Chapter 18

Visualization and Quantification of Microtubule Self-Repair

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Abstract

Since its discovery, several decades ago, microtubule dynamic instability has been the subject of countless studies that demonstrate its impact on cellular behavior in health and disease. Recent studies reveal a new dimension of microtubule dynamics. Microtubules are not only dynamic at their tips but also exhibit loss and incorporation of tubulin subunits along their lattice far from the tips. Although this phenomenon has been observed to occur under various conditions in vitro as well as in cells, many questions remain regarding the regulation of lattice dynamics and their contribution to overall microtubule network organization and function. Compared to microtubule tip dynamics, the dynamics of tubulin incorporation along the lattice are more challenging to investigate as they are hidden in classical experimental setups, which is likely the reason they were overlooked for a long time. In this chapter, we present a strategy to visualize and quantify the incorporation of tubulin subunits into the microtubule lattice in vitro. The proposed method does not require specialized equipment and can thus be carried out readily in most research laboratories.

Key words Microtubule, Defect, Damage, Self-repair, In vitro reconstitution, Quantitative analysis

1 Introduction

Microtubule tip dynamics are an essential aspect of cell physiology: They contribute fundamentally to cell division [1, 2], fibroblast migration during wound closure [3, 4], formation and consolidation of neuronal projections [5, 6], and many other cellular processes [7, 8]. In contrast, even though early observations indicated that the lattice of end-stabilized microtubules is weakened by tubulin loss in the absence of free tubulin [9], the microtubule lattice was generally considered to be a static structure [7, 10, 11].

Recent studies challenge this paradigm: microtubules in a minimal in vitro system continuously lose and incorporate tubulin from the surrounding solution [12], a phenomenon that shares several characteristics with tip dynamics. It depends on the tubulin concentration [12], leads to the transient presence of GTP-tubulin [13–16], and is influenced by—and in turn impacts—the

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localization and activity of other molecular players, such as molecular motors [17–19], severing enzymes [16], and tip-tracking proteins [14, 15, 20]. Interestingly, lattice dynamics appear to preferentially take place at structural defect sites like protofilament transitions [12]. In vitro, the occurrence of lattice defects can be modulated by the tubulin concentration: the higher the concentration, the faster microtubules grow and the more lattice defects they exhibit [21, 22], leading to more pronounced lattice dynamics. In addition, the ability to self-repair by incorporating tubulin from the solution confers resistance to microtubules when exposed to external mechanical stress [23]. Importantly, the incorporation of GTP-tubulin following lattice damage can stimulate rescue events of depolymerizing microtubules [14–16], thus linking the dynamic properties of the microtubule shaft to tip dynamics and highlighting the importance of taking lattice dynamics into account in order to gain a deeper understanding of overall microtubule-associated processes.

Compared to microtubule tip dynamics, lattice dynamics are difficult to visualize and quantify as they are hidden in typical experimental setups. Tubulin subunits lost from the lattice are replaced by fresh tubulin from the solution without altering the overall appearance of the microtubule in fluorescence images. It is thus necessary to employ free tubulin labeled with a different fluorophore in order to distinguish newly incorporated tubulin from the preexisting lattice. The methods used so far for the visualization of tubulin incorporation along the lattice often rely on microfluidic systems to exchange solutions or are based on other specialized equipment that is not readily available in most laboratories [12, 19, 23]. Here, we describe a simple method for the visualization and quantification of tubulin incorporation along the microtubule lattice that can be applied without the need for cost-intensive equipment or technically challenging setups. The goal of this chapter is to add to the standard repertoire of experimental strategies commonly used in microtubule-oriented research groups. In this way, we hope to contribute to lattice dynamics becoming a regular parameter to be considered along with "classical" microtubule properties such as growth rates, catastrophe and rescue frequencies, and microtubule lengths.

2 Materials

1. Double-sided tape, 70 μm thick, precut (Lima Adhésifs, Couzeix, France; *see* **Note 1**).
2. Cutting Plotter CE 6000-40, for precutting the double-sided tape (optional, *see* **Note 1**).

