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Modern methods to interrogate microtubule dynamics

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Microtubules are essential protein filaments required to organize and rearrange the interior of the cell. They must be stiff with mechanical integrity to support the structure of the cell. Yet, they must also be dynamic to enable rearrangements of the cell during cell division and development. This dynamic nature is inherent to microtubules and comes about through the hydrolysis of chemical energy stored in guanosine triphosphate (GTP). Dynamic instability has been studied with a number of microscopy techniques both in cells and in reconstituted systems. In this article, we review the techniques used to examine microtubule dynamic instability and highlight future avenues and still open questions about this vital and fascinating activity.

I. Introduction: microtubule dynamics

In the cell, filamentous assemblies of proteins make up the cytoskeleton. There are three types of cytoskeletal filaments: microtubules, actin, and intermediate filaments.[1] Of these, microtubules are the stiffest,[2–6] and thus are similar to the "bones" of the cell. Yet, unlike the bones of your body, microtubules have an inherent ability to form and fall apart, a process called "dynamic instability".[7–9]

Microtubules have a number of indispensable biological functions: they are the tent-poles that support long extended structures of cells, they are the highways for the long-distance transport system, and they are the tension ropes and compression rods that push and pull chromosomes during cell division.[1] Correct microtubule organization is essential to the processes of cell division, neuronal cell development, differentiation, cellular maintenance, plant cell cellulose deposition, and ciliary beating in the lungs, kidneys, and intestines.[10–15] Failure to create the correct microtubule network in these processes results in cancer, birth defects, brain abnormalities, neuromuscular diseases, kidney disease, fragile plants, and cell death. Dynamic instability is a major mechanism for microtubule organization and rearrangement within cells.

Microtubules are hollow tubes assembled from globular protein subunits.[1] The minimal subunit is the tubulin dimer made from similar, yet distinct, alpha tubulin and beta tubulin subunits (Fig. 1A). Tubulin dimers assemble head to tail to form linear protofilaments. These protofilaments bind laterally to create a sheet that rolls into a hollow tube. Once a short tube is nucleated, filaments can grow by the addition of individual dimers to each exposed end. The differences between the alpha and beta subunits give the filament an overall physical polarity, where the two ends of the filament are different, one with beta tubulin exposed, the other with alpha tubulin exposed (Fig. 1A). The end with the beta tubulin exposed can assemble faster and is termed the "plus-end" of the microtubule. The end with the

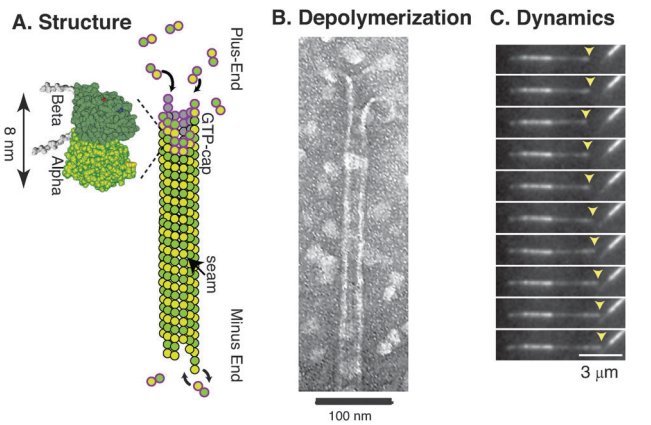

Fig. 1 Microtubule structure and dynamic instability. (A) Tubulin dimers are made from alpha (light green) and beta (dark green) tubulin monomers. Dimers bind head-to-tail longitudinally and side-to-side laterally to create a rhombic lattice that rolls into a hollow filament. Tubulin dimers with GTP in the E-site (violet outline) add to the plus and minus-ends to enable filament elongation. (B) Upon hydrolysis of GTP in the E-site, the dimers undergo a conformational change from straight to bent. During rapid depolymerization, the protofilaments bend back and curl inside-out into rings. (C) Microtubule dynamics can be directly imaged *in vitro* and in cells. In this example, we used TIRF microscopy to image single microtubules *in vitro*.

