**Force fluctuations and polymerization dynamics of intracellular microtubules**

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Edited by Tom C. Lubensky, University of Pennsylvania, Philadelphia, PA, and approved August 16, 2007 (received for review April 3, 2007)

Microtubules are highly dynamic biopolymer filaments involved in a wide variety of biological processes including cell division, migration, and intracellular transport. Microtubules are very rigid and form a stiff structural scaffold that resists deformation. However, despite their rigidity, inside of cells they typically exhibit significant bends on all length scales. Here, we investigate the origin of these bends using a Fourier analysis approach to quantify their length and time dependence. We show that, in cultured animal cells, bending is suppressed by the surrounding elastic cytoskeleton, and even large intracellular forces only cause significant bending fluctuations on short length scales. However, these lateral bending fluctuations also naturally cause fluctuations in the orientation of the microtubule tip. During growth, these tip fluctuations lead to microtubule bends that are frozen-in by the surrounding elastic network. This results in a persistent random walk of the microtubule, with a small apparent persistence length of ~3 μm, ~100 times smaller than that resulting from thermal fluctuations alone. Thus, large nonthermal forces govern the growth of microtubules and can explain the highly curved shapes observed in the microtubule cytoskeleton of living cells.

Microtubules are biopolymer filaments that are essential for many fundamental biological processes including cell division, migration, and transport. Microtubules exhibit highly dynamic growth behavior, with repeated cycles of polymerization and depolymerization (1), allowing the microtubule network to rapidly restructure to perform different tasks. Many of these tasks require the network to behave as a rigid scaffold that can support mechanical loads, such as those generated by motors during cargo transport. Mechanical stability of microtubules is also crucial in guiding directional cell migration and effectively connecting to and pulling apart chromosomes during mitosis. Indeed, each of their varied biological roles requires some degree of mechanical stability. Consistent with this, microtubules are the most rigid of the cytoskeletal filaments; they are ~100 times more resistant to bending than actin filaments. As a result of this high bending rigidity, microtubule shapes are relatively insensitive to thermal Brownian forces: in vitro experiments with isolated microtubules show that they exhibit significant thermal bending fluctuations only beyond a length scale on the order of a few millimeters, known as the persistence length (2). This persistence length is 10–100 times larger than a typical animal cell, which suggests that Brownian fluctuations do not contribute to microtubule bending in cells.

Although microtubules are sufficiently stiff to resist small thermal fluctuations, inside of animal cells they can also experience large, nonthermal forces such as those arising from myosin contractility (3) or other ATP-consuming motor proteins. Indeed, in most adherent cells, microtubules exhibit significant curvature on both short and long length scales, as shown in Fig. 1a. Such bends are ubiquitous in animal cells, and are generally thought to arise after growth, by bending under the action of internal cytoskeletal forces, as shown schematically in Fig. 1a Inset (3, 4). However, the surrounding elastic cytoskeleton has been shown to mechanically reinforce microtubules to withstand these large forces; this suppresses bending, particularly on long length scales (5). As a result, under the action of even large nonthermal forces, bending occurs primarily on short wavelengths of ~3 μm. Such dynamic, short-wavelength bends can be observed to fully fluctuate in time, as shown in the temporal sequence of the shape of a single microtubule in a GFP-tubulin-transfected CHO cell, Fig. 1b (also see supporting information [SI] Movie 1). Bending on longer wavelengths may result from slow, large-scale cellular restructuring, such as that occurring because of the retrograde flow of the entire cytoskeletal network at the leading edge of cells (3). These microtubule bends will significantly affect the location of the distal tips of growing microtubules and therefore affect their well known role in targeting and biochemical signaling (6, 7), in addition to the myriad biological processes that depend on the overall structure of the microtubule network. However, although the architecture of the microtubule network is of central biological importance, the precise origin of microtubule bending and the role of mechanical forces in establishing this network architecture remain unclear.

