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REVIEW ARTICLE

Investigation of *in vivo* microtubule and stress fiber mechanics with laser ablation

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Laser ablation has emerged as a useful technique to study the mechanical properties of the cytoskeleton in living cells. Laser ablation perturbs the force balance in the cytoskeleton, resulting in a dynamic response which can be imaged. Quantitative measurement of the dynamic response allows the testing of mechanical theories of the cytoskeleton in living cells. This review discusses recent work in applying laser ablation to study stress fiber and microtubule mechanics in living endothelial cells. These studies reveal that molecular motors are major determinants of the mechanical properties of the cytoskeleton in cells.

Introduction

In physiological processes such as muscle contraction, embryogenesis, wound healing, and inflammation, tissue cells exert pushing or pulling forces on their surroundings. These forces are generated within the cell's cytoskeleton and transmitted through adhesive contacts to the surrounding tissue. The cytoskeleton is a highly dynamic structure that is continually being assembled and dissembled. Cytoskeletal actin filaments polymerize and push on the inner cell membrane in order to form protrusions that extend over or through surrounding cells and the extracellular matrix.[1] Microtubules also generate pushing forces as a result of their directional polymerization at the cell membrane.[2] Due to their central role in generating intracellular forces,[3] much attention has focused on understanding the mechanical properties of the F-actin and microtubule cytoskeleton.

The flexural rigidity of individual microtubules and actin filaments have been determined from *in vitro* studies of reconstituted cytoskeletal filaments.[4] The *in vivo* environment in which cytoskeletal filaments function is considerably more complex owing to the presence of proteins that interact with the cytoskeleton. For example, cross-linking proteins like alpha-actinin[5] and filamin[6] bind to F-actin filaments and create mesh-like actin networks.[7] In cells, the F-actin cytoskeleton contracts by the action of the motor protein myosin which walks on the sides of actin filaments causing them to slide past each other.[8–11] Similarly, microtubules are crosslinked to other cytoskeletal elements[12–14] and are linked with motor proteins like dynein[15] and kinesin[16] that can generate forces. Recreating the complex interactions present in the cell is difficult *in vitro*. Therefore, recent efforts have focused on understanding cytoskeletal mechanics *in vivo* through the development of novel methods for mechanically characterizing the cytoskeleton. For example, particle tracking methods have enabled the measurement and comparison of rheological properties of the cytoskeleton *in vitro*[17] and in living cells.[18–20]

Laser ablation, first used on living cells in the 1960's,[21] has become a powerful tool for testing mechanical models of force generation in the cytoskeleton.[22–27] The method focuses ultrashort pulses of a laser beam on a microtubule or actomyosin stress fiber in a living cell.[28,29] In a typical system, a femtosecond Ti:sapphire laser delivers 100-fs pulses at 790 nm to 800 nm, 1 kHz repetition rate[28,29] resulting in a pulse energy of 2–5 nJ. The energy is absorbed in the diffraction limited spot ($\sim$300 nm) resulting in vaporization of material and severing of cytoskeletal filaments.

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Insight, innovation, integration

We review the application of the laser ablation technique as a powerful tool in the field of cytoskeletal mechanics. The approach involves severing an actomyosin bundle or a single microtubule. Severing cytoskeletal filaments instantaneously perturbs the cytoskeletal mechanical force balance, and imaging the subsequent dynamic changes in cytoskeletal configuration provides *in vivo* quantitative experimental data for the testing of mechanical models of force generation in the intact cytoskeleton. The key advantage of the laser ablation approach is that experiments are performed in living cells. We expect this technique to be increasingly used by biologists, bioengineers and biophysicists for answering fundamental questions in cell biology.

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The short duration of the pulses (picoseconds or femtoseconds) ensures that the amount of power (energy/time) is high but the total energy delivered is low, which minimizes the damage to the cell due to energy dissipation outside the ablated spot. When the laser is focused through a high-resolution objective, a single microtubule or a single actomyosin bundle can be severed.[30–32] Severing cytoskeletal filaments instantaneously perturbs the cytoskeletal mechanical force balance, and imaging the subsequent dynamic changes in cytoskeletal configuration provides *in vivo* quantitative experimental data for the testing of mechanical models of force generation in the intact cytoskeleton. In this review, we discuss recent work with this approach to develop quantitative models of force generation in actomyosin stress fibers and the microtubule cytoskeleton.

