

Nonequilibrium Bending Fluctuations Reveal the Mechanics of Microtubules and Mitochondria in Living Cells

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Living systems operate far from thermodynamic equilibrium. Movements in cells are always driven by thermal forces but, more importantly, also by motor-generated active forces in the cytoplasm. How intracellular structures respond to these forces depends on their mechanical properties. Intracellular dynamics remain challenging to understand because, in most cases, neither local forces nor local material response properties are known, and imaging of fluctuations is typically not sufficient to measure both. While some subcellular structures such as actin filaments and microtubules, which form the major cytoskeletal networks, have been extensively studied *in vitro*, their mechanical properties in living cells where they are regulated by post-translational modifications and association with regulatory factors remain poorly understood. Here we present an alternative approach that uses nonequilibrium bending fluctuations of rod-shaped structures to characterize their mechanical properties and those of their surroundings and to estimate driving forces. We demonstrate that, when one of the three factors—two material properties and one driving force—is determined independently, the other two can be derived from observed fluctuations. By applying our method to microtubules in living cells we find that polyglutamylation, a post-translational modification enriched on microtubules that need to withstand large deformation forces like those in axons or cilia, increases microtubule stiffness. In contrast, tubelike mitochondria, lipid membrane-bounded organelles which easily deform, yielded a bending stiffness significantly lower than that of microtubules. Our method opens the door to a quantitative understanding of the effects of cellular factors on the mechanical properties of filaments and organelles in cells.

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I. INTRODUCTION

Evolution has produced highly complex and versatile functional biomaterials. These materials are typically active, i.e., they are maintained by constant energy dissipation and they function in the nonequilibrium environments of cells and tissues. Acting collectively, cytoskeletal filaments, the outer cell membrane, and membrane bounded organelles with sheet, tubular, and spherical structures generate the mesoscopic dynamic processes that are crucial for cell functions such as

meiosis and mitosis, cytokinesis, cell locomotion, phagocytosis, and intracellular trafficking [1,2]. To control these functions, structures are constantly assembled, disassembled, and modified, while motors are activated when and where needed. Previous studies have examined the mechanical properties and the nonequilibrium nature of the cytoplasm by introducing external mechanical probes [3–8], which generally provides coarse-grained averages and tends to perturb cell functions. However, both the mechanical properties and the forces driving fluctuations of native subcellular structures within the living cytoplasm remain unknown. Here we present a method making direct use of nonequilibrium shape fluctuations of native filamentous or tubular structural components of living cells to determine their bending elasticity, the elastic response parameters of their surroundings, and the active driving forces.

We first applied our approach to microtubules, tubular polymers constructed by noncovalent association of

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$\alpha\beta$ -tubulin heterodimers. Microtubules are the most rigid of the cytoskeletal polymers and are critical for cell division, intracellular transport and signaling, as well as for the assembly of cilia and flagella [9]. Their chemical and mechanical properties are fine-tuned by the binding of a multitude of microtubule-associated proteins [10] and by post-translational modifications (PTMs) of $\alpha\beta$ -tubulins [11–14]. The consequences of these modifications for microtubule mechanics are yet to be determined. We found that we can well describe the mode spectrum of nonequilibrium microtubule bending fluctuations and derive values for their bending stiffness in the living cells falling within the range of published *in vitro* data [15–17]. Interestingly, we found that glutamylation, a post-translational modification of tubulin, enhanced bending stiffness significantly, providing a possible explanation for its enrichment in microtubule arrays like those in axons and cilia which need to withstand large deformation forces. Our analysis can, in principle, be applied to any filamentous or tubelike intracellular structure, including lipid-membrane-bounded organelles, whose structures and properties are typically regulated in response to cellular metabolism and thus are difficult to isolate or reconstitute [18–21]. In this study, we focused on mitochondria, the cell's power plants that produce ATP via oxidative phosphorylation [22]. Mitochondria maintain some structural characteristics of their prokaryotic ancestors [23], but do not possess a peptidoglycan cell wall. Mitochondria have a double lipid membrane with characteristic deep invaginations of the inner membrane (cristae). Depending on ATP production state, mitochondria can fuse to an interconnected tubular network or split into individual small compartments, accompanied by dramatic changes in cristae folding [24]. While the morphology of their tubular networks and cristate structures are presumed to be correlated with their mechanical properties [20], the latter have not been measured as it has not been possible to isolate or reconstitute tubular mitochondria *in vitro*. We show that our approach also describes the nonequilibrium bending fluctuations of mitochondria well. We found that mitochondria have a bending stiffness significantly lower than that of microtubules, although their diameter is larger. This difference is consistent with microtubules being solid protein polymers while mitochondria are lipid-membrane enclosed tubular organelles.

II. RESULTS AND DISCUSSION

To interpret data, we model filamentous structures in living cells as homogeneous elastic rods embedded in a viscoelastic continuum, with bending fluctuations driven by active forces [Fig. 1(a)] (see the Supplemental Material (SM) for the Langevin equation of motion [25]). We used HeLa cells as a model system and imaged microtubules labeled with SiR-tubulin [26] in a confocal microscope [Fig. 2(a)] [see the SM [25]]. Here we assume that both microtubules and mitochondria experience predominantly lateral forces generated by the local vicinity of actomyosin rather than axial buckling forces generated by MT-associated motors or polymerization-depolymerization. To minimize the chances of axial buckling, we restricted our analysis to microtubule segments located away from the cell edge as discussed in the SM [25]. The instantaneous shape of an individual rod can be characterized

