

PERSPECTIVE

Understanding microtubule dynamics: The synergy of technology, theory, and experiment

J. Richard McIntosh¹ 

This perspective traces the ways in which information obtained with different technologies for sample preparation, imaging, and image analysis has interacted with biochemical information and theories of protein polymerization to form an ever deeper understanding of microtubule dynamics. Essential to this progress has been a more accurate knowledge of microtubule structure, especially at the polymer's tip, where subunits are commonly added and removed. I follow the contributions of light microscopy, i.e., bright-field, polarization, differential interference contrast, and dark-field optics, then fluorescence microscopy with either immunolabeling or fluorescent labeling of tubulin itself. I also trace electron microscopy from its use on thin sections of fixed materials through negative staining of isolated polymers, then on to imaging of fast-frozen samples with cryo-electron microscopy and tomography. The results from these observations are combined with data from biochemistry and x-ray crystallography, image analysis, and atomistic molecular dynamic modeling to build what is likely to be an accurate overview of how microtubules polymerize.

Introduction

Why scientists care about microtubule dynamics

The significance of microtubule (MT) polymerization for cells is demonstrated by the wide range of roles these polymers play in vivo, a subject that has often been reviewed, e.g., [Gudimchuk and McIntosh \(2021\)](#). The growth of MTs in vivo is regulated in part by the site, orientation, and timing of polymerization initiation and in part by factors that control the velocity of polymerization and the frequency of depolymerization. The latter qualities are regulated, at least in part, by MT-associated proteins, particularly those that interact with MT tips, such as the polymerization catalyst CKAP2 (XMAP215) ([Brouhard et al., 2008](#)), and other proteins that speed polymer growth, like doublecortin ([Fourniol et al., 2010](#); [Bechstedt and Brouhard, 2012](#)), CLASPs ([Girão et al., 2020](#)), and the “end-binding proteins” (e.g., EB1) ([Maurer et al., 2012](#)). A solid understanding of MT control by cells will depend in part on understanding the structures that serve as intermediates in the tubulin assembly process. This essay describes technologies, theories, and observations that have led to our current understanding of these matters. To understand the complexity of MT dynamics, the reader is referred to reviews on the physics of the subject (e.g., [Grishchuk et al., 2012](#)).

Historical background

MT dynamics were studied before the fibers in mitotic spindles, first visualized by bright-field light microscopy in the 19th century, had been identified as MT bundles. Measurement of

spindle birefringence in live cells by polarization microscopy ([Inoue, 1953](#)) characterized the lability of these fibers to cold, high pressure, and certain drugs ([Inoué et al., 1975](#)). The existence of hollow fibers in cells was first established by electron microscopy (EM) of cilia ([Fawcett and Porter, 1954](#)). Subsequent studies of mitotic spindles indicated that slender tubular fibers were the origin of spindle birefringence ([Harris, 1965](#)). The prevalence of MTs in eukaryotic cells was established following the widespread use of glutaraldehyde as a fixative to prepare cells for EM ([Sabatini et al., 1963](#)). Their likely importance as factors in cell structure, morphogenesis, and intracellular motions was reviewed by [Porter \(1966\)](#). Observations and experiments on cells showed that MT formation, position, orientation, and stability are all dependent upon the controlled polymerization of soluble subunits ([Tilney and Porter, 1967](#)). The molecule that played this role was discovered by identifying a protein that bound colchicine, a well-known inhibitor of mitotic spindle formation ([Borisy and Taylor, 1967](#); [Wilson and Friedkin, 1967](#)). This protein was named tubulin ([Mohri, 1968](#)), shown to bind GTP ([Weisenberg et al., 1968](#)) and to exist as a dimer of two similar but not identical proteins, α - and β -tubulin ([Feit et al., 1971](#)).

Our growing understanding of MT dynamics

Condensation polymerization 1: Paths for tubulin polymerization

Initial ideas about tubulin polymerization were heavily influenced by a theory of “condensation polymerization” ([Oosawa](#)

¹Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO, USA.

Correspondence to J. Richard McIntosh: richard.mcintosh@colorado.edu.

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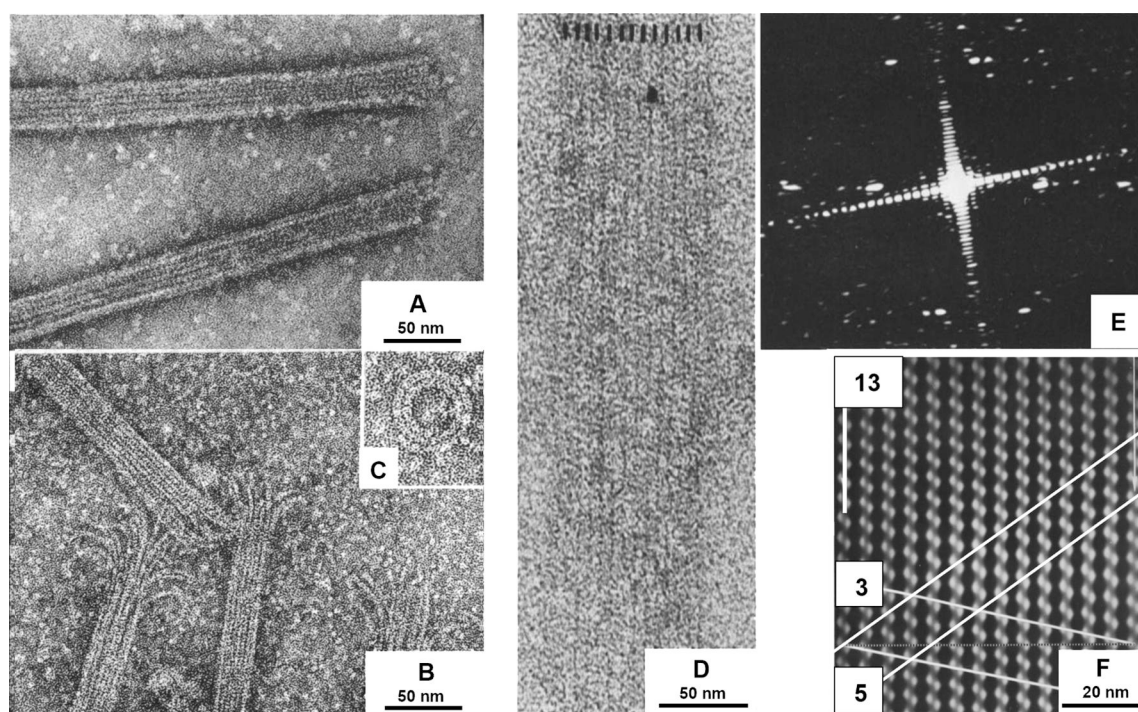


Figure 1. **Negative staining of MTs formed in vitro.** (A–F) 1A, growing polymers. 1B, MTs after minutes of depolymerization, showing frayed MT ends. Fig. 1 C: An oligomer formed during depolymerization. 1D, 13 protofilaments from a flattened MT. 1E, Optical diffraction from Fig. 1 D showing lattice parameters. 1F, Portion of Fig. 1 D, filtered and averaged to improve image signal-to-noise. Tubulins are now clearly seen in an array that resembles a diagram of the tubulin lattice, made by unfolding the cylinder and laying it flat. Each bilobed structure is one tubulin monomer. White lines labeled 3, 5, and 13 indicate lines of helical symmetry. A–C from Kirschner et al. (1974), D–F from Erickson (1974b).