In this article, we show that large-scale microtubule bends result primarily from the coupling of active intracellular forces to the dynamics of microtubule growth; this leads to a dramatically reduced persistence of directional growth. During microtubule growth, nonthermal forces cause directional reorientation of microtubule tips. As a microtubule grows under the action of these fluctuating forces, its growth trajectory is curved, and the resulting microtubule bends are locked in by the surrounding cytoskeletal network. As we show, the fluctuations in tip orientation suggest a persistent random walk trajectory of microtubule growth, in agreement with the microtubule shapes we observe. However, the apparent persistence length observed is ~100 times smaller than the thermal persistence length. Moreover, once a microtubule is grown, the surrounding elastic network reinforces it against bending under the action of these forces. Large nonthermal force fluctuations thus appear to play a central role in establishing the structure of the microtubule network, primarily by affecting the directionality of microtubule growth; these fluctuations also are likely to affect a number of other microtubule-associated processes such as the generation of polymerization forces and intracellular transport.

**Results**

Fourier Analysis of Intracellular Microtubules Shapes. Anomalous distribution of instantaneous bends. To quantitatively investigate the origin of microtubule bending in cells, we used automated contour...
tracking algorithms to trace the shapes of fluorescently labeled microtubules (8). From the instantaneous contours, we determine the local tangent angle $\theta(s)$ of each microtubule as a function of the contour length $s$ along the microtubule. The shape is then decomposed into component Fourier modes (2) by expressing $\theta(s)$ as a sum of cosines:

$$\theta(s) = \sum_{n=0}^{\infty} a_n \cos(ns).$$

Here, $L$ is the total filament length, and $q = n \pi L$ is the wave vector. This technique allows us to determine the amplitude of bending as a function of the length scale over which the bending occurs. The bending amplitude is given by the Fourier amplitude $a_n$, which is proportional to the height of a bend on a wavelength, $\lambda = 2\pi/q$, as illustrated in Fig. 2A Inset. The $n = 0$ mode corresponds to a constant angle and therefore accounts for the average orientation of the microtubule. The $n = 1$ mode corresponds to a smooth curvature in only one direction, whereas a full wavelength bend is given by the $n = 2$ mode. Each of the amplitudes $a_n$ for a single microtubule depends on time because the shape $\theta(s)$ changes with time.

For microtubules in thermal equilibrium, the square of the amplitude, averaged over unconstrained microtubule shapes, is given by

$$\langle a_n^2 \rangle = \frac{1}{L_p} \frac{\langle a_n \rangle^2}{q^2},$$

where $L_p$ is the persistence length, whose value is on the order of several millimeters (2); the angle brackets indicate an average, which can be a time average, over unconstrained shapes of a single, unconstrained microtubule at different times, or an ensemble average, over many different filaments. For intracellular filaments, we can determine an ensemble-averaged variance by analyzing the instantaneous shapes of many different microtubules in fixed animal cells. We find that the $q$-dependence of the Fourier bending spectrum is given by

$$\langle a_n^2 \rangle_E = \frac{1}{L_p} \frac{\langle a_n \rangle^2}{q^2},$$

where the subscript emphasizes the ensemble average; this is shown for microtubules in CHO cells by the brown squares in Fig. 2A. This yields an apparent persistence length, $L_p^o \sim 30 \mu$m, that is $\sim100$ times smaller than expected from thermal fluctuations alone, indicating significantly larger amplitude bending fluctuations. The Fourier bending spectrum of microtubules in COS-7 cells exhibits similar behavior. This unexpectedly large amplitude of bending suggests a nonthermal origin.

Whereas the enhanced fluctuation in cells, relative to thermal equilibrium, is itself not surprising, the observed thermal-like wave vector dependence, $\langle a_n^2 \rangle_E \sim q^{-2}$, is surprising because there is no reason to expect a priori that cellular or motor activity within the composite cytoskeleton should lead to an equilibrium-like distribution of bending amplitudes. In fact, even if the cellular activity were random and characterized by an elevated effective temperature, $\langle a_n^2 \rangle_E = q^{-2}$ should not be expected because, unlike a free microtubule in buffer, the energy of microtubule bending is determined not only by bending rigidity but also by the elasticity of the surrounding matrix.