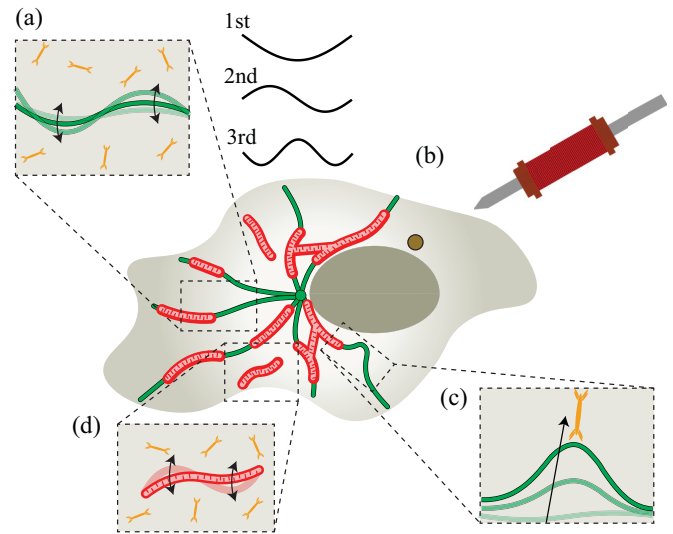


FIG. 1. Schematic illustration of microtubules and mitochondria in cells. (a) Molecular motors, most prominently nonmuscle myosin minifilaments, and other active processes provide a (typically) incoherent background of fluctuating forces, driving transient bending deformations of microtubules that we recorded by confocal fluorescence microscopy. The shapes of the fluctuating microtubules were decomposed into Fourier modes (right). (b) Schematic of magnetic tweezers microrheology to measure cytoplasmic viscoelasticity in HeLa cells. Only microtubules (green) and mitochondria (red) are sketched. The force generated by an electromagnet with a ferromagnetic core ending in a sharp tip near the cell acts on 1- μ m-diameter superparamagnetic beads that were electroporated into the HeLa cells. (c) Occasionally, molecular motors produce strong isolated forces causing sharp localized microtubule bends. (d) Mitochondria also display nonequilibrium bending fluctuations, even when not coupled to microtubules.

by the transverse deflection $u(s, t)$ from a line connecting the ends, which can be decomposed into Fourier modes as a sum of sines [Fig. 1(a)]:

$$u(s, t) = \sqrt{2/L} \sum_n a(q, t) \sin qs, \quad (1)$$

with contour length variable s (see the SM [25]) and time t . Because microtubules in cells are not freely fluctuating, free-end boundary conditions are not applicable as common in *in vitro* studies [27]. Instead, we used a simple Fourier decomposition along the filament contour, which does not require specifying the end constraints and is therefore more general and suited to complex cellular environments. The bending-mode amplitudes $a(q, t)$ parametrizes the shape of rods for a set of discrete wave vectors $q_n = n\pi/L = \pi/L_{\text{eff}}$, where n is the mode number and L and L_{eff} are rod length and effective mode length, respectively.

For a medium with complex shear modulus $G(\omega)$, the frequency-dependent bending mode amplitude $a(q, \omega) = \chi(q, \omega)F(q, \omega)$ is proportional to active forces produced by motors $F(q, \omega)$ for fluctuations slower than ~ 10 Hz in the cytosol, where thermal forces can be neglected [3,4]. Here $\chi(q, \omega) = [\kappa q^4 + \alpha(\omega)]^{-1}$ is the response function and $\alpha(\omega) \approx 4\pi G(\omega)/\ln(AL_{\text{eff}}/d)$ (see Ref. [28]), where $A \cong 2.3$,

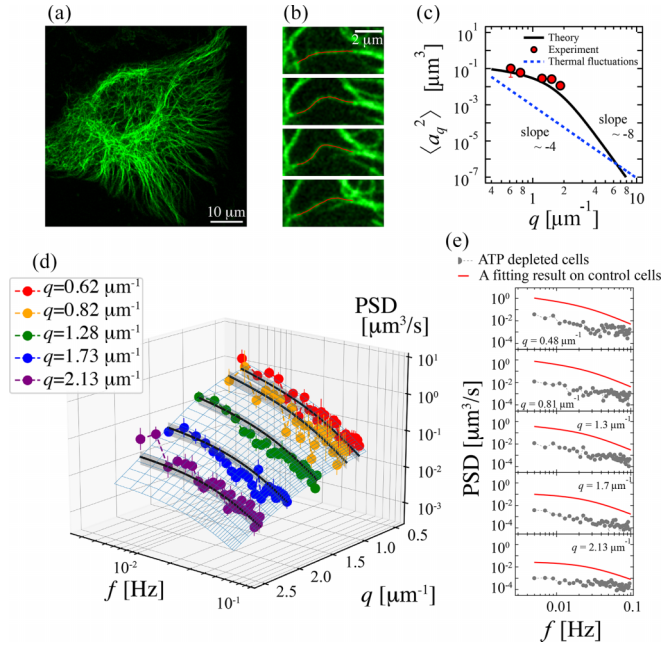


FIG. 2. Bending fluctuations of microtubules in living cells. (a) A substrate-adherent living HeLa cell with microtubules fluorescently labeled using SiR-tubulin (green) (see supplementary movie S1 [25]). (b) Microtubules undergo significant bending fluctuations caused by nonthermal forces. Consecutive images are separated by 20 s. The red overlay is the filament contour approximated by image analysis. (c) Variance of the mode amplitudes plotted vs wave number of 18 microtubules. The mode amplitude variance (red circles) (average of 18 microtubules) is significantly larger than the predicted thermal fluctuations: $\langle \Delta a_q^2 \rangle = 1/(l_p q^4)$ with a persistence length of $l_p \sim 1$ mm (blue dashed line), using a value reported value from *in vitro* studies [15]. Measured variances are consistent with the theoretical expectation (black line) applying the Wiener-Khinchin theorem and using fitting results with Eq. (3) to PSDs shown in (d). (d) Global fit of mode-amplitude power spectral densities of microtubules in HeLa cells. Binned PSDs from 18 microtubules at $q = 0.62, 0.82, 1.28, 1.73$, and $2.13 \mu\text{m}^{-1}$ are plotted as circles. Error bars are $\pm$ SEM. The bin widths at all q points are approximately $\sim 0.3 \mu\text{m}^{-1}$. The gray mesh plane shows a two-dimensional (2D) fitting result of the PSDs with Eq. (3). Black solid lines represent slices of the fitted plane at $q = 0.62, 0.82, 1.28, 1.73$, and $2.13 \mu\text{m}^{-1}$, respectively. The shaded area indicates the uncertainty range of the fit, calculated from the errors in the fit parameters. The upper and lower boundaries of the shaded area correspond to the upper and lower limits of the fit function, considering the parameter errors. (e) For comparison, we tracked and analyzed bending fluctuations of four microtubules in ATP depleted cells. Their mode amplitude fluctuation PSDs (gray circles) were smoothed by binning in q -space at $q = 0.48, 0.81, 1.3, 1.7$, and $2.1 \mu\text{m}^{-1}$. Also plotted are 1D slices of the 2D fit surface to the mode amplitude fluctuation PSDs of microtubules in control cells at $q = 0.48, 0.81, 1.3, 1.7$, and $2.1 \mu\text{m}^{-1}$ (red line). Four microtubules from two different ATP-depleted cells were tracked.