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Laser ablation studies of stress fiber mechanics

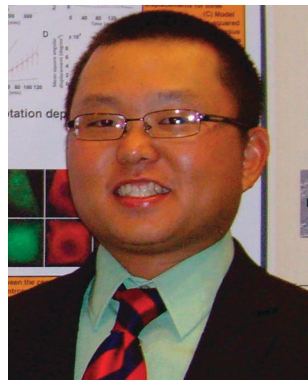
Stress fiber structure

Stress fibers are a specialized type of actomyosin structure that generates tensile forces. These contractile cytoskeletal cables display a continuous staining for actin until their ultimate termination into focal adhesions, see example in Fig. 1A. Numerous studies have reported the presence of stress fibers in the intact endothelium *in vivo*[11,33,34] (and in a few other cell types *in vivo*[35,36]). The magnitude of hemodynamic forces exerted on vascular endothelial cells correlates with the expression of stress fibers in endothelial cells.[34,37,38] The physiological function of stress fibers is to enable strong adhesion between endothelial cells and the basement membrane.[11] This adhesion allows endothelial cells to resist blood flow-induced mechanical stresses including cyclic strain, hydrostatic pressure and shear flow.[11,33,39–42] Endothelial cells respond to applied mechanical stresses by changing their orientation both in tissue[39,40] and culture,[24,42–46] which depends on remodeling of stress fibers.[42]

In addition to F-actin, stress fibers are composed of myosin and a number of different cross-linking proteins. Proteins like α-actinin localize to stress fibers in a punctate pattern as illustrated in Fig. 1B.[5] These punctate spots were originally referred to in the literature as dense bodies as they appeared as electron dense regions similar to muscle Z-lines.[47] Further studies have identified multiple other proteins that localize to stress fiber dense bodies including filamin,[6] fascin,[48] and VASP.[49] Staining for myosin[50,51] or tropomyosin[52] reveals a punctate pattern that is offset from the dense bodies. These findings support the concept that stress fibers are composed of contractile subunits analogous to muscle sarcomeres. The idealized sarcomeric model of the stress fiber is such that each dense body serves as the boundary between adjacent sarcomeres (Fig. 1B and ref. 52). A single sarcomere consists of opposite polarity actin filaments emanating inwards from the respective dense bodies where the barbed ends are located.[53] Bipolar myosin filaments cross-link the opposing bundles of actin filaments.[50] Contractile force is generated as the bipolar myosin filaments translocate in opposite directions towards the dense bodies.

Stress fiber mechanics

There has been considerable interest in understanding the mechanical properties of stress fibers. Katoh *et al.*[54] showed that isolated single stress fibers are contractile in an ATP and myosin dependent manner. While early experimental data on



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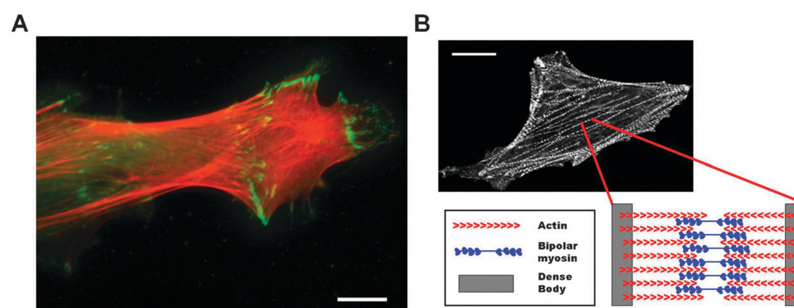

Fig. 1 Structure of the stress fiber. (A) Picture of a bovine capillary endothelial (BCE) cell stained with phalloidin for actin stress fibers (red) and immunostained for vinculin (green) in focal adhesions. (B) Microstructure of stress fibers consists of sarcomeric subunits. Shown is a micrograph of a BCE cell transfected with EGFP-α-actinin which results in a punctate staining of dense bodies. The cartoon schematic illustrates the microstructure of a single stress fiber sarcomere marked by two penultimate dense bodies. Scale bars are 20 μm (reprinted from ref. 23 with permission from Elsevier).

