The consensus mechanics of cultured mammalian cells

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Although understanding cells' responses to mechanical stimuli is seen as increasingly important for understanding cell biology, how to best measure, interpret, and model cells' mechanical properties remains unclear. We determine the frequency-dependent shear modulus of cultured mammalian cells by using four different methods, both unique and well established. This approach clarifies the effects of cytoskeletal heterogeneity, ATP-dependent processes, and cell regional variations on the interpretation of such measurements. Our results clearly indicate two qualitatively similar, but distinct, mechanical responses, corresponding to the cortical and intracellular networks, each having an unusual, weak power-law form at low frequency. The two frequency-dependent responses we observe are remarkably similar to those reported for a variety of cultured mammalian cells measured with different techniques, suggesting it is a useful consensus description. Finally, we discuss possible physical explanations for the observed mechanical response.

cell mechanics | cytoskeleton | mechanotransduction | microrheology

The important role of mechanical and physical cues in determining cell behavior is increasingly recognized. Cell shape can modulate cell differentiation (1), whereas substrate stiffness can affect tissue morphogenesis (2) and myoblast differentiation (3). The mechanisms, however, by which mechanical cues lead to molecular and biochemical responses remain largely undetermined. One approach to studying such mechano-sensing processes is to understand the mechanical properties of cells' constitutive molecules individually. Although protein conformation can respond to locally applied, small-scale mechanical signals such as molecular tension (4), how these signals may be combined to sense larger-scale mechanical properties remains unclear (5). Ultimately, an integrated physical description of cytoskeletal mechanics will be required to connect these molecular and cellular levels of description.

In soft-matter and polymer physics, the mechanical response and dynamics of supramolecular assemblies are determined by using rheology, the study of the frequency-dependent elastic and viscous behavior of deformable materials. Reliably interpreting rheology measurements on living cells, however, has proven notoriously difficult. Only after decades of experimental effort have different cell measurements begun to report comparable responses, although many differences remain (6). The rheology of cells is typically inferred from either deformation in response to an applied force (termed active microrheology) or the Brownian motion of embedded or attached tracer particles (termed passive microrheology). The results of both approaches depend on theoretical models for the deformation geometry or coupling between the tracer and the cell. Passive methods will also be confounded by any non-Brownian tracer motion such as intracellular trafficking or cell crawling. The comparison of cell mechanics measurements based on different methods is currently confounded by such technical effects and the unknown degree of variability among different cell types.

This article seeks the consensus mechanical response of living cells by applying a suite of different microrheology techniques to a single cultured mammalian cell type (Fig. 1). We apply an original technique that we recently developed, two-point microrheology (TPM) (7), to measure cells' dynamic shear modulus.

Fig. 1. Sketch of our four cell rheology techniques. From top to bottom, MTC measures the rocking motion of 4.5- μ m-diameter tracers, adhered to the apical cell surface by integrins, in response to a sinusoidal magnetic torque. TPM measures the correlation of the random motion of pairs of endogenous tracers to infer the Brownian fluctuations of the intervening network. LTM measures the translational Brownian motion of the MTC tracers either phagocytosed into the cell interior or on the apical surface.

Although TPM has the advantage that it does not depend on details of the tracer coupling or assumed deformation geometry, providing a uniquely interpretable and quantitative result, it does not probe the cell cortex. For this reason, we also apply an active method using externally attached magnetic tracers, magnetic twisting cytometry (MTC) (8), and passive methods using the same tracers (either internalized or externally adhered), termed laser tracking microrheology (LTM). When the confounding effects of non-Brownian motion are removed by chemical depletion of intracellular ATP, these four experiments report two distinct frequency-dependent shear moduli, which we conclude correspond to the cortical and intracellular cytoskeletal networks. Comparison of our results with the literature shows many earlier measurements made on a variety of cell types matching one or the other of our two mechanical responses, suggesting that our findings may be rather universal.

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Abbreviations: TPM, two-point microrheology; MTC, magnetic twisting cytometry; LTM, laser tracking microrheology; MSD, mean-squared displacement; SGR, soft glassy rheology.