d is the filament diameter, and κ is its bending stiffness. The power spectral density (PSD) of mode amplitudes is given by:

$$\langle |a(q, \omega)|^2 \rangle = |\chi(q, \omega)|^2 \langle |F(q, \omega)|^2 \rangle = \frac{\langle |F(q, \omega)|^2 \rangle}{|\kappa q^4 + \alpha(\omega)|^2}. \quad (2)$$

For active forces with a single correlation time τ_M , the force spectrum at a point is a Lorentzian [3,4,29,30]. Assuming that force generation by different motors is uncorrelated and that the rod fluctuations are most strongly driven by motors located near the rod, we find for the PSD of the mode amplitudes of the fluctuating rod:

$$\langle |a(q, \omega)|^2 \rangle = \frac{1}{|\kappa q^4 + \alpha(\omega)|^2} \frac{C_{FF} \tau_M}{1 + \tau_M^2 \omega^2} \quad (3)$$

(see the SM [25]). Here C_{FF} is approximated as $C_{FF} \sim \langle f_0^2 \rangle / l_M$ in the limit of high motor density, where $\langle f_0^2 \rangle$ is the mean-squared active force on a single point and l_M the distance between neighboring motors [31]. This result relates the PSDs of the mode amplitudes to physical system properties, i.e., the active force fluctuations, C_{FF} and τ_M , the filament bending stiffness, κ , and the medium viscoelasticity, $\alpha(\omega)$. As q increases, bending stiffness dominates the PSDs. High- q PSDs are thus essential to estimate filament bending stiffness.

Given the typical case of noisy data, it is most reasonable to not extract all three constants and the function $\alpha(\omega)$ from the data but rather use independent measurements to determine some parameters up front. Here we determined cytoplasmic response $\alpha(\omega)$ independently, using embedded magnetic particles [Fig. 1(b)] (see the SM [25]), which allowed us to determine τ_M , κ , and C_{FF} from the data. Independently, we also estimated the cytoplasmic response from localized large-amplitude bends of microtubules [Fig. 1(c)].

We first applied our methodology to active bending fluctuations of microtubules in living cells. Microtubules have a persistence length of ~ 1 mm and therefore thermally fluctuate only weakly in cells [15,16,32], but they exhibit significant active bending dynamics, caused by actomyosin contractility and/or the dissipative polymerization of microtubules [4,33–35]. Bending on length scales $\gtrsim 6 \mu\text{m}$ can also result from slow, large-scale cellular restructuring, such as the retrograde flow of the entire cytoskeletal network [34]. Such bends relax slowly because they are trapped by the cytoskeleton. To observe uncorrelated fluctuations on practical recording timescales (minutes), we focused on length scales shorter than $\sim 6 \mu\text{m}$ [Fig. 2(b)]. To obtain mechanical properties from nonequilibrium bending fluctuations, we analyzed scale-dependent PSDs of microtubule deflection amplitudes. We tracked the shapes of microtubules by confocal fluorescence microscopy of adherent cells [Fig. 2(a)]. A time series of snapshots of conformations of an individual microtubule is shown in Fig. 2(b). The transverse deflection $u(s, t)$ of microtubules (not including center-of-mass movement and global rotation) was evaluated by tracking their backbones (see SM [25]).

We calculated mode amplitude variance as a function of mode number $\langle a^2(q) \rangle$ [Fig. 2(c)] from recordings either in the cell periphery or beneath the nucleus of adherent cells. Fourier amplitudes were calculated frame-by-frame from recordings taken at frame rates of 0.25–0.5 Hz. To quantify average dynamics, we calculated the mean-squared amplitude difference as a function of lag time, $\langle \Delta a^2(q, \tau) \rangle = \frac{1}{2} \langle \{a(q, t + \tau) - a(q, t)\}^2 \rangle_t$. At long lag times τ , microtubule shapes became uncorrelated from their initial shapes, leading to $\langle \Delta a^2(q, \tau \rightarrow \infty) \rangle = \langle a^2(q) \rangle$. In thermal equilibrium, equipartition predicts $\langle a^2(q) \rangle = k_B T / (\kappa q^4) = 1/(l_p q^4)$, with

persistence length $l_p = \kappa/k_B T$ on the order of millimeters [15,16] [Fig. 2(c)]. We found that the bending amplitude variance of microtubules in cells was significantly larger than the predicted thermal fluctuations, consistent with previous reports [34]. For further analysis, we calculated power spectral densities (PSDs) $\langle |a(q, \omega)|^2 \rangle$ of individual mode amplitudes [Fig. 2(d)]. Consistent with the mode-number dependence of the variances [Fig. 2(c)], we found that the PSDs decreased in amplitude with increasing q due to microtubule bending elasticity. Note that the functional form of PSDs changes with q . In addition, we observed a significant drop in PSD amplitudes for all modes in ATP-depleted cells [Fig. 2(e)], which confirms that active forces dominated the bending fluctuations of microtubules for the observed frequencies < 0.1 Hz or times longer than a few seconds.