**Time-dependent bending fluctuations.** To investigate the origin of these anomalous bending amplitudes, and the role of cellular activity, we tracked the time-dependent bending fluctuations of microtubules in living cells transfected with GFP-tubulin. This is illustrated by comparing subsequent shapes of a single microtubule in a GFP-tubulin-transfected COS-7 cell, as indicated by the contours of a single microtubule in Fig. 2B, where each color represents the contour at a different time separated by 8 sec. The Fourier amplitudes of each individual filament were determined for each

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point in time, and the average of fluctuations over time was calculated from the mean-squared amplitude difference as a function of lag time, \( \langle \Delta q^2(t) \rangle = \langle (q_0 + q(t + \tau) - a(t) \rangle^2 \rangle \). As a result of this slow evolution, a bend on a length scale of \( \sim 10 \mu m \) would require \( \sim 1,000 \) see to fully fluctuate, which is longer than the lifetime of a typical microtubule. Thus, whereas instantaneous microtubule bends have short-wavelength fluctuations while the long-wavelength arc is frozen-in on all length scales. Thus, the ATP dependence of the ensemble-averaged variance is similar to that of control cells. However, the time-average of fluctuations of the long-wavelength bends does not depend on ATP, and the ensemble average that we observe for microtubule shapes.

Lateral Fluctuations Have a Nonthermal Origin. To elucidate the origin of the forces that drive the microtubule bending dynamics, we depleted cells of ATP, which will limit any mechanochemical enzymatic activity, such as that of myosin and other motors. ATP depletion has been shown to have only a small effect on the mechanical properties of the cytoskeleton (10) and should not change the magnitude of thermally driven microtubule bending fluctuations. We fixed and stained ATP-depleted CHO cells and analyzed the instantaneous ensemble microtubule shapes, as above. We find that, once they have formed, the presence of these microtubule bends does not depend on ATP, and the ensemble variance is similar to that of control cells. However, the time-dependent fluctuations of these bends are completely arrested, as shown in Fig. 2c. The instantaneous bends do not evolve in time and are completely frozen-in on all length scales. Thus, the ATP dependence of short-wavelength bending dynamics suggests that these fluctuations have a distinctly nonthermal origin, consistent with recent studies of the dynamics of motor-driven force fluctuations in the cytoskeleton (10–12).

Role of Microtubule Growth in Establishing Shape. Force-induced redirection of microtubule growth. The very slow evolution of the long-wavelength bending modes, it is unlikely that these bending fluctuations can account for the development of a network of highly bent microtubules, starting from initially straight filaments. Rather, we hypothesize that the observed spectrum of bent shapes in Fig. 2a results from curved growth trajectories of microtubules, as well as because of the action of fluctuating internal cytoskeletal forces. These active forces not only will lead to lateral microtubule bending fluctuations but also will naturally cause directional reorientation of the microtubule tip, as shown schematically in Fig. 3a. Furthermore, as we show, such orientational fluctuations would naturally lead to the \( \langle a_{\tau}^\tau \rangle \sim q^{-2} \) dependence of the ensemble average that we observe for microtubule shapes.

To test the hypothesis that lateral bending forces can reorient the microtubule tip, we used a microneedle to apply a transverse force to the region behind a growing microtubule tip (Fig. 3b). Microtubule growth could indeed be redirected under the action of such an exogenous force, as shown by the change in direction of the tip of a microtubule highlighted in red in Fig. 3c (also see SI Movie 2). Intrinsic cytoskeletal forces appear capable of redirecting microtubule growth in a similar manner, because microtubules frequently appear to change their direction of growth in response to local bending forces, as shown by the growing microtubule highlighted in red in Fig. 3d (also see SI Movie 3). Moreover, such naturally redirected microtubules give rise to relatively static, long-wavelength bends, as can be seen in the example in Fig. 3d. Analysis of microtubule growth trajectories. Force-induced tip deflections provide a quantitative explanation for the wavelength dependence of the large bending amplitudes shown in Fig. 2a. Specifically, given the elastic environment in which a microtubule grows, lateral forces will lead to directional reorientation that can persist; the resultant directional fluctuations become independent over a distance on the order of a few micrometers. In such a growth process, the tip, defined by position \( s(t) \), has an orientation \( \theta(s(t)) \), which performs a random walk, with \((\theta'(s(t)) - \theta(0)) = 2D\theta(t)\); this results in a spectrum of the form \( \langle a_{\tau}^\tau \rangle \sim q^{-2} \).