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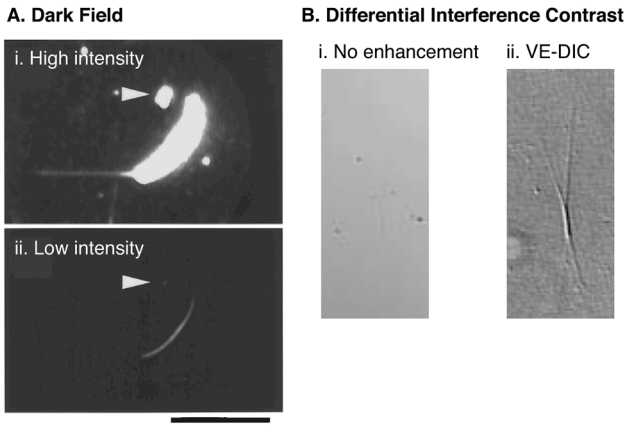

Fig. 2 Transmitted light imaging of microtubule dynamics. (A) Dark field imaging of a microtubule nucleated from an axoneme. Reprinted by permission from *J. Mol. Biol.*, Elsevier Publishing.[101] (i) High intensity light shows both the microtubule and the axoneme. (ii) Low intensity light shows only the axoneme. (B) Differential interference contrast microscopy of a microtubule nucleated from an axoneme. Reprinted by permission from IOSPress.[102] (i) Without video enhancement, only the axoneme is barely visible. (ii) With video enhancement, the microtubules are clearly visible. Scale bar is 10 μm.

alpha tubulin exposed is termed the ''minus-end,'' and it assembles slower.

In addition to growing, microtubules can also stochastically switch to a shrinking state, and then switch back to growing (Fig. 1). This cycling of growing and shrinking has been termed ''dynamic instability''. Dynamic instability is an inherent property of microtubules requiring active tubulin, guanosine triphosphate (GTP) as an energy source, and warm temperatures (20–37 °C).[8] Tubulin proteins are GTPases, and nucleotide binding is required for polymerization. GTP hydrolysis destabilizes the filament enabling shrinkage. Electron microscopy imaging has revealed the structural differences of the ends of microtubules during growth, rapid growth, shrinkage, and rapid shrinkage. Slow growing microtubules have blunt ends, but rapidly growing microtubules have tapered ends.[16,17] Microtubules typically shrink at a much faster rate than they grow, and the ends of shrinking microtubules have peeled back protofilaments that curl backward and break off into GDP-rings (Fig. 1B).[18]

Protofilaments curve backward into rings because GTP hydrolysis is accompanied by a bending of the dimer.[19,20] GDP tubulin in the microtubule lattice is being forced straight because it is docked into the lattice.[17,21,22] At the top of the microtubule, a cap of GTP-tubulin in straight conformations keeps the microtubule stable as it grows.[23] Loss of the GTP-cap is associated with the initiation of shrinkage, termed a ''catastrophe''.[7,24] Re-establishment of the GTP-cap results in a new growth phase, termed ''rescue'' (Fig. 1B).[25]

In cells, microtubule-associated proteins (MAPs) and enzymes regulate dynamic instability to spatio-temporally control microtubule network organization. Together, microtubule filaments with MAPs and enzymes create a responsive network enabling rapid dynamic reorganization that is sensitive to chemical and mechanical cues.

In this review, we will overview recent novel techniques utilized to examine microtubule dynamic instability both inside cells and reconstituted *in vitro*. In cells, microtubule dynamics have been visualized using fluorescence techniques, greatly enhanced by the use of spinning disc confocal microscopy, yet there are limitations to what can be visualized within the cell – specifically where microtubules can be visualized. *In vitro*, direct imaging was established using dark field microscopy and differential interference microscopy. Recent fluorescence techniques have been employed, empowered by the advent of total internal reflection fluorescence (TIRF) microscopy (Fig. 1C). Finally, a handful of technically challenging, yet insightful force-microscopy measurements have been made.

II. Transmitted light microscopy techniques, results, and limitations

Dynamic instability is an inherent property of microtubules. Mitchison and Kirschner[7] revealed the first evidence of microtubule dynamics in 1984 using electron microscopy and immunofluorescence imaging of microtubules at fixed time points. Although microtubule growth and shrinkage was proposed in this groundbreaking paper, dynamic instability was not directly observed until 1986 when Hotani's group first used dark field microscopy to observe individual filaments growing and shrinking (Fig. 2A).[8] Following that work, others employed video-enhanced differential interference contrast (VE-DIC) microscopy that implemented frame averaging to visualize individual microtubules.[9,25] Microtubules are the smallest objects visible by DIC microscopy (25 nm in cross-section), and high intensity lamp light combined with frame averaging is essential to overcome the noise due to low signal and the thermal fluctuations of the individual filaments.