To analyze the measured PSDs, we applied the theoretical model introduced above. In principle, one can obtain all five unknown parameters in Eq. (3), including the function $\alpha(\omega)$ by a global fit of all measured PSDs, but the typical case of relatively noisy data will lead to large uncertainties. For more accuracy, we thus first independently determined the viscoelasticity of the cytoplasm, $G(\omega)$, by active microrheology [3,5,6,36,37]. Previous studies used optically trapped probe particles [3,5,37], but intense laser light can induce photodamage in cells, and probe-particle displacement measurements are perturbed by refractile structures in the cells, such as the nucleus and organelles. To avoid these problems, we used “magnetic tweezers” [Fig. 1(b)]. We embedded 1- μm -diameter magnetic beads in the cytosol of HeLa cells [Fig. 3(a)] and applied magnetic force steps [Fig. 3(b)]. From the displacement, $d(t)$, of the beads, we calculated creep compliance $J(t) = \gamma(t)/\sigma_0 = 6\pi a d(t)/f$, where a is bead radius, $\gamma(t)$ is the time-dependent shear strain, σ_0 the applied constant stress, and f is the applied magnetic force. Creep compliance showed a power-law behavior, $J(t) \propto t^\beta$ with $\beta = 0.45$ (± 0.01) [Fig. 3(c)], indicating a broad spectrum of relaxation times in the cytoplasm as observed before [5]. Viscoelastic response can also be expressed in terms of the frequency-dependent shear modulus $G(\omega)$, related to the time-dependent compliance through a Fourier transform:

$$G^*(\omega) = G'(\omega) + iG''(\omega) = \left(\int_0^\infty dt \frac{dJ(t)}{dt} e^{-i\omega t} \right)^{-1}. \quad (4)$$

We found that the dynamic shear modulus varied with frequency as a weak power law with an exponent of 0.3–0.5, as has been widely observed before in cells [3,5,6,38–41], and the magnitude of $G_M(\omega)$ that we measured agrees with $G_{OT}(\omega)$ reported in the literature [5] [Fig. 3(d)].

We then globally fitted the scale-dependent PSDs [Fig. 2(d)] with a single two-dimensional (2D) plane defined by Eq. (3) with just three free parameters, C_{FF} , τ_M , and κ . Using our rheology results, we fixed the memory function $\alpha(\omega) = k_0 G(\omega)$ using $G(\omega) = \frac{1}{J_0 \Gamma(a+1)} (i\omega)^a$, where J_0 and a were obtained from a power-law fit to the rheology data, $J(t) = J_0 t^a$, and Γ is the gamma function. From the global fit to five PSDs evaluated from 18 microtubules, we found $\kappa = 4.1 (\pm 0.7) \times 10^{-24} \text{ Nm}^2$, $\tau_M = 5.9 (\pm 0.7) \text{ s}$, and $C_{FF} = 4.8 (\pm 1.3) \times 10^1 \text{ pN}^2/\mu\text{m}$. Uncertainties in the fitting parameters come from the variations in the measured PSDs from different

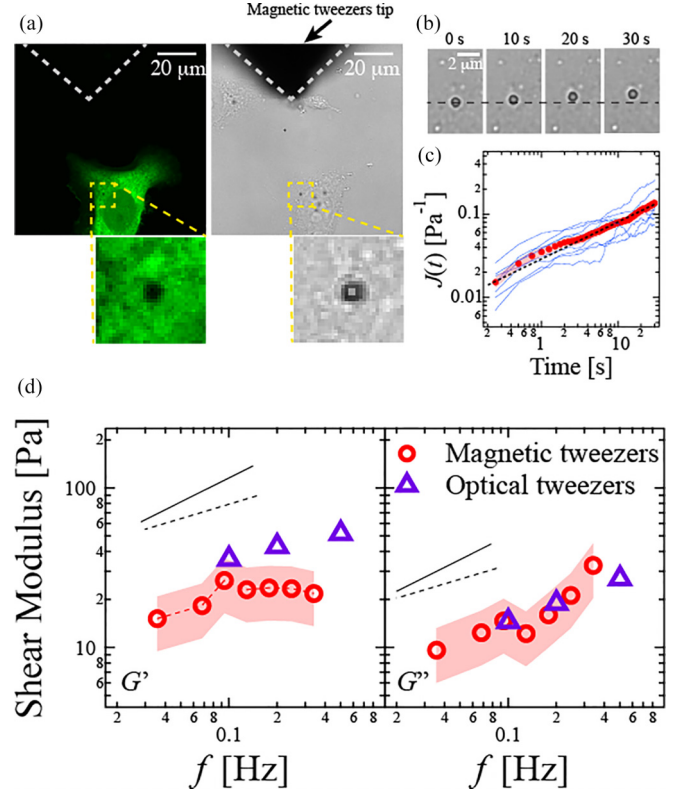


FIG. 3. Probing cytoplasmic viscoelasticity by magnetic tweezers. (a) Confocal and brightfield images of a magnetic tweezers experiment in a substrate-adherent HeLa cell expressing GFP. The dark spot in the fluorescent image at the bead position proves that the bead is successfully introduced in the cytoplasm. The tip of the electromagnet is 45–50 μm away from the magnetic bead and applies ~ 60 pN force for 30 s (see supplementary movie S2 [25]). (b) Time sequence of brightfield images of typical bead displacements towards the tip of the magnet in the cytoplasm with ~ 60 pN force. Beads move ~ 500 nm in 30 s, a small-enough distance to avoid nonlinear response or rupture of the cytoskeleton. (c) Displacement is converted to creep responses $[J(t)]$ (nine measurements from nine different cells). Individual response curves (blue), average (red), and power-law fit (dotted black line). The applied force f is assumed to be constant since the displacement of the beads (~ 500 nm) is negligible compared to the distance between the tip and the bead. (d) Complex shear modulus of the HeLa cell cytoplasm measured from the creep response to force steps (open circles). Creep functions are converted to the complex shear modulus using Eq. (4), containing the real part or storage modulus G' and the imaginary part or loss modulus G'' . Errors are $\pm$ SD. For comparison, values obtained from oscillatory experiments using optical tweezers were taken from a previous study [5]. Slopes of 0.3 and 0.5 are also plotted to guide the eye.