To quantitatively test this hypothesis, we analyze the motion of microtubule tips using CHO cells expressing YFP CLIP-170, a protein that specifically associates with the tips of growing microtubules (13). We tracked the position of microtubule tips in these cells and measured their lateral fluctuations by fitting a short segment of the growth trajectory to a line and determining the deviation from this line as a function of time, \( h(t) \). We find that microtubule tips undergo significant lateral fluctuations as a function of time, as shown by the plot of \( h(t) \) for a microtubule tip in the upper Inset of Fig. 3e (also see SI Movies 4 and 5). These tip fluctuations are qualitatively similar to the lateral bending fluctuations along the microtubule backbone, as shown by the plot of the time dependence of a Fourier amplitude, \( a_\tau \), in the lower Inset of Fig. 3e. Moreover, both tip fluctuations and lateral microtubule bending fluctuations exhibit a diffusive character, as shown by the roughly linear time-dependence of the mean-squared displacement of the lateral deviation, \( \langle (h(t) + r) - h(0) \rangle^2 = 2D \langle s(u) \rangle^2 \), where \( s(u) \) is an average of fluctuations over time. Further confirmation comes from the fact that this curve is nearly identical to the ensemble-averaged variance of grown microtubules (brown squares in Fig. 3f). This suggests that force-induced lateral microtubule bending fluctuations are indeed associated with microtubule tip fluctuations. Because these forces are sustained over a period of several seconds, the microtubule will continue to grow in the new direction defined by the reoriented microtubule tip, and the resulting bend will be stabilized by the surrounding elastic network. Consistent with this, the trajectories of growing microtubules revealed by an overlay of successive positions of YFP CLIP-170-labeled microtubule tips shows that tip trajectories frequently exhibit significant curvature, as shown by the microtubule trajectories highlighted in red in Fig. 3f Inset; this suggests that such behavior is quite general.

To further test this physical picture, we performed a Fourier analysis of the trajectories of hundreds of growing microtubule tips in YFP CLIP-170-expressing CHO cells. As predicted by the tip-reorientation mechanism, these growth trajectories exhibit a roughly \( \langle a_{\tau}^\tau \rangle \sim q^{-2} \) ensemble-averaged Fourier spectrum, as shown by the yellow squares in Fig. 3f. Further confirmation comes from the fact that this curve is nearly identical to the ensemble-averaged variance of grown microtubules (brown squares in Fig. 3f), strongly suggesting that these curved microtubule growth trajectories are indeed the origin of the locked-in long-wavelength bends. Newly grown microtubules are highly bent. If tip fluctuations during growth give rise to the thermal-like Fourier bending spectrum of intracellular microtubules, \( \langle a_{\tau}^\tau \rangle \sim q^{-2} \), this Fourier spectrum should be apparent even in newly grown microtubules that have not had time to undergo significant lateral bending fluctuations. To test this, we depolymerized microtubules by treating COS-7 cells with...
nocodazole for 6–8 h, after which there are no microtubules detected (upper Inset in Fig. 4a). The cells remain alive and well spread, although their morphology is slightly altered. These microtubule-free cells were then washed to remove nocodazole, and after only 5 min the cells were fixed and stained for tubulin. By then, many microtubules have begun to regrow in these cells, as shown in Fig. 4b. The cells remain alive and well spread, although their morphology is slightly altered. These microtubules are highly bent shape, with an approximately q^{-2}-dependent mode spectrum, in <5 min of growth.

Discussion

Intracellular microtubules play a wide variety of fundamental biological roles. In addition to functioning as a mechanochemical scaffold controlling cell shape, microtubules also guide polarized cell migration, act as a highway system for motor-driven transport, and mediate the controlled distribution of DNA and other material between daughter cells.