Direct imaging methods were essential to proving that microtubules have the innate ability to exchange subunits from their tips through dynamic instability as opposed to exchange from the entire filament or treadmilling. Further, these techniques were implemented in cells and in extracts[9,26,27] to show that microtubules exhibit dynamic instability in cellular or cell-derived environments. Follow up experiments of dynamic instability were used to show the effects of drugs, such as vinblastine and Taxol,[28–30] and microtubule-associated proteins, such as tau and MAP2.[26]

Although these methods reveal microtubule dynamics, they have several drawbacks. For dark field imaging, the surface of the cover glass must be exceptionally clean since any protein aggregates on the glass surface will also scatter the light and can make visualization difficult. For VE-DIC, while the electronics to control the contrast, gain, and averaging have become more sophisticated and easier to implement with modern computers and software, the optics for DIC are still problematic because perfect Kohler illumination is needed with a very bright light source. Mercury bulbs were originally employed, since incandescent bulbs are not powerful enough, and modern labs use white light LEDs.

With both dark field and DIC imaging, it can be difficult to distinguish single filaments from small bundles. Further, the effect of DIC is to enhance objects with an altered index of refraction so that the filaments look like a hill or valley with a dark and light side (Fig. 2B). This enhancement has a particular

angle that works best, and only microtubules laying parallel to that direction are visualized. Microtubules at perpendicular angles are invisible, and those at intermediate angles are difficult to observe. Despite these drawbacks, dark field and DIC both offer transmitted light imaging that is less likely to cause photodamage to microtubules during visualization, and they are still implemented in many laboratories today.

III. Fluorescence microscopy

Over the past 30 years, there has been a revolution in fluorescence imaging techniques that have enabled the visualization of microtubules and their dynamics with previously unachievable resolution both *in vitro* and in cells. With fluorescence imaging there are a number of options for visualizing microtubule dynamics including *epi*-fluorescence,[9,31–39] scanning and spinning disc confocal microscopy,[40–47] and total internal reflection fluorescence (TIRF) microscopy.[48–50] Further, dynamic processes can also be resolved using photobleaching,[33,36,38,51] photoactivation,[52] or speckle microscopy experiments.[53]

All fluorescence techniques require the incorporation of fluorescent dyes into the biological structure (for a recent review see ref. 54). For microtubules, this can be achieved employing a variety of methods. Purified tubulin can be directly labeled with small, organic fluorophores using standard procedures.[31] Such fluorescent tubulin can be used *in vitro* directly or in cells through micro-injection.[35] Unfortunately, micro-injection is difficult and slow, enabling only one to a few cells to be visualized. Alternatively, for live cell imaging, tubulin has been labeled with a protein-based, genetically encoded fluorophore, such as green fluorescent protein (GFP)[55–57] or photoactivatable GFP (PA-GFP)[58] leading to numerous important discoveries about microtubule localization and dynamics in cells (Fig. 3).

In cells

We will first describe the employment of fluorescence imaging to cellular systems. Standard *epi*-fluorescence imaging can be used to directly observe single microtubules at the cortex of cells where they are well separated.[9,35] *epi*-Fluorescence can be used in combination with photobleaching or photoactivation to resolve the turn-over of microtubules or microtubule-associated proteins (MAPs) in thicker portions of the cell.[39] Several important facts were gleaned from *epi*-fluorescence imaging including: microtubules form anew from spindle poles;[36,37] microtubules are unstable to cold[9,31] as well as the drugs colchicine and nocodazole.[31] Unfortunately, *epi*-fluorescence has limitations such as being relatively dim when using arc-lamps. Higher intensity lamps and lasers can cause photodamage and toxicity to cells. Further, *epi*-fluorescence has low signal-to-noise due to background from the bulk of the cell and low spatial resolution due to the diffraction limit of light, limiting observations to the very edges of cells.

In order to increase the signal to noise, confocal microscopy imaging gained popularity. In confocal imaging, the background noise is reduced because the emitted fluorescence is passed through a pinhole that blocks light from other focal planes.