3. Glass coverslips, 20 mm by 20 mm (Agar scientific, AGL46S20–15).
4. Glass slides, 24 mm by 60 mm (Fisher scientific, 15165452).
5. Slide and coverslip holders (Sigma Aldrich, Z688568-1EA and Z758108-1EA (or custom-made using a 3D printer).
6. Glass beakers, 500 mL.
7. Glass or plastic beaker, minimum 3 L.
8. Aluminum foil.
9. Parafilm.
10. Tissue paper.
11. Petri dishes, 100 mm diameter.
12. Ultracentrifuge (optional, *see* **Note 1**).
13. Rotor (Beckman TLA100) with ultracentrifugation tubes (Beckman, 357448) (optional, *see* **Note 1**).
14. Water bath equipped with floaters or tube holders.
15. Laboratory shaker.
16. Ultrasonic water bath (Advantage Lab, AL 04-30) (optional, *see* **Note 1**).
17. mPEG-silane, 30 kDa.
18. Biotin-PEG-silane, 10 kDa.
19. PLL-PEG (JenKem Technology, PLL20K-G35-PEG2K).
20. Acetone.
21. 96% ethanol.
22. 37% HCl
23. 2% (v/v) Hellmanex in water.
24. 1 mg/mL neutravidin in 1× PBS supplemented with 10% (v/v) glycerol.
25. 25 mM paclitaxel (Taxol) in DMSO.
26. 1 M DTT (DL-dithiothreitol) in water, pH 7.
27. 0.1 M GTP (guanosine triphosphate) in water (Sigma-Aldrich, G5884).
28. 10 mM GMPCPP in water.
29. 150 mg/mL glucose in water.
30. 5 mg/mL glucose oxidase in water.
31. 1 mg/mL catalase in water.
32. 2% (w/v) methyl cellulose in water.
33. 10% (w/v) BSA (bovine serum albumin) in water.
34. 20× BRB80 buffer: 1.6 M PIPES-KOH pH 6.8, 20 mM $MgCl_2$, and 20 mM EGTA.

35. 15× HKEM buffer: 150 mM HEPES-KOH pH 7.4, 750 mM KCl, 75 mM $MgCl_2$, and 15 mM EGTA.
36. TicTac buffer: 10 mM HEPES, 16 mM PIPES-KOH pH 6.8, 50 mM KCl, 5 mM $MgCl_2$, and 1 mM EGTA.
37. Fluo buffer: 30 mM HEPES pH 7.4, 150 mM KCl, 15 mM $MgCl_2$, 3 mM EGTA, 3 mM GTP, 60 mM DTT, 9 mg/mL glucose, 300 μg/mL glucose oxidase, 60 μg/mL catalase, and 0.75% (w/v) methyl cellulose.
38. Oxygen scavenger cocktail: 20 mM DTT, 3 mg/mL glucose, 100 μg/mL glucose oxidase, and 20 μg/mL catalase.
39. Unlabeled tubulin, tubulin-488 (labeled with ATTO 488-NHS-Ester, ATTO Tec, AD488-35), tubulin-biotin (labeled with sulfo-NHS-LC-biotin, Thermo Fisher Scientific, 21335), and tubulin-565 (labeled with ATTO 565-NHS-Ester, ATTO Tec, AD565-35) purified from bovine brain by three cycles of temperature-dependent assembly/disassembly and by ion exchange chromatography and labeled as previously described [24]. Alternatively, labeled and unlabeled tubulin are commercially available.
40. ImageJ, version 2.0.0-rc-69/1.52p.
41. GraphPad Prism (or a similar software suitable for data analysis).

3 Method

3.1 Preparation of Microtubule Seeds

1. On ice, prepare a mix (100 μL) of ATTO-565-labeled tubulin (5%–20%) and biotinylated tubulin (10 μM total tubulin concentration) in 1× BRB80 supplemented with 0.5 mM GMPCPP.
2. Incubate 5 min on ice, then 15 min at 37 °C.
3. Add 2 μM Taxol and incubate 15 min at room temperature.
4. Pellet the seeds at 50,000 rpm (about 110,000 × g_{av}) for 15 min at 25 °C (optional, *see* **Note 1**). Discard the supernatant and resuspend the pellet in 100 μL of BRB80 supplemented with 0.5 mM GMPCPP and 2 μM taxol.
5. Snap-freeze the seeds in small (5–10 μL) aliquots and store them in liquid nitrogen for up to 2 weeks.