stress fiber mechanics was from *in vitro* experiments with extracted stress fibers,[54,55] a number of recent studies have used laser ablation to measure these properties in living cells.[22–26] Severing a single stress fiber causes a retraction of the severed edges (Fig. 2A and ref. 22). This retraction is due to instantaneous dissipation of all tension in the fiber. Inhibiting myosin eliminates the retraction of the severed edges.[22,23] The first observations of this phenomenon indicated that the

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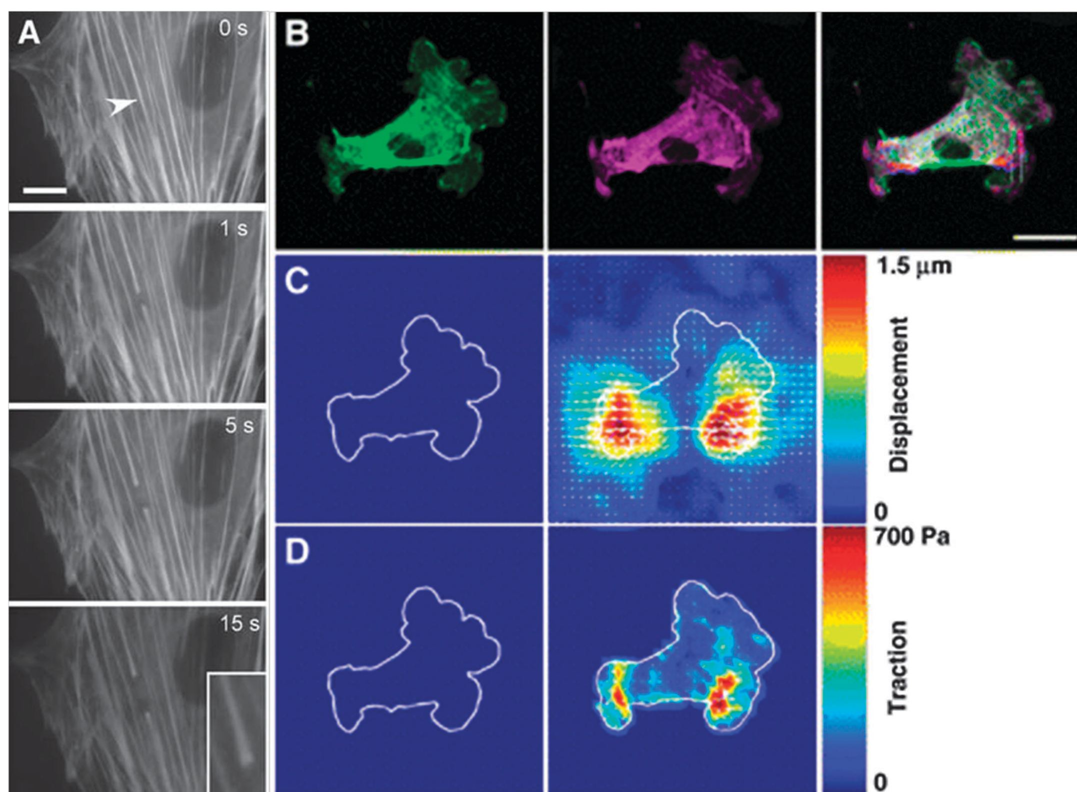

Fig. 2 Incision of stress fibers in living cells using a laser nanoscissor (reprinted from ref. 22 with permission from Elsevier). (A) Severing and retraction of a single stress fiber bundle in an endothelial cell expressing EYFP-actin. As the stress fiber retracted over a period of 15 s, the severed ends splayed apart (inset). (Arrowhead indicates the position of the laser spot; bar = 10 μm). (B–D) Fluorescence microscopic (B) and traction force microscopic (C, D) images showing the effects of stress fiber incision on cytoskeletal organization, global cell shape and ECM mechanics. A single stress fiber was incised in a cell cultured on a flexible polyacrylamide ECM substrate (B–D; stiffness = 3.75 kPa). (B) The actin cytoskeleton is depicted in green before incision (Pre-cut, left) and magenta after incision (Post-cut, middle); when the two images are overlaid (Overlay, right), cytoskeletal regions which did not change position appear white, whereas those that rearranged retain their distinct green and magenta colors. Note that stress fiber incision resulted in global cytoskeletal rearrangements only in the cell on the flexible substrate (B), including wholesale outward translation of the whole cell and cytoskeleton along the main axis of the cut fiber. The two vertical white lines indicate a vertically oriented stress fiber located many micrometers away from the site of incision in the right portion of the cytoskeleton that undergoes large-scale lateral displacement in response to stress fiber ablation (Bar = 10 μm). (C) Change in bead displacements and ECM substrate strain distribution. (D) Change in cell traction forces relaxed into the ECM substrate.