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Fig. 5. Comparison of ATP-depleted (filled symbols) and untreated TC7 cells (open symbols). (A) Typical external LTM data for tracers showing typical cortical (Upper) and intracellular (Lower) responses. The curves are offset by a factor of 4 and aligned at the shortest time point. (B) Averaged MSDs for internalized tracers by LTM; $n = 23$ cells (ATP-), $n = 21$ (control, ATP+). (C) Averaged MSDs reported by TPM (scaled to a 4.5- μ m tracer); $n = 7$ (ATP-), $n = 20$ (control, ATP+). (D) The shear modulus (in Pa) at $\omega = 1000$ rad/s. Error bars are log-normal standard errors.

stiffness discrepancies here, TPM does provide a model-independent stiffness. The mean TPM response for ATP-depleted cells at a frequency $\omega = 10$ rad/s is $|G^*(\omega)| = 38$ Pa, with a cell-to-cell standard deviation of $\Sigma = 1.6\times$, i.e., most responses ranged from 20 to 60 Pa.

Passive Microrheology in Normal Cells Confounded by ATP-Dependent Processes. There are many mechanisms other than Brownian fluctuations that can move intracellular particles. Intracellular trafficking by molecular motors can lead to either directed or random motion inside the cell. Cell crawling can cause spurious tracer motion. Reaction forces from trafficking, cytoskeletal treadmill, or remodeling can “jiggle” the network, also moving tracers. All of these processes require metabolic energy. Because energy dissipation increases with velocity, short lag time motion is likely to be Brownian, and non-Brownian effects will dominate at long times.

Typical microrheology data for our three passive methods, both with and without ATP depletion are compared in Fig. 5. For the shortest lag times, there is little change in any of the observed MSDs, consistent with both thermal driving at short lag times and little change in the frequency dependence of the mechanical response. The long-time superdiffusive behavior ($\approx \tau^\alpha$, $\alpha > 1$) is mathematically inconsistent with the generalized Stokes–Einstein relation (Eq. 2), underscoring its non-Brownian origin. The superdiffusive results for LTM experiments (Fig. 5A and B) show non-Brownian motion with variable crossover times and exponents, α . For TPM measurements (Fig. 5C), the nonthermal motion appears at shorter lag times ($\tau > 3$ ms) and is more reproducible in form, with $\alpha \approx 1.5$. In an earlier paper (10), we modeled similar long-time data in terms of stress fluctuations driven by random force “steps” inside a continuum with weak power-law shear modulus $G^* \approx \omega^\beta$, predicting a superdiffusive behavior $\text{MSD} \approx \tau^{(1+2\beta)}$. The observed superdiffusive exponent is consistent with $(1 + 2\beta_1)$, providing a further check that the frequency dependence observed by TPM does not change significantly with ATP depletion.

Last, we turn to the amplitude of the shear modulus, rather than its frequency dependence, and whether it changes with ATP

depletion (Fig. 5D). Given the large amplitude of non-Brownian motion, using TPM we can only sensibly estimate the modulus of normal cells at our highest available frequency, $\omega = 1,000$ rad/s. These cells yield a value of 160 Pa, whereas TPM of ATP-depleted cells yields a value of 217 Pa, which is not a statistically significant change ($P = 0.17$), even when the results of 27 measurements are averaged. These results suggest that any change in stiffness in our TC7 cells caused by ATP depletion is modest or nonexistent. In contrast, LTM of internalized tracers (assuming Stokes boundary conditions) reports values of 40 Pa (control) and 18 Pa (ATP-depleted) at the same frequency, which is a significant change ($P = 0.003$). Because TPM is insensitive to tracer/network coupling, whereas LTM is not, a natural explanation is that this apparent change with ATP depletion is artifactual, caused by ATP depletion-induced changes to the tracer/network coupling. The difference between LTM and TPM is both large and highly significant (ATP-depleted, $P < 10^{-4}$; control, $P < 10^{-4}$). As stated above, getting an absolute stiffness figure from LTM requires a model, here a Stokes-like boundary condition. The factor of 4–10 $\times$ amplitude difference between LTM and TPM indicates that the Stokes model for LTM measurements is not quantitatively accurate. This finding agrees with published results (11) that show the deformation field around similar internalized tracers is much more localized spatially than predicted by the Stokes model.