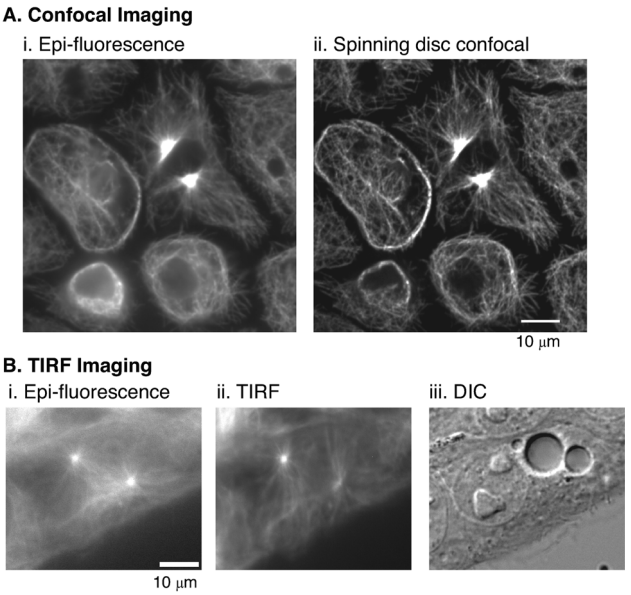

Fig. 3 *epi*-Fluorescence, confocal, and TIRF imaging of fluorescent tubulin in cells. (A) Comparison of (i) *epi*-fluorescence and (ii) spinning disc confocal imaging of S2 cells expressing GFP-tubulin. Courtesy of Thomas Maresca. (B) Comparison of (i) *epi*-fluorescence, (ii) TIRF, and (iii) DIC imaging of LLCPK2 cells expressing GFP-tubulin. Courtesy of Patricia Wadsworth.

The signal was further improved since confocal microscopy uses laser illumination, providing more excitation photons to and receiving more emission photons from the sample. Scanning confocal employs photodetectors, which measure the intensity from a single region, instead of using a camera or your eye for wide-field imaging. A trade-off occurs with the confocal technique, because increased laser power into a small region can lead to more photobleaching and photodamage of the sample, severely limiting the total duration of imaging achievable. Currently, spinning disc confocal microscopy is the method of choice for most cell biologists studying microtubules. In a spinning disc microscope, the pinholes are multiplexed and rotating to enable wide-field imaging to a camera. The laser power is spread throughout the imaging area, reducing photobleaching and photodamage, while maintaining the ability to block out of focus light.

For both *epi*-fluorescence and confocal imaging, two-dimensional images can be taken at varying focal planes in the z direction (depth in the sample) to create slices through the sample. The fluorescence at the focal plane is convoluted with the fluorescence gathered from other planes due to the z-dependence of the point-spread function of the objective. Using algorithms to deconvolve the fluorescence from out of focal plane sources, data can be digitally cleaned up to give clearer images in each plane (for a recent review, see ref. 59). Although the pinholes employed in a confocal microscope can remove much of the background, deconvolution is widely employed to enhance images of live cells taken as three-dimensional stacks over time (truly four-dimensional data sets). Further, the three-dimensional data can enable tracking of microtubule ends in three-dimensions. Many important facts were determined from confocal microscopy imaging including: Taxol, a small molecule chemotherapeutic,

stabilizes microtubules against cold and destabilizing drugs;[41] microtubule dynamic instability parameters differ depending on cell type, cellular process, such as division or migration, and intracellular location;[43,46,60] combined with photobleaching or activation, microtubules were observed to treadmill in kinetochore fibers[45] and plant cells.[61]

Despite these advances, fluorescence techniques are still limited by spatial resolution in *x*, *y*, and *z*. The resolving power within a single *z*-slice is limited by diffraction to half the wavelength of light. Thus, two microtubules within 200 nm of each other will appear as a single filament. In regions of high microtubule density including the mitotic spindle, the axon, and the cilia, imaging individual microtubule dynamics is next to impossible. *z*-Slicing with deconvolution makes the *z*-resolution better, but the limit is still 300–400 nm.[62] Given that a single microtubule is over 10× smaller than the resolution in any direction, these techniques are still missing information.