retraction followed an exponential form such that the rate of translation of the severed edge was high immediately after severing but slowed down and eventually became zero at steady state.[22] Using a Kelvin-Voight mechanical model for viscoelasticity consisting of a spring and dashpot in parallel, Kumar *et al.*[22] modeled the exponential translation of the severed edge. This work was significant because it demonstrated the use of laser ablation for testing mechanical properties of the cytoskeleton *in vivo*. Kumar *et al.*[22] also showed that that stresses of nearly 700 Pa could be dissipated by severing a single actomyosin fiber in endothelial cells (Fig. 2B and C). Thus, the stress fiber is in mechanical continuum with the underlying adhesive substrate.

Mechanical models for stress fiber sarcomeres have typically assumed that velocity-dependent myosin contraction operates in parallel with an elastic element such that expanded or compressed elastic elements partially balance the tension.[24,56,57] Such mechanical models have been used to explain the experimental observation of Peterson *et al.*[58] that stress fiber sarcomeres contract near focal adhesions on MLCK activation.

To understand the origin of the exponential retraction of the severed stress fiber ends, a recent study[23] carried out laser ablation of cells expressing labeled alpha-actinin which marks dense bodies and allows the quantification of stress fiber sarcomere lengths (kymograph in Fig. 3A). On laser severing, the severed end retracted exponentially consistent with the study by Kumar *et al.*[22] (Fig. 3B). At the same time, the sarcomeres in the severed fibers decreased in length; thus, the net retraction of the severed edge corresponded to the contractions of individual sarcomeres (Fig. 3A). Interestingly, the contraction of sarcomeres was not exponential; rather, after an initial near-instantaneous contraction, the sarcomere length decreased linearly with time, and abruptly stopped after the linear retraction (Fig. 3C and D). If a spring element were present in parallel with myosin-based contraction in the sarcomere, then an exponential decrease in sarcomere lengths is expected. In addition, on inhibiting myosin by blebbistatin treatment in a severed contracted fiber, no increase in stress fiber length occurred. These results collectively argue against the presence of an elastic spring that balances part of the stress fiber tension in parallel with myosin forces.

Based on these results, Russell *et al.*[23] proposed a simple mechanical model for the stress fiber sarcomere (Fig. 3E). The results (Fig. 3C) suggest that following severing, the sarcomere initially relaxes elastically, followed by slower myosin-mediated contraction at a constant speed V, which is likely limited by the maximum (unhindered) myosin motor velocity. The abrupt cessation of contraction suggests that either a strong resistance to further contraction is suddenly encountered, which could reflect a rigid steric barrier, or that myosin motors have reached a limiting minimal distance from the dense bodies where the motors can no longer walk on actin filaments. Because myosin motors are sensitive to load, the uniform speed of the contraction phase (on severing) suggests that elastic forces do not increase (and are not relieved) during contraction. Thus, tension exerted at focal adhesions by stress fibers is entirely balanced by myosin activity.