3.2 Coverslip and Slide Preparation

To anchor the microtubule seeds to the glass surface while avoiding nonspecific protein adsorption, it is necessary to coat the coverslips and slides with an antifouling agent (Fig. 1a–e). For this purpose, we employ silane-PEG-biotin; seeds can be attached to the biotin functional group via neutravidin sandwiching, while the PEG prevents nonspecific protein adsorption. When preparing the

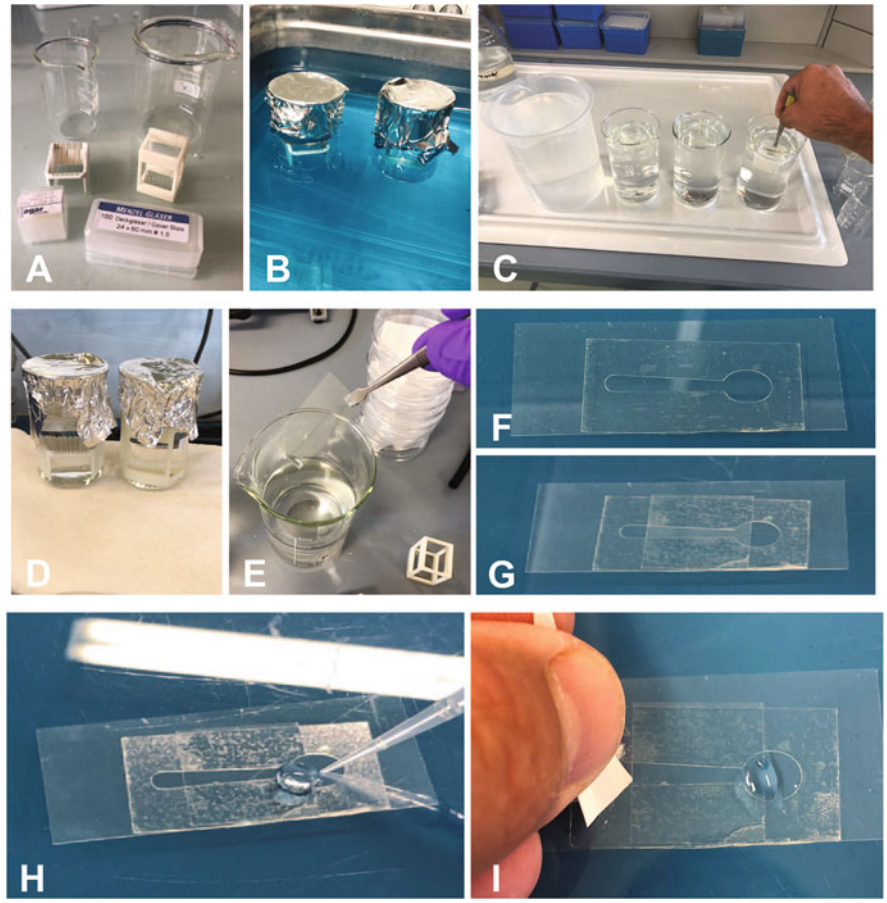

Fig. 1 (**a–e**) Preparation of the coverslips and slides and (**f–i**) flow chamber assembly. (**a**) Place the glass coverslips and slides in holders inside glass beakers filled with acetone and (**b**) clean them in an ultrasonic bath during 30 min. (C) After a 15 min cleaning step in 96% ethanol, wash the coverslips and slides extensively with ultrapure water and blow-dry. (**d**) Incubate the coverslips and slides in a PEG solution for 18 h and (E) subsequently rinse them with 96% ethanol and ultrapure water. A uniform retraction of water from the edges indicates a good PEG coating. (**f, g**) Assemble the flow chamber by sandwiching precut double-sided tape between a coverslip and a slide. (**h**, **i**) Fill the chamber with solution and carry out the perfusion steps by pipetting into one end of the chamber and aspirating with tissue paper from the other end

coverslips and glass slides, it is important to perform the washing steps with care and using extensive volumes of ultrapure water in order to remove any traces of chemicals that may persist on the glass surface and interfere with the PEG coating or the proteins.