In order to accomplish better resolution and enable visualization of single microtubule dynamics in dense regions of the cell, single molecule techniques with implementation of high-resolution tracking have proven successful. One such technique widely used for actin and microtubules is fluorescence speckle microscopy. In this technique, only a small number of tubulin dimers are labeled, either by injecting or expressing very low levels of fluorescent tubulin. Fluorescent speckles represent small clusters of stochastically incorporated tubulin dimers. By imaging speckled microtubules *via* confocal in three-dimensions over time, the spatio-temporal path of a cluster of tubulin in a single microtubule can be tracked. Birth and death of the cluster represents polymerization and depolymerization, respectively. Such techniques have been employed to examine the dynamics of microtubules of the meiotic spindle and cell cortex.[63–65]

In vitro

In vitro studies of microtubule dynamics relied on the transmitted light methods described above for many years, despite their disadvantages. In 2007, many of these limitations were removed when the first fluorescence assays to image dynamic microtubules were reported by several groups. These methods, which were informed by fluorescence methods determined by the Pollard lab to visualize actin dynamics,[66] use TIRF microscopy to eliminate out of focus fluorescence from tubulin dimers in solution. In TIRF imaging, a collimated laser beam hits the cover glass at a glancing angle to totally internally reflect at the glass–liquid interface. Although the majority of the laser power will reflect, a fraction of the photons will tunnel through the boundary into the water and decay exponentially with depth in the *z*-direction into the sample. These decaying photons make up an evanescent wave that enables illumination of the sample within 100–200 nm only at the surface. TIRF microscopy uses the evanescent wave to illuminate fluorescent molecules at the cover glass surface only and ultimately removes fluorescence from the bulk solution. To image microtubule dynamics, microtubule seeds are adhered to the cover glass surface and free tubulin dimers can add from the bulk above the surface to cause growth. Without TIRF imaging, the microtubules are completely obscured by out of plane fluorescence caused by the free tubulin dimers in solution.

In order for TIRF imaging to enable visualization of surface-bound microtubules, free tubulin dimers must be prevented from binding to the glass coverslip and obscuring the filaments. As with much of single molecule imaging, surface chemistry is key, and two different schemes are described in the literature: (1) one using a block-copolymer, pluronic F127, to block the surface,[67–71] and (2) one using poly-ethylene glycol (PEG) to block the surface.[72–74] Both techniques require the adhering of small, stabilized microtubules, called ''seeds'' used to nucleate the growth of the microtubules. Sometimes, as the filament grows longer, the end of the microtubule diffuses and fluctuates; filaments can fluctuate out of the evanescent field of TIRF illumination. In order to decrease perpendicular diffusion, large, steric polymers, such as methylcellulose can be added to increase the viscosity and dampen fluctuations.[72]

Fluorescence techniques have a number of advantages over traditional transmitted light techniques. Better temporal resolution can be achieved because there is no need to average frames to resolve microtubules. Further, the associated proteins and enzymes that affect dynamics can be simultaneously visualized in separate fluorescence colors to reveal the time and location of MAPs during microtubule dynamics further elucidating the mechanism of action for these regulators.

Several important results have been determined using fluorescence techniques to examine microtubule dynamics *in vitro*. The tracking of microtubule ends by EB proteins was recapitulated using two-color imaging,[71,72] and different types of EB proteins have been shown to have differential effects on microtubule dynamics.[75] Other tip-tracking proteins were shown to require EB to tip-track, such as CLIP170, CLASP, and dynactin p150.[70] XMAP215 was shown to be a microtubule growth-regulating protein that can plus-tip track in the absence of EB proteins.[67,68,76]

Microtubule depolymerizers, such as kinesin-8 and kinesin-13, were observed to act at the ends of depolymerizing microtubules to catalyze depolymerization in an ATP-dependent fashion. Further, these depolymerases could either diffuse or walk to the ends of microtubules, depending on the type. Competition between growth promoters, such as the EB proteins, and depolymerases, such as MCAK can lead to enhanced dynamics of the filament.[77] Patronin, a minus-end stabilizing protein that causes short spindles when knocked down, can also compete with MCAK for microtubule minus-ends.[78] TIRF imaging was also used to show that depolymerizing microtubules could collect yeast kinetochore-associated proteins, DAM complex proteins, during depolymerization events, which led to the first direct evidence that depolymerizing microtubules pull apart chromosomes during mitosis.[79,80]