To perform these varied tasks, microtubules continually restructure, undergoing repeated cycles of growth and depolymerization. This dynamic instability is particularly well suited for restructuring into different functional microtubule network architectures because microtubules that fail to grow into a proper location can simply depolymerize over much of their length and then regrow into different functional microtubule network architectures. When the magnitude of the fluctuation is small, and the filaments naturally grow along the same straight path, very little of the cell is explored. This could be accomplished by complete depolymerization of the microtubule back to its origin, followed by regrowth from a nucleation site with a different orientation. However, in animal cells, most microtubules exhibit “recovery” from shrinkage well before full depolymerization, with the same microtubule undergoing repeated shrinking and regrowth into the leading edge. Under the action of only small thermal fluctuations, a microtubule that undergoes such recovery would simply regrow into the same small area of the cell. Indeed, although dynamic instability is particularly well suited to restructuring and self-assembly of the microtubule network, the high rigidity of microtubules would tend to limit such network plasticity. However, in the presence of large, fluctuating nonthermal forces, the resulting tip fluctuations will lead to a dramatic reorientation upon regrowth, and the tip will explore a much broader region of the cell. To illustrate this, we generate growth trajectories using a simple two-dimensional simulation in which a filament begins with a horizontal orientation, growing toward the right. At each time step, the filament tip, at position s, extends by an amount Δs, such that the new tip orientation is given by θ(s + Δs) = θ(s) + Δθ. Under purely thermal fluctuations, Δθ is small, and the filaments naturally grow into nearly straight trajectories, as shown by the blue curves in Fig. 5c. Thus, a microtubule “searching” under the action of thermal fluctuations alone will only explore the small sliver of the cell traversed by these blue filaments. However, under the action of larger nonthermal fluctuations, this “searching” ability of microtubules changes dramatically. When the magnitude of the fluctuations, √<(Δθ)^2>, is increased by a factor of 10, the trajectories exhibit significant curvature, as shown by the red curves in Fig. 5c. These simulated trajectories exhibit remarkable qualitative similarity with...
The actual trajectories obtained from tracking the tips of microtubules in CHO cells, as shown in Fig. 5b. Moreover, simulated trajectories also exhibit a Fourier bending spectrum of \( \langle \alpha^2 \rangle_E \sim \frac{1}{l_0^2} - \frac{1}{q^2} \), where \( l_0^2 \sim \langle \theta^2 \rangle^{-1} \).

We have shown that nonthermal force fluctuations lead to significant microtubule curvature in cells, primarily by causing curved growth trajectories. As a result, microtubules exhibit a persistent random walk trajectory, with an apparent persistence length, \( l_0^* \sim 100 \) times less than that of microtubules in vitro. However, it is important to note that although nonthermal forces cause microtubules to exhibit a small apparent persistence length, their actual rigidity need not be small. Although it is possible that the small apparent persistence length could arise if intracellular microtubules are significantly softer than in vitro, in vitro measurements under a variety of conditions, including with and without microtubule associated proteins, have shown that the rigidity of microtubules varies by at most an order of magnitude (8, 15, 16). Moreover, the lateral bending fluctuations are strongly ATP-dependent, and thus thermal fluctuations alone cannot be responsible. Another possible contribution to the small apparent persistence length is defects in the microtubule lattice that could cause locked-in bends (“kinks”) during growth. However, the nature of tip reorientation events (e.g., Fig. 3d) appears to be inconsistent with a lattice defect mechanism. This could conceivably be tested by removing the surrounding cytoskeleton, although complete cytoskeletal disruption typically results in pathological features such as blebbing and cell retraction. Nevertheless, our results suggest that tip reorientation from force fluctuations alone is sufficient to explain the small apparent persistence length. Indeed, using the approximate mechanical properties of the cytoskeleton, we estimate that the observed persistence length, \( l_0^* \), of order 10 \( \mu \)m, can arise from nonthermal forces of order 1–10 pN, comparable with forces generated by individual motor proteins (see SI Text and SI Fig. 6).

In addition to dramatically increasing the effective plasticity of the microtubule network, fluctuating nonthermal forces also have important implications for force generation by polymerization. Growing microtubules are known to be capable of generating mechanical forces that are important for movement within cells (17). This is typically explained by the Brownian ratchet mechanism, whereby the chemical energy of polymerization is converted into directional force generation through harnessed fluctuations, typically assumed to be of a thermal nature (18, 19). In vitro experiments on thermally fluctuating microtubules have shown this to be an adequate description of the piconewton-level forces generated when a microtubule polymerizes against a wall (16). Polymerization forces in vivo are usually assumed to also be driven
by thermal fluctuations, although neither forces nor fluctuations have been directly measured for intracellular microtubules. Because nonthermal fluctuations are clearly important in causing fluctuations in microtubule tips, they may therefore also be the dominant factor in determining the magnitude of polymerization forces generated in cells. These fluctuations are much larger than thermal fluctuations, and the resulting polymerization forces will be correspondingly larger.

Conclusions

We have shown that microtubules in adherent animal cells have a surprisingly thermal-like bending spectrum, with bends on both long and short wavelengths. However, only short-wavelength bends fluctuate significantly in time, whereas long-wavelength bends are effectively frozen-in. These fluctuations result from active, nonthermal forces within cells and lead to an apparent persistence length that is ≈100 times smaller than the thermal persistence length measured in vitro. This small apparent persistence length arises during growth, when active forces cause fluctuations in the orientation of growing microtubule tips, leading to long-wavelength bends that are subsequently frozen-in by the surrounding elastic network. Thus, microtubules are at once highly rigid biopolymers that are further reinforced by the surrounding network while at the same time being highly deformable under the action of large forces during growth. This has important implications for the mechanical properties and behavior of the microtubule cytoskeleton, including microtubule targeting processes and force generation by polymerization.