and Kasai, 1962). In this view, proteins polymerize by a two-step process: initiation and then elongation through the iterative addition of subunits at the polymer's tip(s). Shortly after the discovery of methods to polymerize tubulin in vitro (Weisenberg, 1972), the structure of the MT lattice was determined by EM of samples prepared by negative staining (Amos and Klug, 1974; Erickson, 1974b). The structures of both growing and shrinking MT ends were also described with this method (Kirschner et al., 1974). Growing MTs were blunt, but shortening polymers appeared frayed, as if strands of tubulin peeled back from the MT tip, occasionally forming circular oligomers as part of the depolymerization process (Fig. 1, A–C). The structures of growing tips were consistent with condensation polymerization, but the disassembly process appeared quite different. Lateral bonds between tubulins in the MT wall severed first, forming strands of tubulin, called protofilaments, which then shortened by breakage of longitudinal bonds. A caveat with all this work was, however, that negative staining involves mixing the structures of interest with a solution of heavy metal, such as uranyl acetate, then drying the sample onto electron-transparent films. In the moments before the heavy metal forms a solid that stabilizes and outlines the protein assembly, solution conditions are far from physiological, so structures might be altered. (Strengths and limitations of each method discussed in this paper are summarized in the Appendix).

The MT lattice as seen by negative staining EM showed the relative position of proteins in the polymer's wall (Erickson, 1974a), but for years, the space resolution was not sufficient to

distinguish α - from β -tubulin (Fig. 1 F). The images and diffraction data could be interpreted as a 3-start left-handed helix of bilobed monomers, a 5-start right-handed helix of dimers, or a 13-start array of dimers in which the strands are straight. Since tubulin formed dimers in solution, with negligible amounts of monomer detected, the results were interpreted to suggest that condensation polymerization of tubulin occurred by the addition of tubulin dimers into “cozy corners” at the MT end, elongating either the 5-start or 13-start helices.

Contemporaneous with these studies, biochemists discovered that tubulin dimers bound two molecules of GTP, only one of which was exchangeable; the exchangeable GTP was required for polymerization (Weisenberg et al., 1968; Kobayashi, 1975), but it was hydrolyzed during the process. Thus, MTs were built largely from “GDP-tubulin,” a form of the protein that polymerizes only under nonphysiological conditions. It was also found that growth occurred at different rates at the two polymer ends; the name “plus” was given to the fast-growing end, and “minus” to the other (Allen and Borisy, 1974). Subsequent labeling studies showed that β -tubulin was exposed at the plus MT tip (Mitchison, 1993). Moreover, at polymerization steady state, MTs could “treadmill,” meaning that the polymers would grow at one end and shorten at the other (Margolis et al., 1978). This striking behavior is not a violation of the second law of thermodynamics, because the energy of GTP hydrolysis is released at some time during the tubulin polymerization process. This energy enables different polymerization properties at the MT's two ends and also endows MT dynamics with the ability to do

mechanical work. For a while, treadmilling was thought to contribute to the roles of MTs in cells; indeed, it formed the basis for a successful model for MT function in the mitotic spindle (Margolis and Wilson, 1981). Subsequent work has shown, however, that MT-binding proteins of many kinds, especially motor enzymes, are more likely to be the agents that affect most MT-dependent cytoplasmic motions.

Studies of MT growth rates following initiation from isolated centrosomes suggested that the MT plus end lay distal to the site of initiation and that minus ends were inactive (Bergen et al., 1980). This polarity of MT orientation was confirmed by an assay that used nonphysiological conditions to polymerize tubulin onto the walls of pre-existing MTs, forming a partial wall whose image in cross section was one or more “hooks.” The direction of hook curvature allowed an assignment of MT orientation (Heidemann and McIntosh, 1980). For many years, it seemed that the MT minus end was inactive for polymerization in vivo, so the field concentrated on plus-end behavior. Subsequent work has shown, however, that MTs in cells can depolymerize at their minus ends, even when associated with a MT-organizing center (Mitchison, 1989). This important area of current work will not be treated in this discussion of plus-end dynamics.

Condensation polymerization 2: Initiation of tubulin polymerization

The second part of condensation polymerization theory is polymer initiation. Work in vitro demonstrated that pure tubulin could initiate polymerization spontaneously, albeit with a lag. This lag was interpreted as the time needed to form oligomers of sufficient size to start end-on tubulin addition (Carlier et al., 1989). MTs so formed contained predominantly 14 protofilaments, but some had 13 (the number most commonly found in cells [Tilney et al., 1973]), and some had 15, with other numbers occurring occasionally. This polymorphism was consistent with the idea that stochastic assembly of dimers formed oligomers large enough to initiate elongation, but the process was imprecise. Structures formed during the early stages of polymerization in vitro were visualized with a double-spray, negative staining method, which immobilized protein assemblies very quickly. Images taken early during in vitro assembly showed many nontubular oligomers of tubulin (Kirschner et al., 1975). Measure of the relative number of different structures over time after initiation of polymerization suggested a progression that included protofilament clusters of many shapes, all of which formed before MTs. However, the relevance of these structures to MT initiation cells was not clear. Which structures were true assembly intermediates, and which were semi-stable branches of the in vitro assembly process could not be determined. Moreover, in vivo, MTs commonly emerge from specialized cytoplasmic objects, such as the “centrosome,” a region near the center of many animal cells that contains a pair of centrioles surrounded by a halo of amorphous material. Exploration by EM of MT arrangements in diverse cell types led to the realization that there was a variety of cytoplasmic objects that could initiate MT growth, including places on the nuclear envelope. All such places were called MT-organizing centers (Porter, 1966).

The molecular mechanism of MT initiation at organizing centers in cells was identified by rigorous use of genetics.

Analysis of mutants in the fungus *Aspergillus nidulans* that failed to form mitotic spindles identified a gene that encoded a new form of tubulin, so-called gamma-tubulin (Oakley and Oakley, 1989). The protein product of this gene was essential for MT initiation in these cells, and homologous proteins were soon identified in essentially all eukaryotic cells. Biochemical, genetic, and structural work showed that γ -tubulin exists predominantly in a conical protein complex called the gamma-tubulin ring complex whose wide end is the right shape to serve as an efficient seed for MT polymerization (Moritz and Agard, 2001). Control of MT nucleation in vivo is now largely studied by identifying the factors that position, orient, and activate γ -tubulin complexes (Aldaz et al., 2005; Liu et al., 2021). This knowledge about MT initiation in vivo is a beautiful example of the power of genetic studies to identify important components of cellular processes that depend on proteins of too low abundance to be evident with normal biochemistry. More recent work has identified another cellular component that contributes to control of MT initiation, the “augmin” complex (Goshima et al., 2008). This protein oligomer binds the γ -tubulin ring complex to the wall of existing MTs, initiating a new MT from an old one, forming what looks like a branch. These MT-associated MTs play an important role in the formation of large mitotic spindles (Travis et al., 2022), but not in the small spindles of fungi.