From the variety of systems and mechanisms examined in the past 6 years, it is clear that fluorescence techniques for dissecting how microtubule dynamics are regulated are just taking off. These techniques are only limited by technical challenges of obtaining protein and labeling, which have numerous strategies. Further, re-examination of established systems, such as dynamic instability

in the presence of tau, using multi-color fluorescence imaging are likely to shed new light on the mechanisms by which these proteins can alter the dynamics of microtubules. This technique is also opening new horizons for increasingly complex systems of regulators – specifically elucidating the interplay between positive regulators that promote growth and negative regulators that promote disassembly. Through systematic reconstitution experiments, we may come closer to understanding how microtubules in the cell are actively regulated spatio-temporally.

IV. Force microscopy

Cellular microtubules are force generators. They push and pull chromosomes to align them in metaphase and pull them apart in anaphase. They push into the actin meshwork at the cell cortex and bump into the cell membrane. They are propelled by axonemal dyneins in cilia and flagella to create fluid flow around tissues in the gut and kidneys. Given their stiff mechanical nature and clear role in force generation, it is natural that a number of studies have recapitulated these cellular roles and measured the forces generated by microtubules during growth and shrinkage.

In order to measure the forces generated by growing microtubules, several studies have employed an optical trap coupled to a dynamic microtubule through a dielectric bead.[81–86] Microtubules are typically grown into a solid barrier while being held by the optical trap on the other end. The optical trap can be held at a constant position relative to the barrier as the microtubule grows into the barrier. As the microtubule interacts with the barrier, the bead will deflect from the trap center. This deflection can be precisely measured by a quadrant photodiode in a conjugate plane to the back focal plane. By calibrating the trap, the restoring force per distance can directly report on the force generated by the growing microtubule (Fig. 4). In order to enhance the interaction of the microtubule tip with the barrier, a second keyhole trap can be employed to position the growing microtubule, and the barrier can be shaped to inhibit the microtubule from slipping.[82,83]

Higher resolution force measurements can be made using the optical trap in force-clamp mode. Force clamping sets the force constant by moving the trap to keep the bead in the same position relative to the trap center. Such adjustments are very fast, since the quadrant photodiode can read and automatically feedback to acoustic optical modulator (AOM) to steer the trapping laser at kiloHertz rates. Force clamping has the advantage of constant force and constant contact of the microtubule with the barrier or substrate during growth and shrinkage.

A number of important and interesting discoveries have been made using these techniques, which helped propel the idea that filamentous growth of microtubules and actin can act by a thermal ratchet mechanism to create force during growth.[81–83,86,87] This thermal ratchet can create pico-Newtons of force, a biologically relevant magnitude of force, comparable to the forces created by motor proteins, such as kinesin-1.[88,89] The Dogterom group revealed the first force-velocity dependence of microtubule growth, showing that growth rates slow down as the forces increase, and that there is a stall force, or maximum force, that a growing microtubule can exert before it stops growing.[87] They also found that high forces resulted in a drop in the elongation time of a filament, caused by dimer starvation

