models have not been validated for high frequencies; and
3. A549 cells lack the cytoskeletal element concentration and organization that characterize fibroblasts.

The viscoelastic properties behavior dependence on indentation depth over more limited frequency ranges (20–400 Hz) of polymer gels and NIH3T3 fibroblast cells has been shown before by Mahaffy et al. (57,58). However, the divergence between low and high frequency as a function of sample indentation has not been presented before. Moreover, to the best of our knowledge, $k_{\text{dynamic}}$ data on the nucleus and periphery regions reported in this work are the first AFM high-frequency- and indentation-dependent (~8 kHz) nanorheological measurements in live cells. This finding has a major impact when comparing quantitative results obtained from quasi-static and high-frequency dynamic methods.

CONCLUSIONS

We have demonstrated the ability of dynamic AFM to quantify the nanorheological properties of live cells and
compared the dynamic and quasi-static methods to under-
stand the viscoelastic response of live fibroblast cells at 
very low frequencies (quasi-static) and at the cantilever 
resonance frequency (dynamic).

We have also found interesting differences between 
viscoelastic measurements made using quasi-static and 
dynamic AFM modes. On the fibroblasts nuclear region 
the local dynamic force gradient is larger than the statically 
measured one for small indentations (0–250 nm), however, 
beyond a certain critical indentation depth (say >250 nm) 
the ratio of force gradients derived from the dynamic and 
static methods increases rapidly due to interactions 
with subcellular components such as the nuclear complex. 
This depth dependency is also seen in the viscous damping. 
However, on the peripheral parts of the fibroblasts the ratio 
of dynamic and static force gradients does not change appre-
ciably with indentation.

Consequently, it is difficult to compare maps of visco-
elastic properties acquired using the quasi-static and 
dynamic AFM modes. Specifically, when these properties 
are mapped over live cells in buffer media using AM-
AFM, the indentation required to maintain constant oscilla-
tion amplitude changes from pixel to pixel because the 
nanomechanical properties on the cell are heterogeneous. 
Specifically, the tip indents the cell much more (300–
500 nm) over the nuclear region and much lesser on the pe-
riphery (~50 nm). The combination of two effects (i.e., high 
frequency vibration and variation of indentation depth) per-
formed while imaging the cell leads to the dynamic reul-
spive force gradient on the nucleus to be generally greater 
than those mapped using standard quasi-static methods.

These results confirm that dynamic AFM methods can in 
fact be used for the quantitative mapping of viscoelastic 
properties of subcellular components of nuclear and peripheral 
regions of live cells. However, the interpretation of 
these properties and comparison with quasi-static AFM 
measurements requires careful consideration of the fre-
quency- and indentation-dependence. We have demon-
strated the frequency- and indentation-dependence of local 
viscoelastic properties of living cells by comparing the force 
gradients determined from dynamic and quasi-static force 
spectroscopy methods, reporting, for the first time to our 
knowledge, measurements for high frequencies with an 
elucidation of the biomechanical role of subcellular compo-
ents. This has significant relevance not only for cell mecha-
nobiology but also for AFM-based imaging and force 
spectroscopy of live cells.

SUPPORTING MATERIAL

One table, seven figures, References (71–73), and Supplemental informa-
tion are available at http://www.biophysj.org/biophys supplemental/ 
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