MT dynamics is not just condensation polymerization

Electron cryo-electron microscopy confirmed differences between the tips of growing and shortening MT

MT tip structures seen by negative staining were confirmed by cryo-electron microscopy (cryo-EM) of rapidly frozen MTs (Mandelkow et al., 1991). In this study, samples were made by “plunge freezing”—a process that immobilizes biological structures within milliseconds and leaves them embedded in vitreous ice (Dubochet, 2007). Such “cryo” images avoided the problems of negative staining and have been accepted by the field as valid representations of the MT tip structure. The images did not support the idea of growth along 5-start helices; MT tips did not show the predicted fivefold steps (Fig. 2 A). Some protofilaments extended short distances beyond others, suggesting that tubulin could add at the tip of any protofilament. The cryo-EM images of depolymerizing MTs appeared much like those seen by negative staining (Fig. 2 B); MT depolymerization proceeded by first breaking lateral bonds, allowing protofilaments to splay out, followed by breaking longitudinal bonds, leading to dissociation of tubulin dimers and oligomers.

Cryo-EM revealed an additional aspect of MT structure, thanks to the fact that some kinesin-like motors bind largely to β -tubulin (Song and Mandelkow, 1993). Samples whose surfaces were saturated with these motors showed that MTs from both neurons and flagella are built with a lattice in which tubulins along the MT’s 3-start helix are all of one kind: either α or β . This result was confirmed by cryo-EM of cytoplasmic MTs in an epithelial cell (McIntosh et al., 2009), suggesting that the structure is universal. It is a surprising structure, because MTs with this lattice and 13 protofilaments are not helically symmetric. They possess a “seam” in which one pair of neighboring

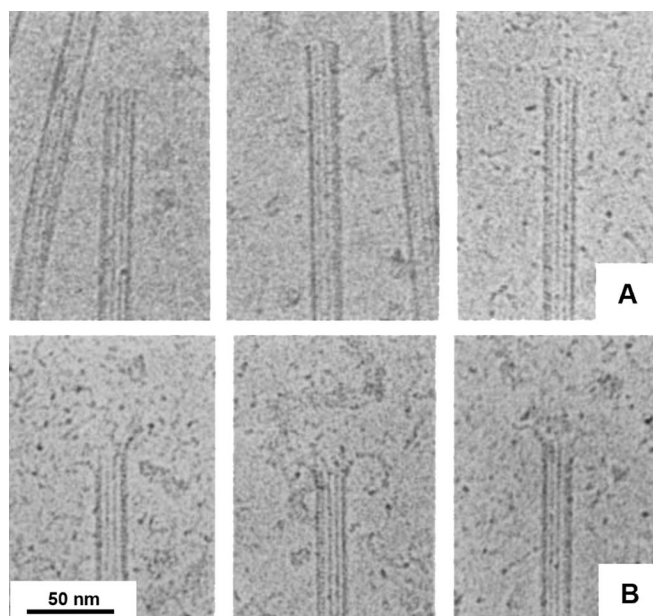


Figure 2. **Tips of dynamic MTs viewed by cryo-EM.** (A) Three examples of the ends of growing MTs. (B) Three examples of the ends of shortening MTs. From Mandelkow et al. (1991).

protofilaments is out of register: α -tubulin lies next to β -tubulin along the 3-start helix. This singularity gives MTs an asymmetry that interferes with polymerization along any lattice line except the 13 protofilaments that make up the MT wall. It seems likely to me that the seam is significant for MT function in cells, e.g., by binding the tail of a specific motor enzyme, but proteins that interact specifically with the seam have not yet been identified.

New ideas about MT polymerization

The rapid dynamics of spindle MTs suggested by the lability seen with polarization optics were confirmed when tubulin was successfully labeled with a fluorophore, microinjected into living cells, then photobleached with a laser to create a subpopulation of protein that lacked visible label (Leslie et al., 1984; Saxton et al., 1984). While interphase MTs in cultured mammalian cells show a halftime for tubulin turnover of >250 s, the mitotic spindle was >10 $\times$ faster, a speed that was impossible to understand with the condensation polymerization model. At about this time, however, our understanding of MT dynamics changed radically with the discovery of “dynamic instability.” Tubulin polymers might elongate by condensation polymerization, but they were different from other protein assemblies in showing periods of growth, followed by stochastic transitions to either a pause in growth or rapid shortening, followed by occasional “rescues” that allowed the polymers to return to growth (Mitchison and Kirschner, 1984; Kirschner and Mitchison, 1986).

Initial evidence for dynamic instability was obtained by immunofluorescence and EM of fixed samples, but direct evidence for this novel phenomenon came from light microscopy using dark-field optics (Horio and Hotani, 1986). This method produces images with an excellent signal-to-noise ratio for samples whose surrounding solution is clear, so the only objects scattering

light into the detector are the structures of interest. The method will detect objects far smaller than the resolving power of a light microscope and allow description of their behavior in aqueous or other solutions. Dynamic instability of MTs was then demonstrated in cultured cells, using differential interference microscopy to visualize MTs in cytoplasm (Cassimeris et al., 1988). These optics, together with video enhancement of contrast, showed the predicted alternation between periods of growth and shortening, although the space resolution of light microscopy was insufficient to describe what was going on at the MT tip. The discovery of dynamic instability was a major achievement in biochemical cell biology. It was an example of studying objects of biochemical interest as individual entities, rather than as ensembles whose properties were viewed in bulk, e.g., by viscosity or spectroscopy. When the behavior of individual MTs was observed, unexpected behaviors became clear. This approach has become a powerful way to study both MTs and related enzymes. Motors are now commonly studied as single enzymes, visualized by their fluorescence, and challenged individually with laser tweezers (Block et al., 2003), rather than as catalysts that simply increase the rate of ATP hydrolysis. This “single-particle approach” has been responsible for important advances in our understanding of biological machines.

Traditional biochemistry did, however, elucidate several aspects of dynamic instability (Caplow and Shanks, 1990). The knowledge that GTP-tubulin was required for polymerization, but that MTs were made predominantly of GDP-tubulin, supported the model that soluble tubulin dimers with GTP bound were naturally straight and suitable for addition to the ends of the straight protofilaments in a MT wall. Tubulin with GDP bound, on the other hand, was hypothesized to be naturally bent, forming curved protofilaments when relaxed to its minimum energy conformation. In the MT wall, the bonds between neighboring tubulins antagonized the tendency of GDP-tubulin to curl. This model accounted for the difficulty of getting GDP-tubulin to polymerize, the curling protofilaments on shortening MTs, and the rapid shortening of any MT that happened to lose the “cap” of GTP-tubulin associated with its growing end (Desai and Mitchison, 1997). The model earned widespread acceptance and was presented in textbooks for many years.

Atomic structures of soluble tubulin oligomers led to a paradox about MT polymerization

Continued study of the components of this model led, however, to questions about its validity. X-ray crystallography of GTP- and GDP-tubulin dimers or oligomers revealed very similar structures; differences that might lead GTP-tubulin to form straight protofilaments and GDP-tubulin to make curls were not observed (Gigant et al., 2000). However, pure GTP-tubulin was difficult to crystallize; its tendency to polymerize inhibited its formation of 3-dimensional (3D) crystals. To solve this problem, investigators used various ways to block polymerization: a drug, like colchicine (Buey et al., 2006), or a protein inhibitor of tubulin polymerization (Ravelli et al., 2004; Pecqueur et al., 2012). Given that GTP-tubulin was crystallized with polymerization-blocking factors, some members of the MT community (myself, included) did not regard these results as definitive; the

inhibiting factors might have put GTP-tubulin into a bent configuration. However, other investigators found by small angle x-ray scattering that the shapes of GTP- and GDP-dimers in solution were very similar (Rice et al., 2008). In addition, studies on the rates of tubulin binding by allo-colchicine, an analogue of the well-studied polymerization inhibitor that binds well to bent tubulin, confirmed the similarity of GTP- and GDP-tubulin in solution (Nawrotek et al., 2011), reviewed in Brouhard and Rice (2014). More recently, atomic structures of *Drosophila* tubulin with either GTP or GDP bound have been determined, and they are essentially the same (Wagstaff et al., 2023), a result confirmed by cryo-EM of tubulin with either nucleotide bound (Zhou et al., 2023). These results pose a problem for the model of dynamic instability that assumes GTP-tubulin forms straight protofilaments and GDP-tubulin forms bent ones. The different phosphorylation states of bound nucleotide might alter tubulin's structure, but the two states are not as different as required to form curved vs. bent protofilaments.