1. To clean the glass surface, place the coverslips and glass slides in holders inside glass beakers filled with acetone.
2. Cover the beakers with aluminum foil and place them in an ultrasonic bath for 30 min.
3. Transfer the holders into beakers filled with 96% ethanol and incubate for 15 min at room temperature.

4. Wash the coverslips and slides extensively by holding them with tweezers and gently dipping them in a large beaker filled with at least 3 L of ultrapure water.
5. Place them in holders inside beakers filled with 2% (v/v) Hellmanex for 2 h, then wash them again extensively with ultrapure water. Blow-dry the coverslips and slides with filtered air or nitrogen.
6. Place the coverslips and slides in a plasma chamber and expose them to oxygen plasma at 80–90 W for 3 min (optional, for improved coating).
7. Prepare 250 mL of solution containing mPEG-silane, 1 mg/mL biotin-PEG-silane (5%–100% biotin-PEG-silane), and 0.1% HCl in 96% ethanol. Place holders with the cleaned coverslips and slides inside beakers filled with the PEG solution.
8. Seal the beakers with parafilm and protect them from light with aluminum foil. Place the beakers on a flat laboratory shaker and incubate at room temperature with gentle agitation for 18 h.
9. Rinse the coverslips and slides with 96% ethanol and extensive amounts of ultrapure water. Blow-dry the coverslips and slides with filtered air or nitrogen and store them in petri dishes sealed with parafilm at 4 °C for up to 2 weeks.

3.3 Flow Chamber

3.3.1 Preparation

Assemble a flow chamber by sandwiching double-sided tape between a glass slide and a coverslip (Fig. 1f, g). The chamber must be open on either end so that solutions can flow through the chamber by pipetting into one end and aspirating with tissue paper from the other end (Fig. 1h, i; *see* **Note 2**).

1. Perfuse the chamber with 100 µL of 50 µg/mL neutravidin in 1× HKEM buffer. Incubate for 30 s to 3 min.
2. Rinse the chamber with 100 µL of 1× HKEM buffer.
3. Rinse the chamber with 100 µL of 0.1 mg/mL PLL-PEG in 10 mM Na-HEPES, pH 7.4.
4. Rinse the chamber with 200 µL of 1× HKEM buffer supplemented with 0.2% (w/v) BSA.

3.3.2 Attaching the Seeds

1. Thaw the microtubule seeds in a water bath at 37 °C.
2. Dilute the seeds (100× to 4000×, depending on the required microtubule density) and flush them into the chamber at high flow rates in order to ensure their proper orientation.
3. Flush 100 µL of microtubule seeds diluted in 1× HKEM supplemented with 0.2% (w/v) BSA into the chamber.
4. Wash out the nonattached seeds immediately using 300 µL of 1× HKEM supplemented with 0.2% BSA.