microtubules and in the complex shear modulus measured by active magnetic-bead microrheology and were estimated by a bootstrapping method (see the SM [25]). Assuming $C_{FF} \approx \langle f_0^2 \rangle / l_M$, where f_0 is an active force of ~ 7 pN, the line density of motors l_M is estimated to be on the order of $\sim 1 \mu\text{m}^{-1}$, which is consistent with imaging data of nonmuscle myosin bundle densities in cells [42]. The parameter τ_M found in this study is similar to the value reported in the literature [3]. The persistence length, $l_p = \kappa/k_B T$, of the microtubules we

tracked in cells was $1.0 (\pm 0.2)$ mm, in the range of values measured in aqueous buffers and actin solutions [15–17]. This consistency validates our alternative method of determining the material properties of microtubules in living cells from active shape fluctuations.

To check for consistency, we evaluated the total mode variance from the scale-dependent PSDs [Fig. 2(c)]. We used a 2D fit plane to the PSDs [Fig. 2(d)] to obtain fitting parameters ($\kappa = 4.1 \times 10^{-24}$ Nm², $\tau_M = 5.9$ s, and $C_{FF} = 4.8 \times 10^1$ pN²/μm). Subsequently, we applied the Wiener-Khinchin theorem,

$\langle a_q^2 \rangle = \frac{1}{2\pi} \int_{-\infty}^{\infty} \langle |a(q, \omega)|^2 \rangle d\omega$, to calculate the total variance for each mode [black solid line in Fig. 2(c)]. We found that experimental data fits theory in the observed q -range.

In the first part of the study, we demonstrate that our framework successfully describes the nonequilibrium shape fluctuations of microtubules, enabling us to calculate microtubule stiffness after independently determining cytoplasmic response. Reversing this logic, we can also use the dynamics of microtubules, once we know their bending stiffness in cells, to probe cytosol mechanics, without introducing foreign particles as mechanical probes, provided that we can use reasonable assumptions about active forces. Using this approach, we fit the shapes of strongly bent microtubules to estimate the cytoskeletal elastic modulus. We selected localized bends on microtubules which were initially straight and relaxed back to a straight shape after a transient deformation [Fig. 4(a)]. Here the magnitude of the force does not need to be known, but it needs to be a lateral point force acting on the microtubule. We observed that microtubules in HeLa cells occasionally experienced large-amplitude transverse bends, likely caused by nearby myosin motors exerting a pointlike force. Similar deformations are known to occur under compressive forces from the cell boundary leading to Euler buckling [15,35]. Fitting the shapes of such strongly bent microtubules yields the zero-frequency shear modulus of the embedding cytoskeleton. The axial extent of a bend is set by the balance between the bending force of the filament and the elastic force of the deformed cytoskeleton [15]:

$$l \cong (\kappa / G'_{\text{bend}})^{1/4}, \quad (5)$$

with the bending rigidity of the microtubule, κ , and the zero-frequency shear elastic modulus of the cytoskeleton, G'_{bend} . G'_{bend} can thus be calculated from the observed length scale l . The bend length l can be extracted from images using [15]:

$$u(x) = \frac{f l^3}{8\kappa} [\sin(|x|/l) + \cos(|x|/l)] e^{-|x|/l}, \quad (6)$$

where f is the force acting in the middle of the bend, $u(x)$ is the amplitude of the localized bend as a function of position x along the filament. We selected localized bends at least 4 μm away from the tips of microtubules to avoid cases of Euler buckling from the cell wall. The shapes of microtubules were well described by Eq. (6) [Figs. 4(b) and S2], which confirms the point-force assumption. We found a bend length $l = 0.64 \pm 0.1$ μm [Fig. 4(c)]. With the value of $\kappa = 4.1 \times 10^{-24}$ Nm², we find $G'_{\text{bend}} = 24 \pm 17$ Pa. The good agreement between the real part of $G_M(\omega)$ and G'_{bend} demonstrates consistency. As a control, we used a drug to weaken the actin cytoskeleton. Cytoskeletal elasticity in the periphery of the