Materials and Methods

Cell Culture. COS-7 cells (African green monkey kidney-derived) were obtained from American Type Culture Collection and cultured in 10% FBS DMEM. YFP CLIP-170 CHO cells (a kind gift of Yulia Komarova, University of Illinois at Chicago) were cultured in 10% FBS DMEM. YFP CLIP-170 CHO cells (a kind gift of Yulia Komarova, University of Illinois at Chicago) were cultured in 10% FBS DMEM without glucose and sodium pyruvate. For visualization of static microtubule shapes, cells were fixed in 0.25% glutaraldehyde/0.5% Triton X-100 for 1 min, followed by 1% glutaraldehyde for 10 min. Cells were then stained with a mouse anti-a-tubulin primary antibody DMA, followed by rhodamine-conjugated anti-mouse IgG (both from Calbiochem), using standard procedures. For studies of the dynamics of intracellular microtubules, confluent monolayers of cells were incubated for 24–48 h with an adenoviral vector encoding EGFP-tubulin (5).

Cells were sparsely plated onto glass-bottomed 35-mm dishes (MatTek) and allowed to adhere and spread overnight. For ATP-depletion studies, cells were incubated in 2 mM sodium azide/10 mM deoxyglucose in phosphate-buffered saline for 30 min.

Microscopy. All images were acquired on a Leica DM-IRB epifluorescence microscope equipped with a heated stage to maintain cells at a temperature of 37°C, and a ×100 N.A. 1.4 oil objective. Sequences of images of fluorescent microtubules in cells were captured by using an Andor iXon du887bf electron multiplying CCD or a Hamamatsu C7190-21 electron bombardment CCD camera controlled by MetaMorph software (Molecular Devices). To minimize background fluorescence, cells were imaged in Hank’s balanced salt solution (Invitrogen) supplemented with 10 mg/ml BSA (Sigma). Microtubule growth trajectories were altered by applying a local force to cells with Femtotip microneedles controlled with a micromanipulator (Eppendorf, Westbury, NY).

Image Analysis. Microtubule contours were analyzed with an automated image analysis algorithm written in IDL and described in detail elsewhere (8). Briefly, the position coordinates of the microtubules were extracted from each image with subpixel accuracy (20 nm or 0.15 pixels) by thresholding, skeletonization, and subsequent position refinement from fits of Gaussian intensity profiles along perpendicular cuts across the filament. To track microtubules in the dense cytoskeleton of living cells, we extended the tracking algorithm with a graphical interface. The Fourier amplitudes of the microtubule contour were determined as described in ref. 2. The variance of the instantaneous Fourier amplitudes from ensembles of =200 microtubules in fixed cells was determined by binning data according to wave vector into bins of size ≈0.1 μm−1 and subsequently determining the variance by direct calculation or from the variance of a Gaussian fit to the distribution of amplitudes, the latter helping to avoid errors associated with occasional filament mis-identification. For the time-dependent fluctuations of the Fourier amplitudes of individual GFP-microtubule, data from ≈20 filaments was averaged by using Origin (OriginLab). To track the tips of microtubules in YFP CLIP-170 CHO cells, we processed images using a Gaussian bandpass filter and then determined tip location using an intensity-weighted center-of-mass tracking algorithm (20). For Fourier analysis of these tracks, an evenly sampled track contour was first obtained by a local average over tip locations. The variance of the ensemble of Fourier amplitudes was then analyzed as described for the instantaneous Fourier amplitudes of microtubules.

We thank T. J. Mitchison, D. J. Needleman, M. L. Gardel, and G. H. Koenderink for helpful discussions. This work was supported by National Science Foundation Grants DMR-0602684 and CTS-0505929, Harvard Materials Research Science and Engineering Center Grant DMR-0213805, and Harvard Interdisciplinary Graduate Education and Research Training on Biomechanics Grant DGE-0221662. F.C.M. was partially supported by the Foundation for Fundamental Research on Matter. C.P.B. acknowledges the hospitality of Vrije Universiteit.