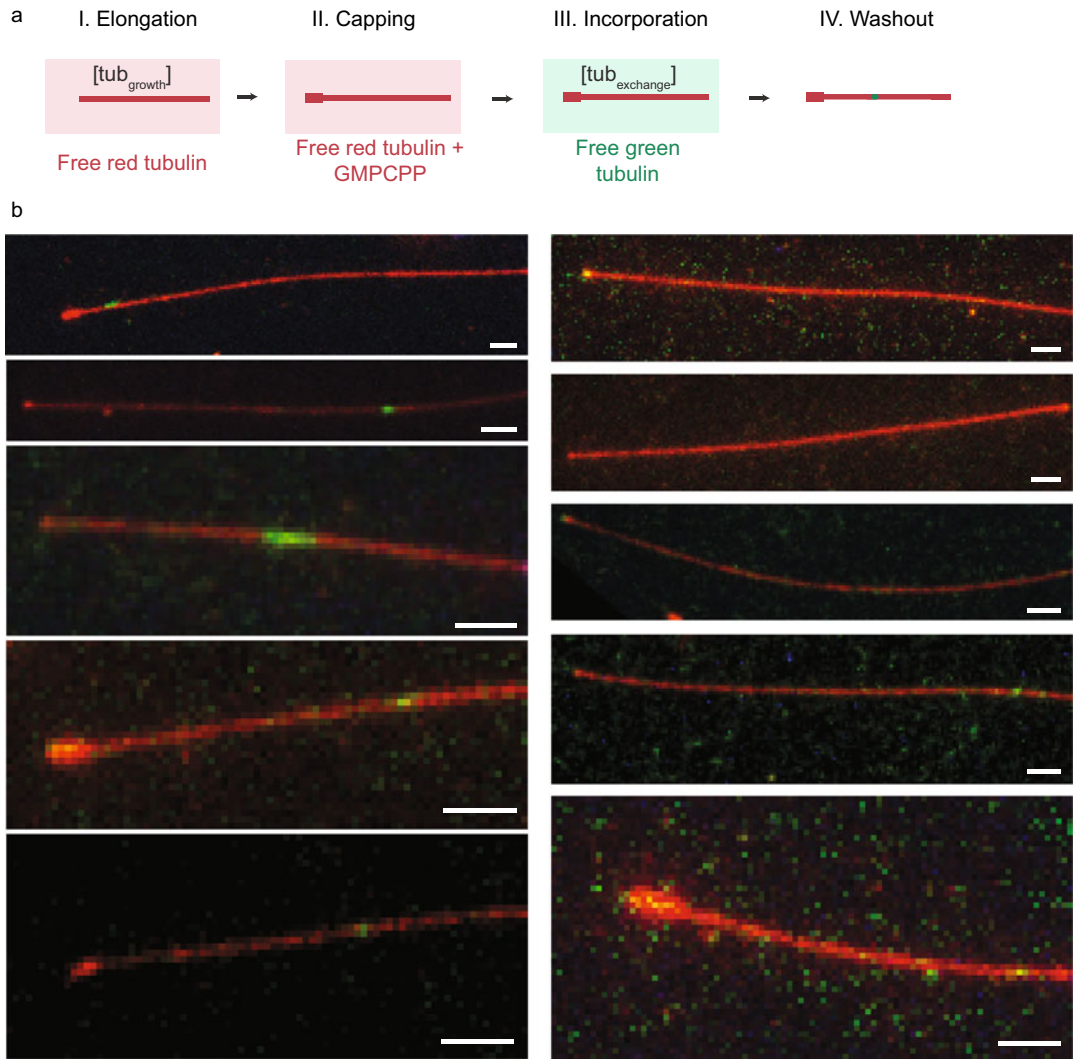

Fig. 2 (**a**) To visualize tubulin incorporation along the microtubule lattice, microtubules are elongated from neutravidin-attached seeds in the presence of red-labeled tubulin (tub_{growth}), capped with GMPCPP, exposed to free green tubulin ($tub_{exchange}$), and imaged after tubulin washout. (b) Left: examples of tubulin incorporations (green) into microtubules (red). Right: examples of microtubules without detectable tubulin incorporations. Scale bars: 3 μm

3.3.3 Elongation

Assemble dynamic microtubules from the seeds (Fig. 2a, I) (the tubulin concentration and incubation time can be varied; microtubules grown at high tubulin concentrations exhibit a higher frequency of lattice defects [12, 21, 22], *see* **Note 3**). Elongation and the following steps (capping and incorporation) may be carried out under different buffer conditions (*see* **Note 4**).

1. Flush a mix (150 μL, prewarmed to 37 °C for 1 min) containing 10–32 μM of tubulin (5% labeled with ATTO-565) in TicTac buffer supplemented with 1 mM GTP, an oxygen scavenger cocktail, 0.1% (w/v) BSA, and 0.25% (w/v) methyl cellulose into the chamber.
2. Incubate at 37 °C for 20 min.