Discussion

A Two-Network Consensus Mechanical Response. The most significant result of this work is the observation of two distinct viscoelastic cellular mechanical responses, which are both well represented by a simple power-law form (Fig. 2). On the basis of our own data and comparisons with the literature (particularly ref. 6), we conclude this phenomenon is caused by two networks in spatially separate compartments corresponding to the cortical/lamellar and deep intracellular space. Moreover, the literature shows (Fig. 4) that a wide variety of mammalian cell types probed with several different techniques give results corresponding to one or the other frequency dependence, suggesting that ours may be a useful consensus description. Because the concentration, organization, and types of cytoskeletal polymers are expected to vary among cell types, the apparent universality of the responses is somewhat surprising. This result suggests either the strong conservation of mechanical properties (e.g., because of their criticality for proper cell function) or a generic physical description that allows networks with different structural protein species or concentrations to naturally have such similar mechanics. Last, the presence of at least two networks with qualitatively similar responses indicates that researchers must be very careful to test that different tracers are reporting comparable responses before pooling multitracer results into an ensemble average. For example, external LTM measurements on *Dictyostelium* also display two distinct classes of tracer behavior (20), as we found in mammalian cells.

Unlike the consensus above regarding the frequency dependence and compartmented nature of the mechanical response, there appears to be little agreement on the absolute stiffness of either structure. Although some of this discrepancy may reflect real cell-type differences in stiffness (without corresponding differences in frequency dependence), it seems more likely to be caused by errors in modeling the cytoskeletal deformation field and structure near tracer probes. Perhaps counterintuitively, reliably measuring cells’ absolute stiffness at a single frequency is more difficult than measuring the frequency dependence of its viscoelastic response. Clearly more work is needed to resolve these issues. TPM does have the advantage of providing a model-independent stiffness value, but only for the interior network and then only in ATP-depleted cells. Most cells range from 20 to 60 Pa, on the low end of literature estimates. Given our finding that ATP depletion has little apparent effect on the

mechanical response, it seems likely that untreated normal cells have comparable intracellular stiffness. This finding may be somewhat surprising given the expected biological ramifications of this treatment (disturbed ion homeostasis, myosin bonds going into rigor, and eventual depolymerization of the actin cytoskeleton). The seeming independence of cell rheology to these effects should be investigated further with specific cytoskeletal disruptors and motor inhibitors. Last, our work confirms the expectation that non-Brownian, ATP-dependent processes significantly confound passive microrheology methods, limiting their utility in normal cells to high frequencies.

Possible Physical Origins of Cellular Mechanical Response. Over the last few decades, several conceptual models of the cytoskeleton have been proposed to explain different cell mechanical phenomena. The “sol-gel” model conforms to the conventional notion that the cytoskeleton’s mechanical response is caused by a gel of filamentary polymers pervaded by a fluid cytosol (21). The “tensegrity” model seeks to explain experiments indicating that cell stiffness is roughly proportional to intracellular stress (22). Most recently, the “soft glassy rheology” (SGR) model (23) has been proposed to explain cells’ weak power-law rheology (8).

The major theoretical challenge for the cell mechanics field is to develop a model that simultaneously accounts for all of the cell phenomena and known cell physiology, i.e., one that displays a stress-dependent stiffness and weak power-law rheology and is consistent with cell ultrastructure. By this standard, the existing models appear inadequate. Counter to the early idea that the cytoskeleton is simply a gel, no synthetic material has been found that simultaneously displays cell-like stress-induced stiffening and power-law rheology. The tensegrity model, corresponding to a macroscopic network of opposed elastic struts in tension and compression, does not naturally predict power-law rheology (24). The SGR model, although it neatly explains the power-law response, does not capture stress-induced stiffening. SGR materials, such as foam and toothpaste, generally yield and flow above a critical stress (25), the opposite of the cellular response (26).

Theory aside, researchers have sought a “minimal *in vitro* cytoskeleton” that reproduces the cell mechanical response with purified cytoskeletal proteins or to identify synthetic “model” materials with broadly similar mechanical behavior. Solutions of entangled actin filaments (7) and actin gels cross-linked with simple, irreversible cross-links (27) have a frequency independent elastic response at low frequencies. In contrast, F-actin networks with the protein cross-links α -actinin (28) and filamin A (29) display a more cell-like weak power-law rheology, indicating that these networks undergo structural changes that relax stress on long time scales. Of these two systems, only the filamin/actin networks also stiffen in response to deformation (30), making them the only material, to our knowledge, to simultaneously show both power-law rheology and cytoskeleton-like stiffening. The filamin/actin gel is not a perfect model, however, as its power-law exponent, β , of ≈ 0.10 , is significantly lower than that found in either cellular network. On the other hand, these experiments suggest that generically, filamentary network gels with protein cross-links can both stiffen in response to stress and have power-law rheology. Future experiments using different cross-links (and perhaps different filaments) may well produce *in vitro* gels that replicate the essential features of cytoskeletal mechanics.