An interesting discovery from the laser ablation experiments with sarcomeres was that the linear contraction distances of sarcomeres were exponentially distributed. This means that in

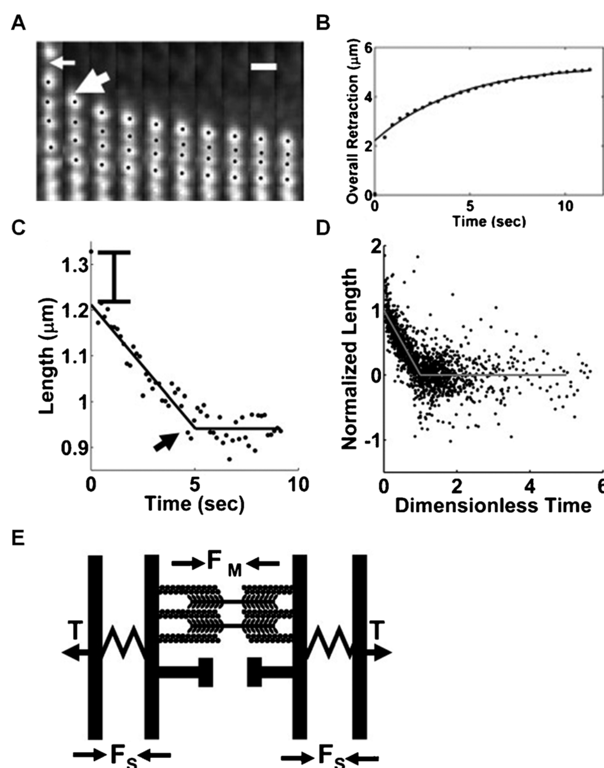

Fig. 3 Sarcomeres contract in a severed fiber (reprinted from ref. 23 with permission from Elsevier). (A) A kymograph showing the results for one half of a severed fiber cut at the thin arrow. Notice that sarcomeres contract and that the contraction is not uniform in every sarcomere (thick arrow) (Scale bar, 1.4 μm and time between frames is 840 ms). (B). The distance the severed edge moves follows an exponential form; the solid line is a least-squares regression fit of the retraction model to the data. (C) Representative example of sarcomere length change in a severed fiber. The contraction occurs in two distinct phases: first a quick initial drop (marked on plot) followed by sustained contraction at nearly constant speed. After some time the sarcomere reaches a steady state length (arrow) and remains there for the remainder of the experiment. (D) Pooled data from contraction of 28 sarcomeres in 18 cells was normalized and plotted together excluding initial sarcomere lengths. A clear linear trend is visible in the normalized data. (E) Proposed mechanical model for the sarcomere. The tension, T, in the stress fiber is only determined by myosin forces, F_M, in series with a stiff elastic element, F_S. An impenetrable barrier prevents further sarcomere contraction at some minimal sarcomere length.

the intact fiber, the most probable state of the sarcomere is one of complete contraction (*i.e.* at the 'barrier'). This observation can be explained by considering that sarcomere lengths in a given fiber may fluctuate randomly owing to myosin activity fluctuations. Assuming that the sarcomeres continue to contract on average (except that contraction is not possible beyond the barrier), Russel *et al.*[23] predict an exponential distribution of contraction distances (this is discussed in more detail in ref. 23). Interestingly, contraction of several sarcomeres in series in a severed fiber can lead to an exponential retraction of the severed, translating end of the fiber if the contraction distances are exponentially distributed.[23]

Since then, Russell *et al.*[59] demonstrated in another study that sarcomere lengths indeed fluctuate in the endothelial cell.

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They and others[59–61] observed continuous F-actin polymerization in focal adhesions; newly formed F-actin is added into the stress fiber and translated into the cell interior. In addition, neighboring sarcomeres can fuse along the length of stress fibers. Continuous addition of F-actin at the ends, fusion in the interior, and fluctuations in myosin activity all contribute to maintain sarcomere lengths in the cell.

Another recent study used the laser ablation technique to understand the mechanical differences between peripheral and interior stress fibers in cells.[25] Peripheral SFs have been shown to remain intact on inhibition of Rho Kinase (ROCK), an enzyme that participates in regulating the phosphorylation of myosin light chain (MLC); however, these SFs dissolve on direct MLC kinase inhibition. Interior stress fibers follow the inverse behavior.[62–64] Tanner *et al.*[25] found that peripheral SFs have different viscoelastic properties from interior fibers; the two sub-populations also play distinct mechanical roles. Severing peripheral fibers caused disengagement of terminal adhesions and contraction of the whole cell, while severing interior fibers produced only minor changes in cell area. However, severing interior fibers in the absence of peripheral fibers caused similar whole-cell contraction as occurred on severing peripheral fibers, consistent with the idea that dissolving peripheral fibers resulted in interior fibers bearing additional tension. These studies highlight the utility of laser ablation as a technique that can be used to spatially map mechanical properties of the cellular cytoskeleton.