3.3.4 Capping

Grow GMPCPP caps at the microtubule ends in order to protect microtubules from depolymerization (Fig. 2a, II):

1. Prepare a mix (150 μL) containing 10–14 μM of tubulin (100% labeled with ATTO-565) in TicTac buffer supplemented with 0.1% (w/v) BSA, 1 mM GTP, an oxygen scavenger cocktail, and 0.25% (w/v) methyl cellulose.
2. Prewarm the mix to 37 °C for 1 min and flush it into the chamber. Incubate at 37 °C for 15 min.

3.3.5 Incorporation

For tubulin incorporation, use the same buffer as for growing microtubules, supplemented with the desired concentration of tubulin (typically between 7 and 26 μM, *see* **Note 3**; Fig. 2a, III). Use ATTO-488-labeled tubulin to distinguish incorporated tubulin from the pre-grown microtubules (100% labeled, to facilitate visualization of tubulin incorporations).

1. Prepare a mix (150 μL) containing 7–26 μM of tubulin (100% labeled with ATTO-488) in TicTac buffer supplemented with 0.1% (w/v) BSA, 1 mM GTP, an oxygen scavenger cocktail, and 0.25% (w/v) methyl cellulose (if needed, kinesin or other MAPs may be diluted in HKEM).
2. Prewarm the mix to 37 °C for 1 min and flush it into the chamber. Incubate at 37 °C for 15–30 min.

3.3.6 Imaging

Before visualizing incorporated tubulin, it is necessary to wash out the free tubulin in order to reduce the background signal (Fig. 2a, IV):

1. Prepare a mix (150 μL) in TicTac buffer supplemented with 0.1% (w/v) BSA, 1 mM GTP, an oxygen scavenger cocktail, and 0.25% (w/v) methyl cellulose. Prewarm the mix to 37 °C for 1 min before flushing it into the chamber.
2. Image the microtubules with a (TIRF or epifluorescence) microscope (Fig. 2b; *see* **Note 5**).

3.4 Image Processing and Analysis

Import the images as image stacks into ImageJ. For every microtubule to be analyzed, average over several images using the Z Project function (Fig. 3a). To quantify the amount of tubulin incorporation and compare between different conditions, we recommend analyzing (1) the incorporation frequency, (2) the incorporation

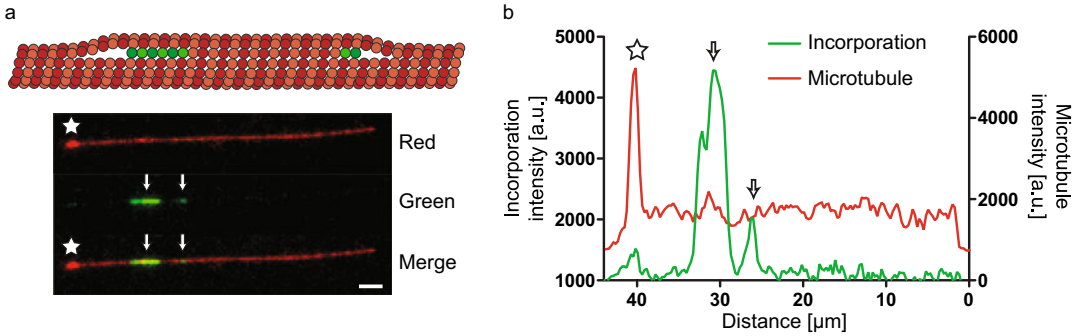

Fig. 3 (**a**) Schematic (top) and fluorescence image (bottom) of a capped (star) microtubule with two tubulin incorporation sites (arrows), averaged over five frames. Scale bar: 3 μm. (**b**) Fluorescence intensity of the microtubule (red, cap indicated by the star) and tubulin incorporation sites (green, arrows), respectively, measured along the length of the microtubule shown in (**a**). Incorporation sites were identified where the fluorescence intensity was at least 2.5-fold higher than the background fluorescence

length, and (3) the incorporation intensity. To distinguish incorporation sites from the background, it is necessary to set a fluorescence intensity threshold.