These results identified a significant issue for tubulin's polymerization mechanism. If GTP-tubulin is bent in solution and straight in the MT wall, subunit straightening is a significant part of the polymerization process.

Some images from cryo-EM suggested that MTs grow from "bent" tubulin

Chretien and colleagues used isolated centrosomes to initiate the polymerization of purified brain tubulin, making radial arrays of growing MTs attached to specimen supports suitable for rapid freezing and cryo-EM. This group saw MT tips that were neither flat nor jagged; they displayed a few, gently curving protofilaments that extended far beyond their neighbors (Chrétien et al., 1995) (Fig. 3). While these protofilament clusters were noticeably less curved than the protofilaments at the tips of depolymerizing MTs, the fact that they were curved at all was consistent with the curved structure of GTP-tubulin in solution. An initial model from this group proposed the formation of tubulin sheets that subsequently curled into a tube, but further work led them to suggest a mechanical equilibrium between the natural curvature of tubulin, which favored curved protofilaments like those at the tips of depolymerizing MTs, and an orthogonal curvature that resulted from lateral associations between adjacent protofilaments whose relative position resembled the structure of a MT wall (Jánosi et al., 1998; Chrétien et al., 1999). As more protofilaments joined a cluster, it would straighten enough to join an existing MT wall. Presumably, GTP-tubulin formed stronger lateral bonds (more tendency to cluster) than GDP-tubulin, allowing it to polymerize, whereas the weaker lateral associations between protofilaments containing GDP-tubulins would allow them to bend and depolymerize. Some years earlier, Kirschner had proposed that sheets of tubulin protofilaments could curl up to form a tube, but as mentioned before, he was studying polymer initiation, whereas the Chretien group was looking directly at MT elongation.

Chretien's model for tubulin polymerization stood for some years beside the model that assumed GTP-tubulin was straight. Possible reasons why the mechanical equilibrium idea was not universally accepted were as follows: (1) the Desai-Mitchison

model was so satisfying and (2) few other labs saw long, curving protofilament extensions at the tips of growing MTs (Arnal et al., 2000). They were rarely, if ever, seen in vivo or by other groups doing microscopy of dynamic MTs.

Electron tomography of MTs in vivo disagreed with both theory and cryo-EM in vitro

A few years later, three research groups began to look at the tips of MTs elongating in vivo, using multiple tilted views of each tip to provide data necessary for 3D reconstructions by axial tomography. Initial work from the McEwen lab examined the kinetochore-associated MTs in cultured animal cells in mitosis. These studies identified MTs with either flared or blunt ends at each kinetochore, but none of the ends showed the long, curling extensions seen by the Chretien group in vitro (VandenBeldt et al., 2006). The diversity of tip morphology at a single kinetochore was thought enigmatic, because the authors assumed that MTs with blunt tips were growing and those with flared tips were shortening, as proposed by Desai and Mitchison (1997). Since both morphologies were found on each kinetochore, there did not appear to be a correlation between MT tip structure and kinetochore motion. A study from the McIntosh lab used the same imaging technology to look at the plus ends of kinetochore MTs, but they employed a radial sampling of the 3D image of each MT tip. Every plus end was viewed in thin slices that contained the MT axis, rotated over multiple orientations from +60° to -60° around that axis. With this method, a large majority of kinetochore MT ends were seen to be flared (McIntosh et al., 2008). This result too was surprising, because during metaphase chromosome oscillations, not all kinetochore MTs would be shortening. Moreover, metaphase kinetochore MTs display "flux" toward the spindle poles (Mitchison, 1989), a phenomenon that requires polymerization at the kinetochore and depolymerization at the pole. Thus, plus-end tips of kinetochore MTs growing in vivo resembled those on shortening MT in vitro.

Soon thereafter, a group led by Johanna Hoog used electron tomography to study fission yeast cells, exposed first to conditions that disassembled MTs, then to physiological conditions so the MTs would regrow (Höög et al., 2011). These 3D images of growing tips showed that they were more flared than expected, given tip structures seen in vitro (Mandelkow et al., 1991); they were more like the tips of MTs shortening in vitro, and none of the tips showed the long, gently curving protofilaments seen by Chretien. This result was supported by images from several labs (Zovko et al., 2008; Kukulski et al., 2011) and by a detailed study from the McIntosh lab that used the same imaging technology to examine the tips of spindle MTs from six different species. The biology of the samples assured that these MTs were growing at the time of fixation. (McIntosh et al., 2018) (Fig. 4).

Further studies of MTs growing in vitro agreed with the in vivo work

The McIntosh group also applied cryo-electron tomography (cryo-ET) to MTs growing in vitro by polymerization of purified tubulin. Polymerization was initiated from the tips of A sub-tubules in axonemes, isolated from the alga, *Chlamydomonas*. These "seeds" contain 13 protofilaments, the number commonly found in cells. The growing MTs were plunge-frozen in liquid

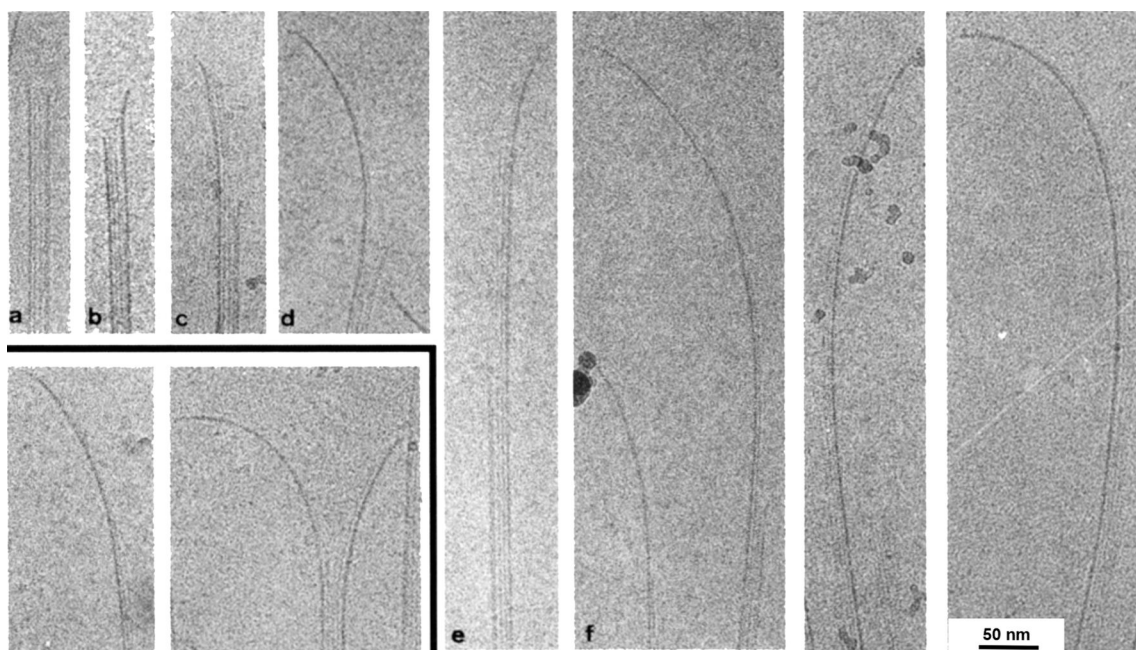


Figure 3. **Tip structure of MTs growing from centrosomes in vitro, as seen by cryo-EM.** (a–f) Showing multiple examples of long, curving protofilament bundles. From Chrétien et al. (1995).

ethane, the standard method for rapid immobilization of structures for detailed study by cryo-ET. The resulting 3D images confirmed a flared morphology at the tips of growing MTs (McIntosh et al., 2018) (Fig. 5). Moreover, the long, gently curving protofilament cluster seen by the Chretien group was observed only after treatment with paclitaxel, and then in only a few cases (Gudimchuk et al., 2020).