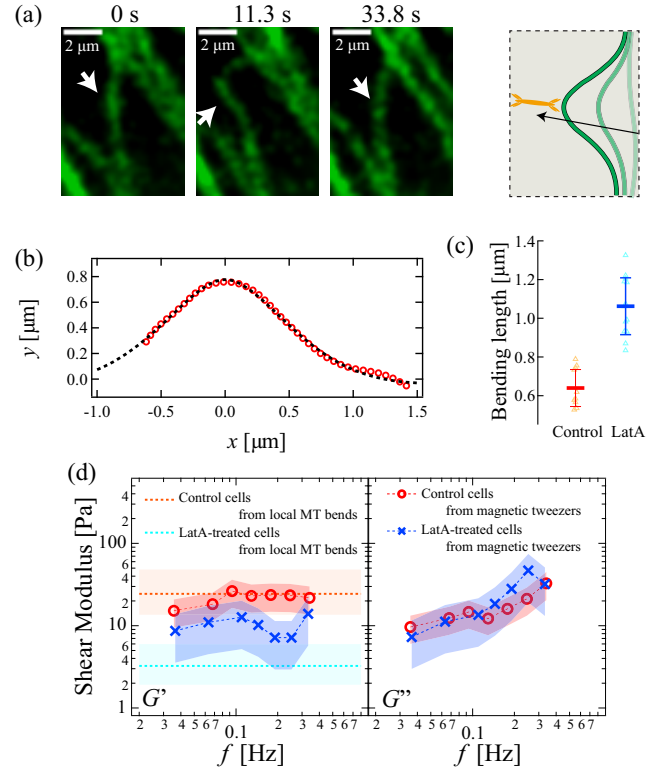


FIG. 4. Localized microtubule bends in living cells. (a) Snapshots of a microtubule showing a transient localized bend in a HeLa cell stained with an SiR-tubulin (see supplementary movie S3 [25]), together with a schematic image. The microtubule was initially straight and relaxed back to a straight shape after a transient deformation. (b) The most strongly bent shape of a section of the microtubule shown in (a) (red dots), with a fit of the predicted shape. (c) Characteristic axial length of localized microtubule bends in HeLa cells with and without Latrunculin A treatment. We tracked 11 microtubules and 13 microtubules for cells without and with Latrunculin A, respectively. Error bars are $\pm$ SD. (d) Real (G') and imaginary part (G'') of the complex shear modulus of the HeLa cell cytoplasm with and without Latrunculin A treatment, measured by magnetic tweezers. Storage moduli estimated from the axial extent of localized microtubule bends are plotted as horizontal dotted lines in the left panel. Error bars are $\pm$ SD.

surface-adherent HeLa cells presumably largely stems from actin networks. We pretreated HeLa cells with 1.5 μM latrunculin A for 15 min to (partially) disrupt the actin network. Bend length increased to 1.06 ± 0.15 μm in latrunculin A-treated cells [Fig. 4(c)]. The resulting elastic modulus again roughly agreed with the real part of $G_M(\omega)$ measured by magnetic tweezers in latrunculin-treated cells [Fig. 4(d)].

Having established that we can noninvasively determine microtubule mechanical properties *in vivo*, we proceeded to study the regulation of microtubule properties in the living cells. In particular, we investigated the impact of poly-glutamylation of tubulin. Glutamylation is a widespread post-translational modification of tubulin, occurring in organisms ranging from ciliates to humans. It involves the addition of chains of glutamates to the C-terminal tails of tubulin. It is the most abundant tubulin post-translational modification in the human brain [43]. Glutamylation is introduced by the