1. Incorporation frequency: To analyze the incorporation frequency, concatenate all microtubules in a random order and measure the distances between incorporation spots. Calculate the incorporation frequency as the inverse of the distances.
2. Incorporation length: We recommend measuring the fluorescence intensity along the incorporation site by manually drawing a line and using the Plot Profile function in ImageJ (Fig. 3b), then exporting the fluorescence intensity data into a suitable program for analysis (e.g., GraphPad Prism) and fitting a Gaussian error function to the intensity drop-off at each end. The half maxima can then be defined as the beginning and end of the incorporation site, respectively.
3. Incorporation intensity: To compare the intensities of incorporation sites between different conditions, make sure to use the same imaging settings and labeling ratios. Additionally, you may use a fluorescent calibration standard. If you aim at quantifying the approximate number of incorporated tubulin dimers at a site, compare the intensity of the incorporation site to a reference stretch that contains a known number of fluorescently labeled tubulin dimers (*see* [12], Fig. S8).

4 Notes

1. Depending on the available equipment, the proposed method may be further simplified at various points. For example, the double-sided tape can be cut into stripes using a scalpel

(or ordered precut into stripes) if no plotter is available. Using an ultrasonic bath for coverslip and slide cleaning is optional; if no ultrasonic bath is available, we recommend putting a few drops of acetone on a tissue paper and thoroughly wiping the glass surface by hand before placing the coverslips and slides immersed in acetone on a laboratory shaker for 30 min. Pelleting the microtubule seeds helps to removed non-polymerized tubulin and short chunks, but if no ultracentrifuge is available, the seeds can be used without prior pelleting.

2. Once filled with solution, the chamber should not be allowed to dry out. During the incubation steps, in order to avoid drying out the chamber, leave excess liquid at the open ends and place the chamber next to a small petri dish filled with water inside a closed container.
3. Both microtubule growth and tubulin incorporation can be performed at different tubulin concentrations. During microtubule growth, the tubulin concentration determines the growth rate and the frequency of lattice defects, thus modulating the occurrence of sites along the lattice where preferential tubulin incorporation takes place. Once grown and capped, microtubules can be exposed to varying tubulin concentrations, with higher concentrations leading to more frequent as well as longer incorporation stretches. When leaving the microtubules with no free tubulin in the surrounding solution, tubulin loss from the lattice eventually leads to the disintegration of the microtubules. In this configuration, the setup can be used to specifically investigate tubulin loss as opposed to incorporation, e.g., by determining the lifetime of microtubules under different conditions. Even tubulin concentrations below the critical concentration for nucleation can, however, prevent microtubules from falling apart. We observed microtubules to remain stable for concentrations as low as 7 μM.
4. Tubulin incorporation into the microtubule lattice can be observed under various buffer conditions. It is therefore possible to adapt the described method to the specific requirements of the experimenter. For example, incorporation experiments can be carried out in BRB80 buffer instead of TicTac buffer if no other molecular players (such as MAPs) are present. If changing the buffer conditions, it may be necessary to adapt the tubulin concentrations accordingly.
5. Make sure to use high intensities and/or long exposure times to achieve good signal-to-noise ratios of the incorporation sites. Take multiple images (we recommend at least 3–5 images) of the same microtubule, as averaging over multiple images further improves the signal-to-noise ratio.