Laser ablation study of microtubule mechanics

Microtubules play critical roles in normal cell functions including the formation of mitotic and meotic spindles, stabilization of protrusions, and polarization of cells.[65] Despite a thermal persistence length in the millimeter range, microtubules have been widely reported to exist in bent shapes in cultured cells.[65,66] Polymerizing microtubules have been shown to buckle upon impinging on an immovable boundary in *in vitro* studies[2,67] and *in vivo* experiments.[68–70] Such buckled microtubules have been widely assumed to be under compressive loading; this also suggests that pushing microtubules may center the centrosomal array of microtubules in cells.[71–74]

Sources of forces that could bend microtubules include polymerization of a microtubule against an immovable boundary,[2,66] actomyosin contractility,[69,75] and microtubule based motors interacting with the cell cortex.[65,76–81] Recently Weitz and coworkers proposed that buckled microtubules could support larger compressive loads if they were laterally supported by an elastic medium.[66] Odde and coworkers investigated the mechanism involved in microtubule buckling through direct observation of buckling microtubules in living cells.[65] By performing fluorescence speckle microscopy, they found that buckling microtubules move in the direction of the cell periphery, instead of towards the cell center, arguing against purely polymerization driven buckling. Furthermore the authors found that the F-actin surrounding buckling microtubules was stationary which suggests that the actomyosin activity or actin retrograde flow may not play a substantial role in buckling microtubules. The authors hypothesized that the anterograde-driven buckling is indicative of microtubule based molecular motors.[65]

A recent study by Wu *et al.*[27] further investigated the role of dynein forces in the microtubule force balance. Wu *et al.*[27] reasoned that a bent microtubule severed with laser ablation would straighten if the bends were due to compressive forces propagating from the microtubule tip. Instead, the segments near the newly created minus ends consistently increased in curvature after severing, whereas the new plus ends depolymerized rapidly along the original contour of the microtubule (Fig. 4A and ref. 27). In dynein-inhibited cells, segments near a free minus end did not show any increase in curvature following severing; instead, the microtubules straightened rapidly, on timescales of a few seconds (Fig. 4B).

These results from the microtubule laser ablation experiments were surprising because they indicated that compressive stresses are not dominant contributors to the microtubule force balance away from the cell periphery. Instead, they suggest a model in which dynein motor forces pull along the length of the microtubule toward the plus end thereby putting the microtubule under tension, although the microtubule tips near the cell periphery still be under compression due to polymerization forces.[66] Another important conclusion from this study was that dynein linkages to the cytomatrix impose a large frictional resistance to lateral motion of the microtubules compared to dynein-inhibited cells.

Based on these results, Wu *et al.*[27] formulated a mechanical model in which transient dynein linkages with the MT result in a tensile force on the microtubule as well as lateral friction opposing the motion. This model predicts the increased buckling of minus-ended microtubules on severing. Importantly, simulations of dynein motors pulling on a radial array of microtubules using parameters estimated from the single MT severing experiments predict that dynein pulling can center the centrosome *in vivo* on time scales similar to those observed in cells. The simulations also explain why centering by microtubule pushing can occur *in vitro* without dynein,[72] but only under conditions (low friction and short microtubules) where buckling does not occur. Under conditions found in animal cells, with longer microtubules and much higher frictional resistance, both simulations and experiments suggest that compressive buckling forces are insufficient to center the centrosome.

In summary, the laser ablation experiments uniquely allowed the testing of mechanical models of microtubule bending in cells. Dynein based forces provide a unifying explanation for the shapes of individual microtubules in the cell and how these shapes are consistent with tension-driven centering of the centrosome by a radial array of microtubules.