The flared tips on growing MTs were interpreted with a straightforward model in which curved protofilaments elongate by the addition of curved GTP-tubulin to their tips. These slender and flexible oligomers of tubulin would, of course, oscillate rapidly in thermal motion, allowing them occasionally to straighten and form lateral bonds with their neighbors. As tests of the model, this group studied MT tip shapes at a range of growth speeds (different tubulin concentrations or with the

addition of a polymerization catalyst); predictions of the model agreed quantitatively with the structural data obtained. Perhaps more convincing, data from many curving protofilaments showed that their average curvature increased linearly from near zero at the MT wall to $\sim 23^\circ/\text{dimer}$ at the protofilament tip (Fig. 5 G). This curvature is very similar to protofilament curvature predicted from the structure of crystallized GTP-tubulin dimers. From these studies, it appeared that MTs grow by the addition of bent GTP-tubulin to the tips of curved, flexible protofilaments. For a timeline of key advances of microtubule research, see Fig. 6.

Concerns about all structural results so far

All this structural work is, however, susceptible to criticism, given the experimental methods used. MTs are labile to both cold

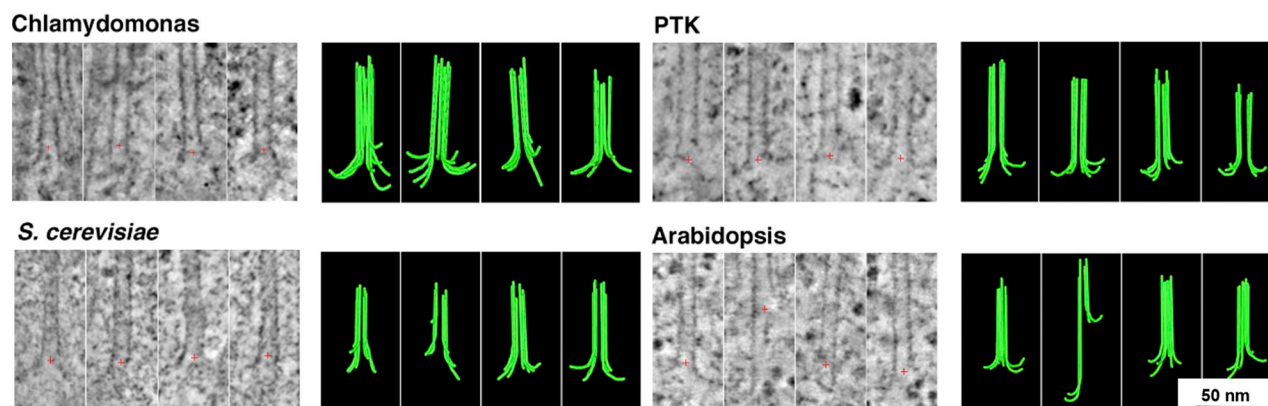


Figure 4. **Tips of MTs from four species growing in vivo.** Grayscale images are slices from electron tomograms of MT tips, and green drawings are 3D models drawn by hand, using rotary sampling of the 3D reconstruction of each MT tip. From McIntosh et al. (2013).

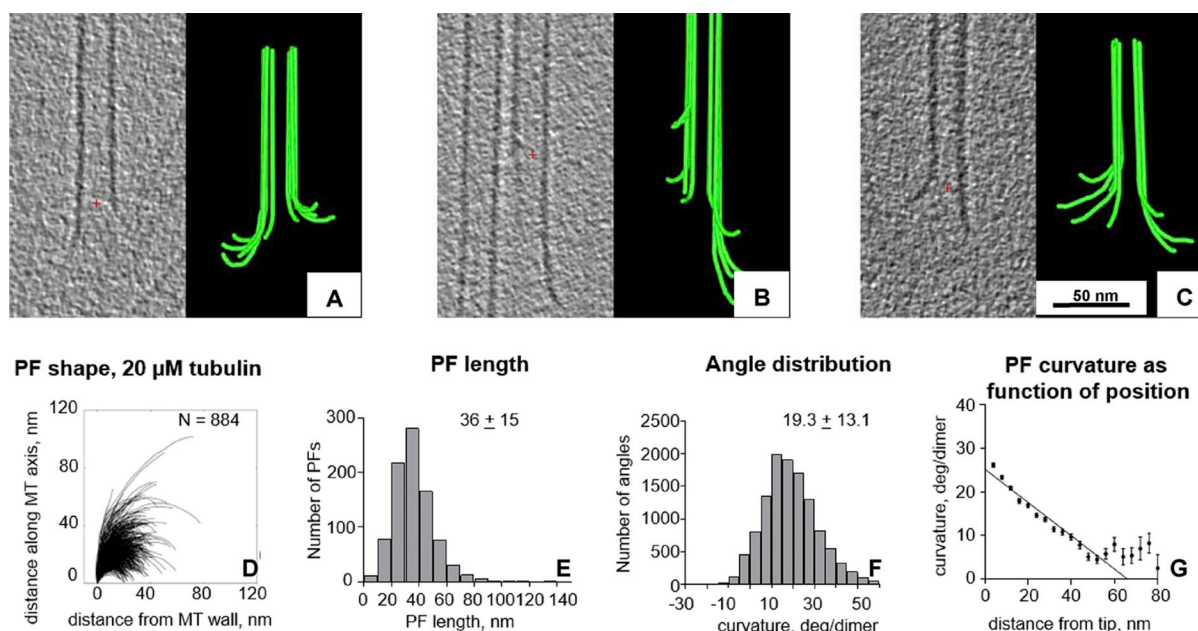


Figure 5. **Tips of MTs growing in vitro.** (A–C) (Left) Slices through cryo-tomograms of MT ends (grayscale images) and (right) models drawn on protofilaments seen in slices at different orientations around the MT axis. Bar = 50 nm. (D) Curves representing the shapes of many protofilaments. (E and F) Distribution of their lengths and (F) distribution of their curvatures. (G) Average curvature in degrees/dimer of many protofilaments as a function of distance from the protofilament's tip. From McIntosh et al. (2018).

treatment (e.g., $\sim 0^\circ\text{C}$) and hydrostatic pressures (e.g., greater than ~ 300 bar; Tilney et al., 1966). The MT ends imaged in vivo were found in samples that had been prepared by high-pressure freezing, followed by freeze-substitution fixation, a method that consistently yields good preservation of most cellular structure (McDonald et al., 2007). The pressure treatment is brief (~ 5 msec before freezing), but it is high enough ($\sim 2,000$ bar) to induce MT depolymerization. Although freezing occurs “rapidly,” as a result of jets of liquid nitrogen that come within ~ 10 msec of the onset of pressure increase, there is no guarantee that such freezing is fast enough to preserve the structure of labile tubulin protofilaments at a growing MT tip. Molecular motions are commonly measured in microseconds or less. Since both high pressure and cold temperatures induce MT depolymerization, and since shortening MTs are well known to have a flared morphology, it is plausible that the flares seen on “growing” MTs in all these studies are a result of preparation-induced depolymerization. This issue was addressed by the McEwen group by examining additional cells prepared for EM by chemical fixation with glutaraldehyde. MT tip morphologies seen at kinetochores in these samples were essentially identical to those seen after high-pressure freezing and freeze substitution (VandenBeldt et al., 2006).