The origin of the power-law rheology in these biopolymer gels is currently unknown. It does not seem to resemble synthetic materials having power-law rheology, which can be divided into two classes: SGR materials and critical gels. Unlike SGR materials (23), the *in vitro* gels do not flow under stress (29), nor do they consist of particles densely crowded together. In critical gels, the power-law response is caused by either a scale-free fractal structure (physical gels) (31) or a power-law molecular weight distribution for its constituents (chemical gels) (32). The fractal gels are generally very

fragile, unlike the biopolymer gel. Moreover, as chemical critical gels are based on a percolation phenomenon, their behavior is very sensitive to changes in cross-linker concentration, whereas the biopolymer gel’s behavior was not (30).

Power-law rheology implies that the microscopic processes responsible for stress relaxation in a material have a broad distribution of characteristic times (or activation rates). A long-standing idea in the cell mechanics field is that such stress relaxation could be caused by discrete unbinding events between or conformational changes in structural proteins (33–35). Although we can imagine that the broad distribution of rates in cells is caused by a variety of polymer species, the biopolymer gel studies indicate that a single cross-link species can generate power-law rheology. Presumably the distribution of activation rates relates to heterogeneity of the network structure, molecular aggregation/bundling, or a distribution of internal stresses. In a recent numerical study (36), we examined the rheology of simplified networks with cross-links that display force-activated serial domain unfolding (4) or unbinding. Under deformation, networks with unfolding domains evolved to an unusual state where many cross-links had near critical forces, allowing thermal activation to unfold them, and leading to power-law rheology. We further speculated that this arrangement, with many molecules organized on the cusp of a conformational change, is well suited to act as a biochemical sensor of network deformation. Although more detailed simulations and *in vitro* experiments need to be undertaken, these results suggest that relatively simple polymer physics-based models may be able to explain the observed cell mechanical response and suggest mechanisms for mechano-sensing (5, 37).

Our mechanical measurements on cultured cells suggest a surprisingly universal consensus mechanical response consisting of two regions with similar, but quantitatively distinct, power-law rheology at low frequencies. Our results underscore the utility of using multiple cell rheology methods in conjunction. This approach should facilitate the quantitative interpretation of future studies, including those that dissect the mechanical responses’ molecular determinants by using pharmacological and genetic methods.

Methods

Cell Culture and ATP Depletion. Cells were cultured with DMEM (GIBCO) supplemented with 10% bovine calf serum (HyClone), 50 mg/ml Gentamicin (Sigma), and penicillin-streptomycin (75 units/ml and 75 μ g/ml) solution (American Type Culture Collection) at 37°C and 5% CO₂. Cells were transferred to Petri dishes with glass coverslip bottoms, coated with collagen I (BD BioCoat; BD Biosciences, San Jose, CA), and incubated overnight before experiments. On an inverted optical microscope (DM-IRB; Leica, Deerfield, IL), the stage, oil-immersion condenser, and 100 $\times$ numerical aperture = 1.4, oil-immersion objective were heated to 37°C, and the atmosphere above the cells was maintained at 5% CO₂. Cell viability for up to 8 h on the microscope was confirmed.

For ATP-depletion experiments, the medium was exchanged for serum- and glucose-free DMEM (GIBCO) with 0.05% sodium azide (Aesar) and 50 mM 2-D-deoxyglucose (Sigma) 1–2 h before data collection. Depletion was verified with a luciferase-based assay (Promega) and showed 98% reduction of ATP. MTC and LTM tracers were attached or internalized before ATP depletion. Visualization of F actin showed no noticeable change to the density or distribution of F actin upon ATP depletion, other than a slight reduction in the number of stress fibers.