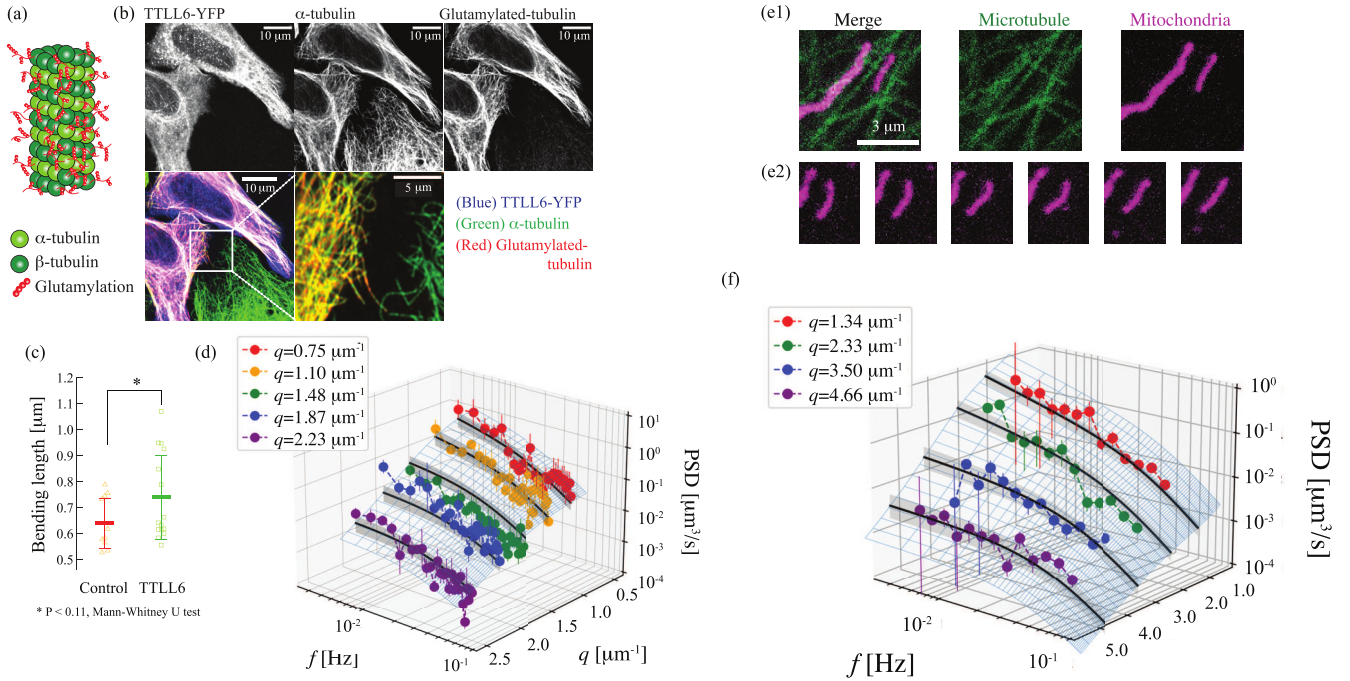


FIG. 5. Bending stiffness of polyglutamylated microtubules. (a) A schematic illustration of tubulin glutamylation. (b) Immunofluorescence images of HeLa cells expressing TTLL6-YFP. Top row: Individual fluorescence channels. Bottom row, left: Superposition of images for TTLL6-YFP (blue), microtubules (green), and glutamylated microtubules detected by GT335 (red). Right: Zoom into marked area. Microtubules (green), glutamylated microtubules (red). (c) Lengths of localized microtubule bends in HeLa cells expressing TTLL6 (see supplementary movie S4 [25]). The asterisk denotes a significant difference from the control group based on a Mann-Whitney U test ($P < 0.11$). (d) Global fit of PSDs of mode amplitude fluctuations of microtubules in HeLa cells expressing TTLL6. Results from 15 microtubules were smoothed by binning at $q = 0.75, 1.10, 1.48, 1.87$, and $2.28 \mu\text{m}^{-1}$. Error bars are $\pm$ SEM and bin widths $\sim 0.3 \mu\text{m}^{-1}$. A 2D fitting plane using Eq. (3) is plotted as a gray mesh. Black solid lines represent slices of the fitted plane at $q = 0.75, 1.10, 1.48, 1.87$, and $2.28 \mu\text{m}^{-1}$, respectively. The shaded area indicates the uncertainty range of the fit, calculated from the errors in the fit parameters. The upper and lower boundaries of the shaded area correspond to the upper and lower limits of the fit function, considering the parameter errors. (e) Top row: HeLa cell with fluorescently labeled microtubules (SiR-tubulin, green) and mitochondria (HC-1, magenta) (see supplementary movie S5 [25]). Bottom row: Mitochondria display strong nonthermal bending fluctuations. Consecutive images are separated by ~ 19 s. (f) Global fit of PSDs of mode amplitude fluctuations of mitochondria in HeLa cells. Results from five movies were averaged by binning at $q = 1.34, 2.33, 3.50$, and $4.66 \mu\text{m}^{-1}$. Error bars are $\pm$ SEM and bin widths $\sim 1 \mu\text{m}^{-1}$. A 2D fitting plane using Eq. (3) is plotted as a gray mesh. Black solid lines represent slices of the fitted plane at $q = 1.34, 2.33, 3.50$, and $4.66 \mu\text{m}^{-1}$, respectively. The upper and lower boundaries of the shaded area correspond to the upper and lower limits of the fit function, considering the errors in the fit parameters.

tubulin tyrosine ligase like (TTLL) enzyme family and has important functions in neuronal development and axonemal assembly [11,12,44]. Glutamylation is highly enriched in microtubule arrays with slow turnover that are subject to high mechanical stress such as those in axons, beating cilia, and parts of the mitotic spindle [45–49]. The modification itself, however, does not stabilize microtubules directly [50]. The consequences of this PTM for microtubule mechanics in cells have remained unknown. We transfected HeLa cells with TTLL6-YFP, a polyglutamylase that adds long glutamate chains to α -tubulin [44]. TTLL6 is enriched in cilia and is important for the biogenesis and function of this organelle [51]. HeLa cells had low levels of glutamylation in the absence of the overexpressed enzyme [Fig. 5(a)]. We confirmed successful polyglutamylation in cells overexpressing TTLL6-YFP by immunofluorescence with the GT335 antibody that recognizes post-translationally added glutamates [Fig. 5(a)]. To measure effects on microtubule bending stiffness, we used both methods described above, fluctuation mode analysis and shape analysis of localized bends. We stained microtubules

with SiR-tubulin in TTLL6-YFP-transfected cells and imaged localized bends. In cells with strong expression of TTLL6-YFP, we observed direct binding to microtubules. We excluded these cells from analysis since the binding of the enzyme to the microtubule might affect its properties. We selected more weakly expressing cells without TTLL6 localization to microtubules (Fig. S3). We found that the length of localized bends significantly increased to $0.74 \pm 0.16 \mu\text{m}$ [Fig. 4(c)]. From this result, we estimated the bending stiffness of polyglutamylated microtubules, κ_{TTLL6} , using the ratio of the cytoplasm elasticities measured in control cells (G'_{con}) and transfected cells (G'_{TTLL6}) applying the relation, $\kappa_{\text{TTLL6}} \cong \kappa_{\text{con}} G'_{\text{TTLL6}} l_{\text{TTLL6}}^4 / (G'_{\text{con}} l_{\text{con}}^4)$, derived from Eq. (5), where κ_{con} , l_{con} , and l_{TTLL6} are defined as the bending stiffness of microtubules in control cells, the characteristic lengths of localized bends for control cells and for cells overexpressing TTLL6, respectively. With $l_{\text{TTLL6}}^4 / l_{\text{con}}^4 \approx 1.79$ and the ratio $G'_{\text{TTLL6}} / G'_{\text{con}}$ from microrheology (Fig. S4) we found that the bending stiffness of polyglutamylated microtubules ($7.8 \times$

10^{-23} Nm^2) was significantly larger than that found in control cells ($4.1 \times 10^{-24} \text{ Nm}^2$).

Microtubule bending fluctuations in transfected cells were then recorded and five mode amplitude PSDs were fitted with a single 2D plane [Eq. (3)] [Fig. 5(c)], using a fixed memory function $\alpha(\omega) = k_0 G(\omega)$ from microrheology (Fig. S4) in TTLL6-YFP transfected cells. We found $\kappa = 7.0 \pm 1.7 \times 10^{-24} \text{ Nm}^2$, $\tau_M = 6.2 \pm 1.1 \text{ s}$, and $C_{FF} = 6.4 \pm 2.4 \times 10^1 \text{ pN}^2/\mu\text{m}$. Both experiments thus suggest that polyglutamylated microtubules are ~ 1.7 times stiffer than microtubules in control cells. Recently, K. P. Wall *et al.* [49] reported that the bending stiffness of *in vitro* reconstituted taxol-stabilized microtubules increases with polyglutamylation due to the interactions of the α -tubulin C-terminal tail with the tubulin body, supporting our *in vivo* measurements, although it is likely that additional *trans* effects occur in cells due to the recruitment of microtubule-binding proteins to polyglutamylated microtubules [45,52–55]. The fact that force fluctuations increased in amplitude for the polyglutamylated microtubules is qualitatively expected since a more rigid inclusion in a fluctuating viscoelastic network will be exposed to more force [56,57].