Other applications of laser ablation in cell and tissue mechanics

In addition to testing mechanical models of cytoskeletal force generation, laser ablation has been a useful tool for determining the effect of force on intracellular protein dynamics. Lele *et al.*[82] used laser ablation to sever stress fibers, and then performed fluorescence recovery after photobleaching (FRAP) experiments to measure protein exchange dynamics in the associated focal adhesion. Using this method, the authors demonstrated

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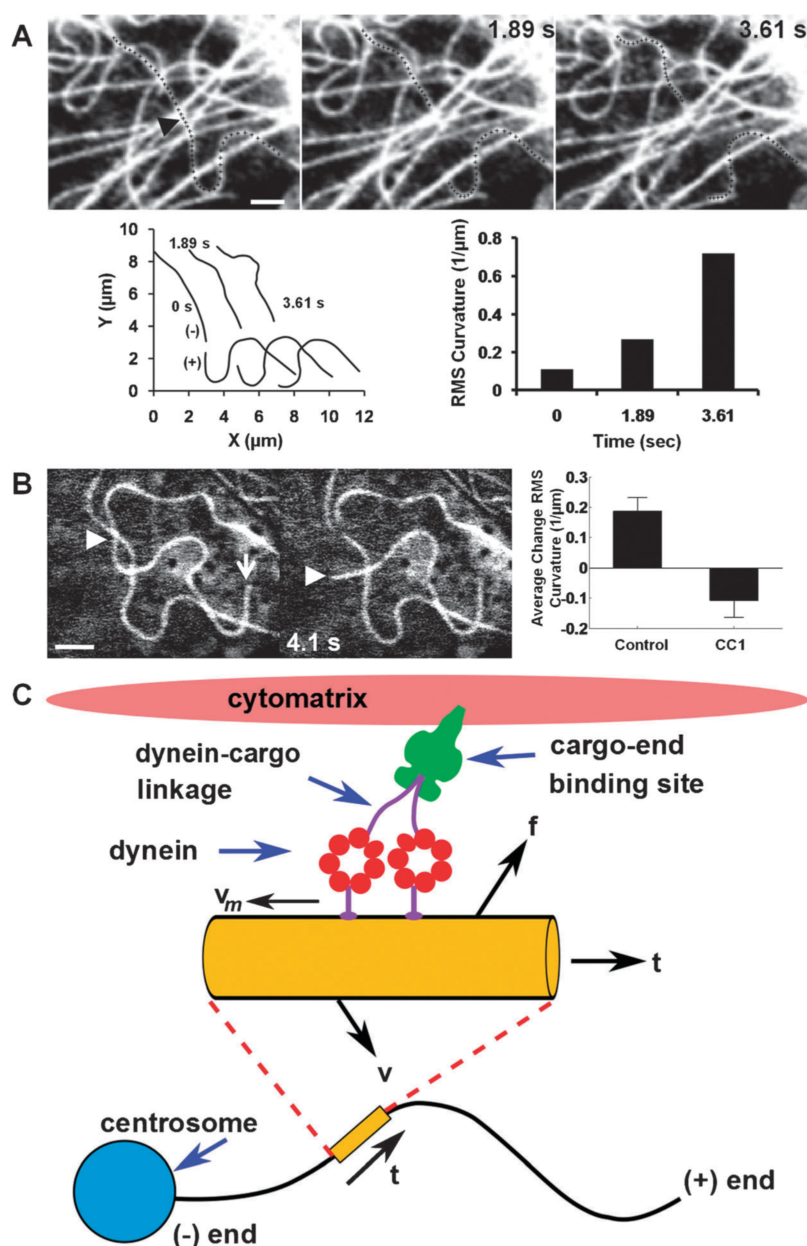