For the studies of the MT tip structure in vitro, no pressure was applied, so this factor is irrelevant. Plunge freezing in liquid ethane, cooled to its freezing point by liquid nitrogen, has long been regarded as the most reliable way to prepare biological structure for study in frozen-hydrated samples. When freezing is sufficiently fast, there is no time for water molecules to move into a lattice; instead, water “super-cools,” becoming ever-more viscous (Dubochet, 2007). At about -70°C water becomes a glass-

like solid. However, at the freezing rates obtained with plunge freezing ($\sim -10^5$ deg/sec), there is at least 1 msec before the sample cools from 37°C to the temperature of immobilization. This time is still quite long for molecular motions, casting some doubt on the ability of rapid freezing to preserve the structure of MT tips. Thus, the results from cryo-ET are also suspect. This issue was addressed by the McIntosh group in two ways: (1) chemical fixation of MTs polymerized in vitro was used both before freezing and as the sole method for structural preservation; and (2) negative staining was used to make samples that experienced neither cooling nor cross-linking fixation. Electron tomographic reconstruction of these growing MT tips yielded images of protofilament curvature that were quantitatively very similar to material that was simply fast-frozen (McIntosh et al., 2018).

There is, however, a cryptic limitation to the reliability of this electron tomography. Images of MT tips in vivo are plagued with structural “noise” contributed by fixed cytoplasm that surrounds the regions of interest (Fig. 4). Protofilaments, as seen in an EM, are slender, faint, and curvaceous, so tracking their shape and extent is not easy. Most groups doing this work used thin (~ 4 nm) slices from their tomograms, about the same thickness as the protofilaments themselves, to cut away as much distracting material as possible, but even then, tracing the protofilaments was difficult. One paper included a discussion of possible tracking errors and used multiple people to make traces of the same MT ends, seeking reliable data about the shape of the slender protofilaments (McIntosh et al., 2020). Nonetheless, all protofilament traces were subject to operator error. Even the work in vitro, where cytoplasm was no longer present, was plagued by image noise (Fig. 5). The dose of beam electrons that a cryo-specimen can tolerate without loss of resolution is limited,

Research Timeline

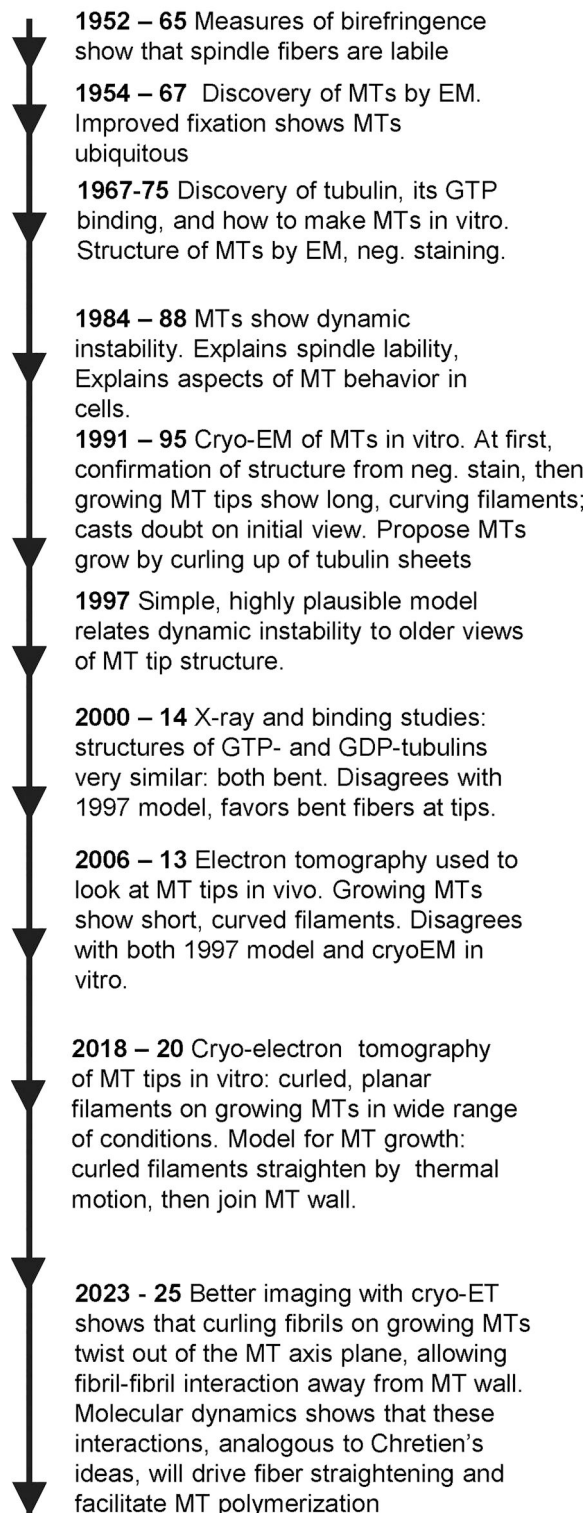


Figure 6. **Chronological account of major advances leading to our current understanding of MT growth.**

meaning that cryo-images include considerable “statistical noise” from the low total dose of imaging electrons. Moreover, electron scattering by ice and protein is similar, so contrast is low, even when the objective lens is set to give phase contrast. In

this work, tomographic slices thicker than ~5 nm contained enough noise to obscure the protofilaments, in spite of using the best noise-filtration protocols then available (nonlinear, anisotropic diffusion). Therefore, most data on protofilament shape were obtained by cutting tomographic slices that contained the MT axis, then rotating the plane of sampling around the MT axis to visualize protofilaments as they flared away from the axis at different orientations (Fig. 5). This method led to models of protofilaments that were planar and approximately regularly spaced at intervals of ~28°.

Insights into protofilament dynamics from molecular modeling and modern cryo-ET

While empirical work on the shapes of dynamic MT tips was ongoing, several groups were applying methods of molecular dynamic modeling to obtain evidence about the impact of bound nucleotides (GTP or GDP) on the equilibrium shapes of tubulin dimers and protofilaments. Some of these studies describe changes in tubulin as a result of removing stathmin or another inhibiting molecule from its complex with tubulin, as seen by crystallography, then simulating tubulin's structure as it relaxed to its new, minimum energy conformation. Such changes should predict the shapes of tubulin dimers or protofilaments at the tips of growing and shortening MTs (Graffmüller and Voth, 2011; Igaev and Grubmüller, 2018; Fedorov et al., 2019). Recently, this work has been aided by improved 3D structures for tubulin in the MT lattice (Zhang et al., 2018; Manka and Moores, 2018; LaFrance et al., 2022), which have provided a more accurate starting point for predictions of how the hydrolysis state of bound nucleotide might affect tubulin's structure changes during polymerization. This work, based on atomistic molecular dynamics, has predicted the most likely structural changes for both GTP- and GDP-tubulins as they add to or come off from a MT tip (Fedorov et al., 2019; Igaev and Grubmüller, 2022).