References

1. Mitchison T (1988) Microtubule dynamics and kinetochore function in mitosis. Annu Rev Cell Biol 4:527–549
2. Grishchuk EL, McIntosh JR (2006) Microtubule depolymerization can drive poleward chromosome motion in fission yeast. EMBO J 25:4888–4896
3. Mikhailov A, Gundersen GG (1998) Relationship between microtubule dynamics and lamellipodium formation revealed by direct imaging of microtubules in cells treated with nocodazole or taxol. Cell Motil Cytoskeleton 41:325–340
4. Ganguly A, Yang H, Sharma R, Patel KD, Cabral F (2012) The role of microtubules and their dynamics in cell migration. J Biol Chem 287:43359–43369
5. Tanaka EM, Kirschner M (1991) Microtubule behavior in the growth cones of living neurons during axon elongation. J Cell Biol 115:345–363
6. Sakakibara A, Andor R, Sapir T, Tanaka T (2013) Microtubule dynamics in neuronal morphogenesis. Open Biol 3:130061
7. Bowne-Anderson H, Hibbel A, Howard J (2015) Regulation of microtubule growth and catastrophe: unifying theory and experiment. Trends Cell Biol 25:769–779
8. Brouhard GJ (2015) Dynamic instability 30 years later: complexities in microtubule growth and catastrophe. Mol Biol Cell 26:1207–1210
9. Dye RB, Flicker PF, Lien DY, Williams RC (1992) End-stabilized microtubules observed in vitro: stability, subunit interchange, and breakage. Cell Motil Cytoskeleton 21:171–186
10. Mitchison T, Kirschner M (1984) Dynamic instability of microtubule growth. Nature 312:237–242
11. Théry M, Blanchoin L (2021) Microtubule self-repair. Curr Opin Cell Biol 68:144–154
12. Schaedel L, Triclin S, Chrétien D, Abrieu A, Aumeier C, Gaillard J, Blanchoin L, Théry M, John K (2019) Lattice defects induce microtubule self-renewal. Nat Phys 15:830–838
13. Dimitrov A, Quesnoit M, Moutel S, Cantaloube I, Poüs C, Perez F (2008) Detection of GTP-tubulin conformation in vivo reveals a role for GTP remnants in microtubule rescue. Science 322:1353–1356
14. Aumeier C, Schaedel L, Gaillard J, John K, Blanchoin L, Théry M (2016) Self-repair promotes microtubule rescue. Nat Cell Biol 18:1054–1064
15. de Forges H, Pilon A, Cantaloube I, Pallandre A, Haghiri-Gosnet AM, Perez F, Poüs C (2016) Localized mechanical stress promotes microtubule rescue. Curr Biol 26:3399–3406
16. Vemu A, Szczesna E, Zehr EA, Spector JO, Grigorieff N, Deaconescu AM, Roll-Mecak A (2018) Severing enzymes amplify microtubule arrays through lattice GTP-tubulin incorporation. Science 361:eaau1504
17. Dumont ELP, Do C, Hess H (2015) Molecular wear of microtubules propelled by surface-adhered kinesins. Nat Nanotechnol 10:166–169
18. Liang WH, Li Q, Rifat Faysal KM, King SJ, Gopinathan A, Xu J (2016) Microtubule defects influence kinesin-based transport in vitro. Biophys J 110:2229–2240
19. Triclin S, Inoue D, Gaillard J, Htet ZM, DeSantis ME, Portran D, Derivery E, Aumeier C, Schaedel L, John K, Leterrier C, Reck-Peterson SL, Blanchoin L, Théry M (2021) Self-repair protects microtubules from destruction by molecular motors. Nat Mater. https://doi.org/10.1038/s41563-020-00905-0
20. Aher A, Rai D, Schaedel L, Gaillard J, John K, Blanchoin L, Théry M, Akhmanova A (2020) CLASP mediates microtubule repair by promoting incorporation into damaged lattices. Curr Biol 30:2175–2183
21. Chrétien D, Metoz F, Verde F, Karsenti E, Wade RH (1992) Lattice defects in microtubules: protofilament numbers vary within individual microtubules. J Cell Biol 117:1031–1040
22. Chrétien D, Fuller F (2000) Microtubules switch occasionally into unfavorable configurations during elongation. J Mol Biol 298:663–676
23. Schaedel L, John K, Gaillard J, Nachury MV, Blanchoin L, Théry M (2015) Microtubules self-repair in response to mechanical stress. Nat Mater 14:1156–1163
24. Hyman A, Drechsel D, Kellogg D, Salser S, Sawin K, Steffen P, Wordeman L, Mitchison T (1991) Preparation of modified tubulins. Methods Enzymol 196:478–485