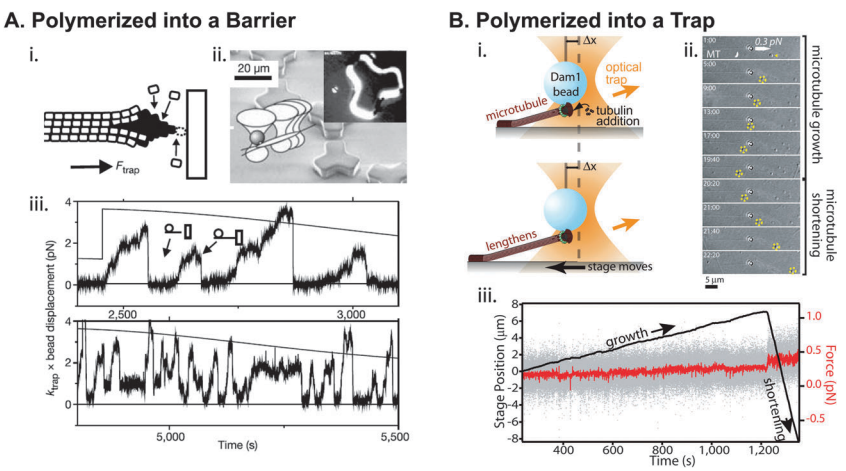

Fig. 4 Force microscopy methods to measure microtubule dynamics *in vitro*. (A) Microtubules polymerized into a barrier. (i) Tubulin dimers add on to a microtubule that is grown into a barrier. Microtubule polymerization results in a force generated against the barrier, as the opposite microtubule end is held in a trap. (ii) Experimental set-up showing a microtubule being grown into a barrier. The microtubule minus end is attached to a dielectric bead that is held in a trap. Shallow keyhole traps along the length of the microtubule prevent the microtubule from sliding against or over the barrier. Inset shows DIC image of experimental set-up. (iii) Example traces of bead displacement over time in the absence of (top) and presence of XMAP215 (bottom). Reprinted by permission from Macmillan Publishers Ltd: *Nature*.[82] (B) Microtubules polymerized into a trap. (i) A dielectric bead coated with the kinetochore protein, Dam1, that binds microtubules is held in a trap. A microtubule is adhered to the chamber surface at its minus end and the plus end interacts with the Dam1-coated bead. In these experiments, the stage is moved in response to microtubule growth and shrinkage to maintain a constant force on the microtubule plus end. (ii) DIC image showing a Dam1-coated bead coupled to microtubule growth and shrinkage. Here the stage moves in response to changes in microtubule length, keeping a constant force on the dynamic microtubule. Stage motion is observed by following an object bound to the surface, initially identified here with yellow arrowhead. (iii) Example trace showing stage position (black) and force (gray is instantaneous trace, red is average trace) over time. Reprinted with permission from Elsevier.[103]

at the end of the filament.[90] More recent work on the force generation during dynamic instability of multi-microtubule bundles showed that the forces of individual microtubules are additive and that high loads can lead to induced catastrophe of the entire bundle.[84]

Improvements to the technique have enabled high resolution tracking of growing and shrinking microtubule ends. The Dogterom group demonstrated that, in the presence of XMAP215, multiple tubulin dimers bind at the growing ends of microtubules and multiple dimers were simultaneously removed during shrinkage.[82] The steps of addition and subtraction were much larger in the presence of XMAP215, implying that XMAP215 pre-assembles small protofilaments that bind to ends or induce cooperative binding of dimers.[82] The Hunt lab showed that there is a high degree of rapid, nanometer-scale activity for growth and shrinkage on the order of 1–2 dimers in length.[83] Theoretical models showed that the complex nature of microtubules as an actual two-dimensional filament results in a high rate of incorporation and loss of dimers in the kiloHertz range, which was directly visualized first by these high-resolution force studies.[86]

Cellular microtubules not only push on objects, but they also pull objects through depolymerization. This activity is especially important in cell division where the chromosomes are pulled apart during anaphase to create the two new nuclei of the daughter cells. *In vitro* experiments have reconstituted this activity using a minimal model of Dam1 kinetochore protein coupled to a dielectric bead with tension applied to a depolymerizing microtubule end.[91,92] The system uses a stabilized seed of microtubule tightly coupled to the cover glass, and the free, polymerizing end coupled to a bead in an optical trap *via* Dam1 (Fig. 4B). By applying compression to the system, they have shown that the Dam1 ring can slide back along the microtubule. Dam1 sliding is stopped by the growing microtubule end and the portion of the filament attached to the cover glass. When tension is applied, the microtubule actually grows for longer at the same speed, implying that tension can control microtubule growth in mitosis.[93] Similar results were observed for kinetochore particles that can also bind to and couple microtubule ends to trapped beads.[94] This study showed that kinetochore particles have a slip-bond interaction with microtubule ends, much like a finger trap toy. Such slip-bonds could stabilize correct bi-oriented attachments of kinetochore microtubules from opposite sides and destabilize syntelic attachments of microtubules to the same pole.[94] On the other side of the spindle, astral microtubules work to push and pull the spindle from the sides to keep it centrally positioned in the dividing cell. Work from the Dogterom group showed that cytoplasmic dynein bound to a wall-barrier inhibited growth and triggered catastrophe of microtubules grown into the barrier. Interestingly, the dynein stayed attached to the shrinking microtubule and was able to create a pulling force.[95] Using this mechanism, cortical dynein acts to position the spindle in the bud neck of dividing yeast cells using both the pushing forces of growing microtubules and the pulling forces of shrinking microtubules.