These studies consistently predicted that protofilaments released from bonds to lateral neighbors would bend outward from the MT axis, but the bending would not be confined to planes, as described in empirical work with cryo-ET (McIntosh et al., 2018). Indeed, simple reasoning suggests that protofilaments are likely to bend out of the plane that contains the MT axis; for them not to do so, they would have to be flexible in the two dimensions of the plane that contains the MT axis but rigid in the perpendicular direction; not impossible but surprising. Gudimchuk pointed this out to me as I was tracing protofilaments in cryo-ET images of MT tips in vitro. I spent significant time trying to track curving protofilaments out of the plane that contained the MT axis but was unsuccessful. In the noisy cryo-tomograms then available, any nonplanarity of these slender tubulin strands was invisible. However, subsequent improvements in both direct electron detecting cameras for intermediate voltage electron microscopes and new algorithms for noise filtration have changed the situation significantly.

Recent structural and theoretical studies

Cameras that serve as direct detectors of beam electrons are now in common use for cryo-EM (Vesler et al., 2013). Their sensitivity allows the detection of essentially every beam electron,

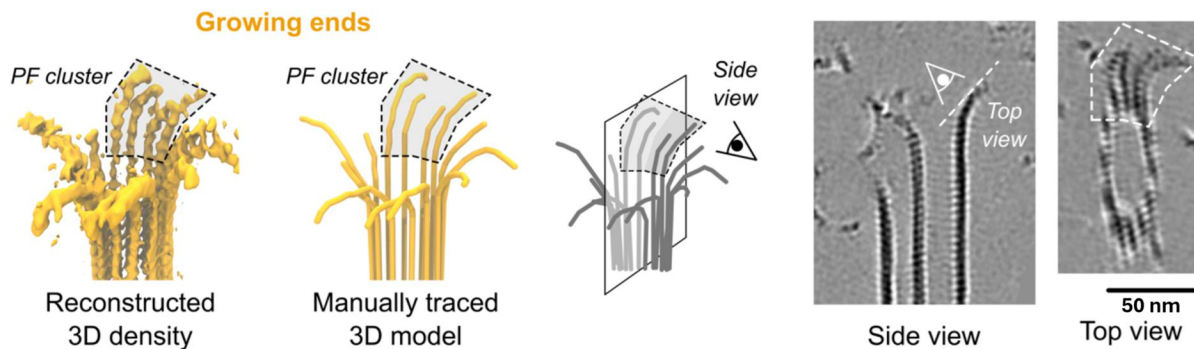


Figure 7. **Electron tomography of the ends of MTs that are elongating.** Images on the left are from a complete tomographic reconstruction. Grayscale images on the right are slices from a tomogram. Note the low noise in the background, compared with Fig. 4. From van den Berg et al. (2023); Kalutskii et al. (2025).

and their high accuracy and efficiency of information transfer to readout means that their image quality at low dose is very good (Peng et al., 2022). Their readout rates are fast enough to allow multiple images to be obtained during a single exposure long enough to collect a total dose that includes enough electrons for good imaging statistics. Algorithms now available can compare each of these images with its neighbors, allowing alignment and therefore correction for specimen motion during exposure to the beam. More importantly, new algorithms for reducing image noise, due to both electron scattering from background ice and statistical noise inherent to low-dose imaging, have greatly improved image signal-to-noise (Buchholz et al., 2019). With these improvements, MT ends imaged by cryo-ET can be viewed in slices thick enough to reveal a projection of an entire tip in one view (van den Berg et al., 2023; Iyer et al., 2025). In these images, it is immediately apparent that the protofilaments on both growing and shortening MTs are not planar (Fig. 7). Although they start to flare out from the MT axis in planes containing that axis, they deviate from those planes far enough to encounter a neighboring protofilament at significant distances from the MT wall. This observation has led to a model for MT growth in which the flaring protofilaments at the tip of a growing MT cluster as an essential part of the assembly process (Kalutskii et al., 2025).

Chretien and his colleagues suggested that protofilament clustering induces protofilament straightening. In the context of recent structural work, we can now apply this idea to short protofilaments rather than the long, gently curving ones seen by Chretien and colleagues. Clustering will bias the curved shape of bent protofilaments toward a straighter configuration, as shown by calculation and discussed in Kalutskii et al. (2025). This is certainly an effective way to change the minimum energy shape of protofilaments toward a configuration that can join the MT wall, but clustering also makes a bundle that is stiffer than a single protofilament and therefore less likely to straighten by Brownian movements. Both the predictions from molecular dynamic modeling and the recent structural work confirm that GTP-tubulin displays more protofilament clusters than GDP-tubulin, supporting the idea that clustering is an important part of polymerization. However, the calculations carried out so far (which represent at most ~ 0.2 ms) have not covered enough time to model real polymerization. From current empirical and

theoretical work, we can say that both clustering and Brownian movement contribute to protofilament straightening, but the relative importance of these (and perhaps additional, not yet recognized factors) remains to be assessed.

The importance of polymerization mechanism for MT function in vivo

As mentioned in the introduction, controlled MT polymerization allows cells to regulate aspects of cell mechanics and intracellular motions. However, a subtle aspect of polymerization deserves attention. The lattice that forms can do mechanical work during both polymerization and depolymerization.

As tubulin joins the MT lattice, energy is stored in the structure that forms. Evidence for this property is in the facts that both GTP- and GDP-tubulin form bent protofilaments, yet tubulin in the MT wall is straight; the conformation of polymerized tubulin is strained by the bonds that form during assembly. The growing polymer can do mechanical work, thanks to the strength of these bonds (Hotani and Miyamoto, 1990). Several groups have measured the force a growing MT can exert (Gittes et al., 1993; Janson and Dogterom, 2004), reviewed in Grishchuk et al. (2012), Vleugel et al. (2016). The resulting values of ~ 2 pN/MT end suggest that polymer growth can be a useful mechanical component of cellular morphogenesis.

MTs can also exert forces during disassembly, so long as the load is properly attached to the MT surface (Grishchuk et al., 2005). The amount of force a shortening MT can generate has been measured in several ways, but the largest force documented with laser tweezers and a force clamp (to hold a MT-tethered microsphere during depolymerization) is 25–30 pN (Volkov et al., 2013; Driver et al., 2017). Assuming that this force is distributed among 13 protofilaments and that the loss of one tubulin dimer causes a change in position of 8 nm, 1.8×10^{-20} J of work can be done with depolymerization event. This corresponds to about 2.6 kcal/mole of free energy change, a change that cannot come from the entropy of ordered subunits going into solution; MTs are cold-labile, so the entropy change for tubulin dissociation is negative. The energy for this force and the work it can do is stored in the MT lattice. The ultimate source of this energy is almost certainly the energy liberated by hydrolysis of tubulin-bound GTP, but the details of how this energy is

divided among the intermediates of hydrolysis, product release, and strain in the tubulin molecule are still subjects of active research. Tubulin and actin share the property of hydrolyzing a bound nucleotide triphosphate during polymerization. As a result, they are uniquely capable of influencing structural and motility events in cells (Wagstaff et al., 2023). Cells can capitalize on this stored energy to do work, such as to move a chromosome during mitosis (Grishchuk and McIntosh, 2006).