MTC and LTM. Measurements used ferromagnetic beads (4.5 μ m diameter, Spherotech, Libertyville, IL) coated with RGD-peptide (Integra, Plainsboro, NJ), attached to the cell exterior via integrin receptors or phagocytosed into the cell interior. For MTC, beads were magnetized vertically after cell attachment by using a $\approx 1,000$ -G magnetic field pulse lasting ≈ 100 μ s. Beads were selected

visually in bright field; cells with multiple beads were rejected. Tracers were illuminated with a red laser diode ($\lambda = 638$ nm), keeping absorbed laser power $<1/3$ mW to minimize heating effects (estimated to be $\approx 1^\circ\text{C}/\text{mW}$) and imaged on a quadrant photodiode module (OSI Optoelectronics, Newbury Park, CA) with a $4.5\times$ auxiliary magnification and dark-field Fourier filter. Bead displacement resolution is ≈ 0.2 nm for lag times <100 μs (shot noise limited) and ≈ 1 nm for lag times >10 ms (vibration limited).

In MTC experiments, externally attached beads were rocked by a 10-G (peak) oscillatory horizontal magnetic field. The magnet current and bead-position signals were simultaneously digitized at 50 kHz (National Instruments, Austin, TX). The amplitude and phase shift of the bead displacement were determined in real time by using a LABVIEW-based digital lock-in. The displacement amplitude is inversely proportional to the shear modulus at the driving frequency, but also depends on details of the bead-cell contact, which is assumed to contribute a frequency-independent prefactor. To avoid errors caused by time dependence of this prefactor during frequency sweeps, we drive the bead with a sum of two sinusoids, sweeping one and using the other as a reference (at 5 Hz). The ratio of the two amplitudes is nearly time-independent, allowing the more precise determination of the frequency dependence of G' and G'' with single cells.

For passive LTM experiments, no field was applied and the random bead deflection signal was digitized at rates up to 50 kHz. Trajectories were digitally filtered to remove narrow-band vibration signals, and MSDs were computed. At lag times where the motion is predominantly Brownian, G' and G'' can be computed from the MSD by using the generalized Stokes–Einstein relation,

$$\langle \Delta r^2(\omega) \rangle = k_B T / \pi i \omega G^*(\omega) a, \quad [2]$$

and numerical methods as described. The same algorithms were used to convert literature MSD or creep compliance data as needed.

TPM and Multiparticle Tracking. Cells were imaged with shadow-cast differential interference contrast microscopy with illumination provided by a pulsed, fiber-scrambled 2-W diode laser

(SDL-2460; Spectra Diode Labs, San Jose, CA; $\lambda = 808$ nm). Images were focused 2–4 μm into the 6- to 10- μm -thick TC7 cells. For each cell, $\approx 35,000$ images were recorded over a 25-min period, at rates of 50 and 1,000 frames per s with a digital complementary metal oxide semiconductor camera (Phantom 4; Vision Research, Wayne, NJ). The cell was illuminated with a 20- to 30- μs laser strobe, and the image scale was 96 nm per pixel. A few hundred ≈ 0.5 - μm endogenous particles (presumed by morphology to be primarily lipid granules and mitochondria) were found within each cell and ≈ 2 μm depth of focus, yielding $\approx 10^7$ positions per single cell data set with 5- to 8-nm spatial resolution. Tracers in nuclei or thin lamellae were omitted. Algorithms for particle tracking (38) and computing two-point correlations (7) have been described. Briefly, the random motion of each pair of tracers during a lag time τ is decomposed into components along and perpendicular to their line of centers. The parallel components are multiplied together, partitioned in r , and averaged to determine a statistical covariance, $D_{rr}(r, \tau)$. The covariance values with $2 < r < 8$ μm are multiplied by r , further averaged together, and rescaled to resemble a MSD. This MSD can be converted to shear moduli (39) as with LTM above.

Log-Normal Statistics. Our measurements appear log-normal-distributed, meaning that their logarithms are Gaussian-distributed. When combining or comparing results from different tracers, we compute the mean, standard deviation, and significance tests by using log-transformed data. The mean of the log-transformed values is exponentiated to yield the reported average value, M . The standard deviations, σ , of the log-transformed data are exponentiated to yield “multiplicative” log-normal standard deviations, Σ . For example, values of $M = 5$ and $\Sigma = 2\times$ would imply that $\approx 70\%$ of the data (1σ confidence interval) would range from 2.5 to 10 ($M/\Sigma - M^*\Sigma$).

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