Overall, these techniques are very useful for measuring the force-dependent aspects of microtubule dynamic instability. They have the advantages of achieving high spatial and temporal resolution. Force microscopy can apply and measure forces in both the growing and shrinking directions of the microtubule. Despite these advantages, progress is slow because the technique is difficult, and only a few research groups have been able to perform these experiments. Finally, these methods are only available to measurements *in vitro* because they have not been optimized to enable force measurements in living cells or tissues.

V. Future directions and open questions

Despite the long history of studying microtubules and their dynamic instability, there are still important open questions. One direction for future studies would be to increase the complexity of current studies to include both stabilizers and destabilizers *in vitro* to begin to dissect how each can tune the dynamics of microtubules. Some recent work has begun to investigate these questions, examining depolymerizing kinesins with end-binding proteins using TIRF imaging,[77] but there are many more variations that should be explored.

For cellular studies, there are still many open questions about the dynamics of microtubules in dense regions where individual filaments cannot be discerned. Work using photoactivation or photobleaching has revealed the motion of many microtubules, such as in the mitotic spindle, but these report bulk dynamics and are not as informative as individual filament dynamics. Recent advances in speckle microscopy combined with spinning disc confocal and high-resolution tracking may be the answer for revealing the activities of microtubules in dense regions. Super-resolution fluorescence techniques, such as Photoactivation Localization Microscopy (PALM)[96–98] or Stochastic Optical Reconstruction Microscopy (STORM)[99,100] have yet to resolve microtubule dynamics due to current limitations in labeling and the time required to collect enough frames for reconstruction. In PALM, the label is a photoactivatable or photoswitchable genetically encoded fluorophore. Because the same fluorophore cannot be turned back on once it is photobleached, this makes repeated images of microtubules to measure dynamics difficult. In STORM, the technique uses photoconvertible dyes that can turn on and off repeatedly, which could allow for microtubule dynamics to be monitored. Future advances of these techniques will likely open new avenues of microtubule dynamics in crowded regions of cells that are currently inaccessible.

Finally, although microtubules undergo dynamic instability in many contexts, there is another mode for microtubule dynamics: treadmilling. Unlike dynamic instability where microtubules grow from two ends of a stable nucleation site, like a seed, in treadmilling, microtubules grow at the plus end and depolymerize at the minus-end. Treadmilling microtubules are not rare. In the mitotic spindle, kinetochore fibers connecting the spindle poles to kinetochores are undergoing ''flux.'' In flux, microtubules grow at the plus-end at the kinetochore, and they depolymerize at their minus-ends at the spindle pole. This behavior, revealed by photobleaching and photoactivation studies, indicates that the two ends of kinetochore microtubules

are not stable or embedded, but free to add and remove subunits. This activity enables alignment of the chromosomes in metaphase and ultimately the shrinking of kinetochore fibers pull apart chromosomes in anaphase.

For the spindle, one might expect that a gradient of microtubule regulators could create a treadmilling effect. For instance, if microtubule stabilizing and polymerizing agents were located near the kinetochore, but destabilizers were at the poles, this could set up a situation where the microtubules would grow at the kinetochores and shrink at the poles. In plant cells, treadmilling microtubules are prominent in the cortical microtubule array. It is difficult to imagine how a gradient of stabilizing and destabilizing agents could be orchestrated, since growing plus-ends are immediately next to shrinking minus-ends. The plant cell system gives us some hope that a stable, treadmilling system of microtubules could be established *in vitro* with wholly reconstituted agents. This is a clear open problem in the understanding of microtubule dynamics, and *in vitro* reconstitution will likely reveal important information about controlling the two ends of a single filament separately.

In conclusion, microtubule dynamic instability has been studied using a variety of microscopy methods. We know that the microtubule growth and shrinkage events can be coupled to force generation, and this is likely used in cells. Finally, *in vitro* reconstitution not only reveals that dynamic instability is an inherent activity of purified tubulin, but that it can also be modulated through associated proteins and enzymes. Despite the past three decades of work, there are still many open questions about how this intriguing and beautiful exploit functions, weaving between the disciplines of biology, chemistry, and physics.

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