Fig. 4 Minus end microtubules increase in bending after laser severing. Representative images highlighting changes in shape after severing a single microtubule in living cells. (A) Increased bending of minus ended microtubules after severing near the nucleus. The black arrowhead indicates the position of the cut and the severed microtubule is highlighted by small crosses. Microtubule shapes in the images were measured in MATLAB (see plots of severed microtubule shapes; the newly created plus and minus ends are indicated) and the root mean square (RMS) curvature was calculated. The minus-end microtubule (recognized as minus ended from the lack of significant depolymerization compared to the newly exposed plus end) showed a 7-fold increase in mean curvature over the visible segment length. However, the plus-end segment depolymerized but showed no measureable change in curvature. These observations are consistent with the hypothesis of minus-end directed motors pulling on the microtubule. (B) Straightening of a bent microtubule in a dynein-inhibited cell. The white arrow indicates the plus end of the microtubule, and the arrowhead tracks the severed end. The microtubule straightens significantly on timescales of a few seconds, supporting the hypothesis that there is an additional frictional force contributed by dynein. The plot compares the change in RMS curvature near severed minus ends in control and dynein-inhibited cells. Data is from at least 10 cells for each condition; the statistical significance is at $p < 0.01$. Error bars indicate standard error of the mean (SEM). The pooled data strongly supports the hypothesis that dynein promotes bending of microtubules in living cells, and that in the absence of dynein, microtubules straighten upon severing. Scale bars are 2 μm. ((A) and (B) reprinted from ref. 27 with permission from the American Society for Cell Biology). (C) Cartoon of a dynein motor indicating how the minus-directed motor bound to the cytomatrix exerts a force towards the microtubule plus end. Individual dynein molecules walk towards the microtubule minus end at a speed $\boldsymbol{v}_m$ (along the local tangent direction, $\boldsymbol{t}$) that depends on the opposing force $\boldsymbol{f}$. Each segment of the microtubule moves relative to the cytoskeleton with a velocity $\boldsymbol{v}$.

that dissipating stress fiber tension resulted in an increase in the dissociation rate constant of zyxin, but not vinculin, in focal adhesions of endothelial cells. The advantage of using laser ablation in these experiments was that severing a stress

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fiber instantaneously dissipates tension at the focal adhesion, and FRAP experiments before and after ablation allow reliable comparison of protein exchange dynamics. Another study showed that severing of individual stress fibers caused a loss of zyxin from both SFs and focal adhesions. This study also demonstrated that retraction of the severed stress fiber can actually nucleate new adhesion sites along the retracting fiber.[26]

To investigate forces acting at the junctional network of disc epithelial cells, Farhadifar *et al.*[83] perturbed cell monolayers by ablating cell-cell junctions with a UV laser beam. Measuring the contracting area and distance of the network after laser ablation gave a good estimation of parameters in their model which explained the packing geometry of epithelial cells in monolayers. Tinevez *et al.*[84] performed local laser ablation of the cell cortex in L929 fibroblasts to test whether bleb formation is pressure-driven. Indeed, a bleb grew from the site of ablation immediately, which confirmed the hypothesis that bleb growth was a direct consequence of cytoplasmic pressure. Mayer *et al.*[85] ablated the actomyosin meshwork in polarizing *C. elegans* zygote at different positions (regions with or without cortical flow) and in different directions (orthogonal or parallel to the cortical flow). The results showed how cortical flow was associated with anisotropies in cortical tension, and further answered the question how actomyosin contractility and cortical tension interact to generate large-scale flow.

Laser ablation studies are increasingly being used in creative ways to answer fundamental questions related to microtubule function. Maiato *et al.*[86] severed kinetochore fibers (K-fibers) by laser ablation in *Drosophila* S2 cells and showed that the severed K-fibers kept growing *via* MT plus-end polymerization at the kinetochore. The minus-end generated by severing was pushed away from the kinetochore and eventually captured and transported to the spindle pole. These observations revealed that even in centrosome-containing cells, kinetochore-driven K-fiber formation is an important mechanism that contributes to spindle assembly during normal mitosis. Another study used laser ablation to disrupt microtubule interactions with the bud neck in budding yeast.[87] This caused mitotic exit suggesting that cytoplasmic microtubules enable the monitoring of the spindle location and mitotic exit in the dividing cell in the event of positioning errors. Another study showed that ablating the centrosome does not inhibit axon extension and growth, revealing a role for acentrosomal nucleation of microtubules in early neuronal development.[88] Laser ablation has also been used to study microtubule dynamics. With laser ablation, Colombelli *et al.*[89] generated severed microtubules at a predefined location in the cell, which allowed them to measure shrinkage rate, growth rate and rescue frequency simultaneously with high temporal and spatial specificity in live cells. Similarly, Wakida *et al.*[90] used laser ablation to show that the microtubule depolymerization rate in living PTK2 cells is location dependent.

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