Concluding remarks

This small piece of history is a poignant example of how the interplay between technology, observation, and theory can lead to significant scientific progress. We have seen how improvements in imaging technologies and specimen preparation have taken the field from bright-field light microscopy of fixed spindle fibers through polarization optical observations of dynamic MT clusters in a living mitotic spindle, to images of single MTs, either in cells or in vitro. We have seen progress from single-projection images of negatively stained samples captured by EM at ~4-nm resolution, to similar images obtained from frozen-hydrated samples, in which physiological structure is more likely to be retained. Force measurements with optical tweezers have shown the ability of MTs to generate force when depolymerizing and polymerizing, revealing directly the energy stored in the MT lattice. Then with electron tomography, 3D images of single MT tips have been obtained, first of frozen then freeze-substitution fixed samples in cells, then of frozen-hydrated samples in vitro. At this stage, data from crystal structures of tubulin and computational methods for predicting molecular behavior from initial structures and first principles led to predictions about MT tip structures that were different from those seen by cryo-ET, but with improved methods for both image capture and processing, then better methods for noise filtration, cryo-images have come to correspond to the atomistic predictions. It seems that we are approaching a valid description of the pathways by which MTs grow and shorten.

A current model poses that curved GTP-tubulin dimers add to the tips of curved protofilaments that are in the Brownian motion, causing them both to straighten and to cluster. Clustering changes the minimum energy shape of tubulin to approach a straight configuration, a change that works with thermal vibrations to let protofilaments join the MT wall. In the images supporting these ideas, there is still the possibility of freezing artifact, so instruments like atomic force microscopy or microscopy with short-wavelength light (Karl et al., 2018), both of which can work in aqueous solutions at physiological temperatures, may provide important new insights. Nonetheless, we now have a structural model into which to fit the various regulatory factors that control tubulin polymerization in vivo, such as end-binding proteins that can bind to both the MT lattice (Zhang et al., 2015) and curving protofilaments at growing MT tips (Guesdon et al., 2016). Likewise, TOG-dependent polymerization catalysts of the X-MAP215 class, CLASPS (Girão et al., 2020), and doublecortin (Fourniol et al., 2010; Bechstedt and Brouhard, 2012) can be considered within the structural context in which they act. This will facilitate understanding their contributions to increased rates of tubulin addition,

protofilament straightening, and the enhanced bonding between protofilaments.

As one expects, however, there are still many unanswered questions about MT dynamics: (1) What are the exact contributions of protofilament clustering and Brownian motion to the pathway for straightening GTP-tubulin as it polymerizes? (2) How do the steps in hydrolysis of tubulin-bound GTP affect the structural transitions associated with polymerization? (3) Do posttranslational modifications of tubulin control any aspect of polymerization, or are they involved solely with depolymerization and modulation of MT binding to associating proteins and organelles? (4) How can a MT polymerization catalyst, like proteins of the X-MAP215 class, speed depolymerization and MT growth? (5) Is information stored in and transmitted by structural changes in the MT lattice? And (6) are there pathways for information flow directly from cell surface receptors to the factors that regulate MT growth? These, among other issues, should keep this field lively for years to come.

Appendix

Optical instruments

Bright-field optics with achromatic, oil immersion lenses identified fibers in the mitotic spindles of cells that had been fixed and stained (~1890). Limitations: fixation artifacts and resolution limits.

Polarization optics showed that spindle fibers are birefringent and exist in living cells. Experiments using these optics showed spindles to be labile to cold, pressure, and colchicine but are enhanced by glycols (1950s–1960s). Limitations: space resolution.

Fluorescence microscopy with labeled antibodies described the distribution of MTs in fixed interphase cells, providing information about probably MT functions (1972 on). It also contributed to the discovery of dynamic instability (1984). Direct labeling of tubulin with fluorophores and photobleaching revealed the extraordinarily rapid turnover of spindle MTs, supporting the dynamic instability model (1984). Labeling with green fluorescent protein has enabled extensive work on MT arrangements and dynamics in cells (1995 on). Limitations: fixation artifacts and nonspecific labeling for immunofluorescence, perturbation of protein behavior by labels (chemical or fluorescent protein chimeras).

Dark-field optics demonstrated dynamic instability directly in vitro (1986). Limitations: resolution. Differential interference contrast optics demonstrated dynamic instability directly in cells and showed chromosome motion in vitro as a result of MT shortening (1988). Limitations: resolution.

Laser tweezers measured the forces generated by MT growth and shortening (2005 on). Limitations: misbehavior of isolated proteins due to coupling with beads.

Biochemical methods

Light scattering (turbidity) and viscosity: the common methods for measuring tubulin polymerization in test tubes. Limitations: observes protein behavior in bulk.

X-ray diffraction: coherent scattering from crystals of tubulin has provided atomic structures for tubulin with different ligands bound and in association with different proteins. This work

provided atomic structures of tubulin dimers and evidence for the structural similarity of tubulin with either GTP or GDP bound. Limitations: modifications of proteins, either to obtain crystals or during crystallization.

Small angle x-ray scattering: it revealed the structural similarity of soluble tubulin with either GTP or GDP bound. Limitations: low space resolution.

Rates of ligand binding: rates of drug binding and exchange for GTP- and GDP-tubulin are very similar, suggesting similarity of their structures in solution. Limitations: looks only at structure of ligand binding site.

Electron optical instruments

EM of fixed, plastic-embedded cells described a hole in the fibers within cilia and thereby identified MTs (1955). EM showed that birefringent spindle fibers were MTs (1965), and glutaraldehyde improved the fixation of cells for EM and led to the discovery of MT ubiquity in eukaryotic cells (1963). Limitations: fixation artifact.

EM of negatively stained tubulin polymers in vitro revealed the arrangement of tubulin in the MT wall (the lattice) and showed that the tips of MTs had different structures during growth and shortening (1974). It also showed that one MT end adds or loses tubulin faster than the other (1974). It contributed to the discovery of dynamic instability (1984). Limitations: heavy metal artifacts, resolution limitations.

EM of frozen-hydrated MTs confirmed both the structure of the MT lattice and the differences in MT tip structure for growing and shortening MTs (1991). The binding of MT-associating proteins to the lattice revealed the ubiquity of the lattice seam (1994). Limitations: possible freezing artifacts.

Electron crystallography of flat tubulin crystals gave information about the atomic structure of tubulin (~1996). Limitations: possible distortion of tubulin in its flattened state.

Improvements in methods for image capture and averaging led EM to provide the resolution needed to see the atomic structure of tubulin in the MT wall (~2005). Methods for aligning and averaging images of many single particles have led to knowledge about the structure of many MT-associated proteins. (~2015) and finally of tubulin itself (2023). Limitations: possible freezing artifact.

EM of many tilted views enabled axial tomography of both fixed-embedded and rapidly frozen samples, providing 3D information about the structure of MT tips, revealing the flared morphology of growing and shortening MTs (2006 on). Limitations: asymmetric resolution, due to missing wedge of data.

Electron tomography with improved methods for image capture and noise filtration provides 3D information about MT tips with sufficient signal-to-noise ratio to enable visualization of the whole MT tip in 3D, revealing the nonplanarity of bending protofilaments on both growing and shrinking MTs (2023 on). Limitations: asymmetric resolution, as above.

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