In principle, our methodology is applicable to any fluctuating high-aspect ratio subcellular structure. We therefore extended our analysis to the bending fluctuations of filamentous mitochondria *in vivo* to obtain an estimate of their mechanical properties. The majority of mitochondria bind to microtubules, which suppresses their bending fluctuations [Fig. 1(d)]. We selected mitochondria minimally interacting with microtubules by simultaneously imaging microtubules and mitochondria using SiR-tubulin and HC-1 [Figs. 1(d) and 5(e)]. Bending fluctuations of these mitochondria in the living cells were tracked, and the mode amplitude PSDs were fitted with a single 2D plane using Eq. (3) [Fig. 5(f)], with a fixed memory function $\alpha(\omega) = k_0 G(\omega)$ obtained from active magnetic-bead microrheology (Fig. 3). From the global fit to four PSDs calculated from five movies, we found $\kappa = 8.5 \pm 1.3 \times 10^{-25} \text{ Nm}^2$, $\tau_M = 4.2 \pm 0.2 \text{ s}$, and $C_{FF} = 7.4 \pm 2.3 \times 10^1 \text{ pN}^2/\mu\text{m}$. The motor timescale τ_M , measured from the fluctuations of mitochondria, is comparable to that measured from microtubule fluctuations, implying a shared origin of active fluctuations. The bending stiffness of mitochondria turned out to be considerably lower than that of microtubules, although they have an ~ 20 -times-larger radius than microtubules. While this bending stiffness is directly measured and not model dependent, some assumptions about the internal structure of mitochondria need to be made to estimate elastic properties of the constituent materials. As a simple starting point, we approximated the mitochondria as tubes with an outer radius $r \sim 250 \text{ nm}$ and a wall thickness $\Delta r \sim 10 \text{ nm}$, the bending stiffness of which is taken to be $\kappa = \pi E \{r^4 - (r - \Delta r)^4\}/4$ with the Young's modulus E [58]. This modeling approach resulted in an estimate for the Young's modulus of the mitochondrial wall of $\sim 1800 \text{ Pa}$, which is similar to the value inferred from elastocapillary mechanical instability in mitochondrial fission [59]. Note that modeling mitochondria as simple tubes with an elastic wall grossly simplifies the real structure of the mitochondrial wall, consisting of a double lipid bilayer, the inner one of which forms large invaginations, the cristae, with a periplasmic space in between and a high

concentration of proteins inserted in the lipid membranes and in the periplasmic space. Our result indicates that the elastic properties of the mitochondrial wall are in the same ballpark as those of the outer cell wall including actin cortex and lipid membrane [38]. Our results are also roughly consistent with published AFM indentation experiments [60], which were difficult to interpret, however, since the mitochondria were indented through the outer cell wall. Interestingly, the line density of force fluctuations (C_{FF}) experienced by mitochondria and microtubules is of the same order of magnitude, despite their difference in diameters, which is expected to affect the forces they experience. This similarity is consistent with both being primarily moved by the surrounding actomyosin network rather than by motors directly attached to their surfaces. At closer inspection, a slightly higher activity was observed for mitochondria, which is somewhat unexpected since a less rigid inclusion in a fluctuating network will experience less force if the surrounding force production is constant. The observation of larger force fluctuations could possibly be explained by higher local motor activity due to the mitochondrial ATP production or by a higher motor density around mitochondria due to their larger diameter compared to microtubules.

III. CONCLUSIONS

In this study, we investigated nonequilibrium shape fluctuations of filamentous and tubular structures in cells and show how these fluctuations can be used to noninvasively characterize material properties of these structures and of their surroundings. We demonstrated that our framework successfully describes the nonequilibrium shape fluctuations of microtubules and mitochondria, enabling us to calculate their stiffness. A direct characterization of microtubule properties in living cells is important because it gives access to the study of the biological regulation of their mechanical response by post-translational modifications or binding of regulatory agents. Conversely, we show that microtubules can be used as an endogenous mechanical probe for cytoplasm mechanics, avoiding the introduction of mechanical probes into cells. We find that the elastic response of the cytoskeleton measured from localized microtubule deformations agrees with microrheology results, both for control cells and latrunculin A-treated cells. We also find that polyglutamylated microtubules are more rigid than unmodified ones. Such an increased mechanical stability is consistent with the abundance of glutamylated microtubules in stable cellular structures. In contrast, acetylation, a tubulin modification also associated with stable microtubule structures, was reported to decrease microtubule stiffness *in vitro* [61] but to enhance cell stiffness [62]. Our approach is applicable to other filamentous cellular structures. We applied the method to mitochondria, which frequently adopt rod-shaped geometries and form networks in cells, but are impossible to extract from cells in their filamentous state for characterization. Focusing on tubular mitochondria detached from microtubules we found that their bending stiffness is significantly lower than that of microtubules. The obtained value is consistent with the fact that mitochondria are membrane-bounded organelles filled with a viscoelastic cytoplasm. The observed increase of force fluctuations might be due to an increased availability of ATP near

the organelles. In summary, the observation of fluctuations of intracellular structures in living cells provides a direct window into the complex context-dependent mechanical properties of these structures, the active forces in the cytoskeleton, and the response properties of the cytoplasm as well as the regulation of these parameters.

An important future goal of research would be to understand the heterogeneity of conditions in the living cell. Microtubule stiffness, active forces generated by molecular motors, and viscoelastic properties of the surrounding cytoplasm are all expected to spatially vary. In this introduction of our alternative approach, we averaged over these variations to extract average parameter values and to capture global changes under a strongly driven PTM. In future work, we aim to apply fast super-resolution microscopy and combine fluctuation analysis with measurements of localized large bends on the same microtubules to obtain simultaneous local estimates of viscoelasticity and stiffness. This could help reveal variations in bending rigidity of individual filaments depending on the types and amounts of bound microtubule-associated proteins and other post-translational modifications. Furthermore, this framework could be extended to study other PTMs and MAPs, including their binding and unbinding effects.

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K.N., A.R.M., and C.F.S. conceived the project. K.N. and F.C.M. developed the model. K.N., R.F., A.R.M., and C.F.S. designed the experiments. K.N., S.R., and A.P. performed the experiments. K.N. analyzed and visualized the data. All authors contributed to the writing of the paper.

DATA AVAILABILITY

The data that support the findings of this article are not publicly available upon publication because it is not technically feasible and/or the cost of preparing, depositing, and hosting the data would be prohibitive within the terms of this research project. The data are available from the authors upon reasonable request.

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