Imaging GTP-Bound Tubulin: From Cellular to In Vitro Assembled Microtubules

Hélène de Forges*,†,1, Antoine Pilon*‡,‡, Christian Pous∗,†,2 and Franck Perez∗,†,2

*Institut Curie, Paris Cedex 05, France
†CNRS UMR144, Paris Cedex 05, France
‡EA4530, Dynamique des microtubules en physiopathologie, Faculté de Pharmacie, Université Paris-Sud, Châtenay-Malabry, France
§Unité d’Hormonologie et Imunoanalyse, Pôle de Biologie Médicale et Pathologie, Hôpitaux Universitaires Est Parisien, APHP, Paris, France
*Laboratoire de Biochimie-Hormonologie, Hôpitaux Universitaires Paris-Sud, APHP, Clamart, France

CHAPTER OUTLINE

Introduction ............................................................................................................ 141
10.1 Imaging GTP Islands in Permeabilized Cells .....................................................142
  10.1.1 Permeabilization ........................................................................ 143
  10.1.2 MB11 Staining........................................................................... 144
  10.1.3 Cell Fixation and Colabeling ........................................................ 144
10.2 Imaging GTP Caps and GTP Islands Using Centrosome-Based Microtubule
  Assembly or Endogenous Microtubule Elongation in Permeabilized Cells ..........145
  10.2.1 Cell Plating, Culture, and Treatment ............................................ 145
  10.2.1.1 Prior to Assembly of Centrosome-Nucleated Microtubules .. 145
  10.2.1.2 Prior to Extracellular Microtubule Elongation....................... 145
  10.2.2 Microtubule Growth .................................................................... 145
10.3 Imaging GTP Islands in Microtubules Assembled In Vitro .........................147
  10.3.1 Microtubules Assembled in Suspension with GTP or GMPCPP ....... 147
  10.3.1.1 Separate Preparation of GMPCPP and GTP Microtubules ... 147
  10.3.1.2 Mixed GMPCPP and GTP Microtubules and Staining
     with MB11 Antibody........................................................................ 147

1,2Equal contributions
Abstract

Microtubules display a very dynamic behavior, and the presence of the guanosine-triphosphate (GTP) cap at the plus ends of microtubules is essential to regulate microtubule dynamics. Dimitrov et al. (2008) showed that GTP–tubulin is present not only at the plus ends but also in discrete locations along the microtubule lattice. These GTP islands were proposed to contribute to rescue events. Studying the localization of GTP–tubulin in microtubules is essential to better comprehend some core aspects in the regulation of microtubule dynamics. In this chapter, we recapitulate essential tools to study the GTP–tubulin using the recombinant antibody MB11 from permeabilized cells to in vitro assays.

Reagents

AMPPNP (Sigma, Ref: A2647)
Alexa 488-labeled goat antihuman antibody (Jackson Laboratories, Ref: 709-165-149)
Anti-tubulin antibody (DM1A; Sigma, Ref: T6199)
Anti-GTP–tubulin antibody (MB11)
ATP (Sigma, Ref: A3377)
Attofluor chamber (Invitrogen)
β-Mercaptoethanol (Sigma, Ref: M6250)
Casein (Sigma, Ref: C6905)
Catalase (Sigma, Ref: C40)
Dithiothreitol (DTT; Sigma, Ref: D9163)
D-Glucose (Sigma, Ref: D-3179)
DMSO (Sigma, Ref: 276855)
EGTA (EuroMedex, Ref: 1310-B)
Ethanol (Carlo Erba, Ref: 528151)
Gelatin (Sigma, Ref: G1393)
Glucose oxidase (Sigma, Ref: G2133)
Glycerol (bidistilled 99.5%; VWR ProLabo, Ref: 24388.295)
GMPCPP (Gena Biosciences, Ref: NU-405L)
GTP (Sigma, Ref: G8877)
Kinesin heavy chain (KHC) motor domain (Cytoskeleton, Inc., KR01)
Methanol (Carlo Erba, Ref: 414819)
NHS ester dyes Cy3 and Cy5 (GE Healthcare, Ref: PA23001 or PA25001)
Nocodazole (Sigma, Ref: M1404)
Paraformaldehyde (EuroMedex, Ref: 15710)
PIVES (EuroMedex, Ref: 1124)
Taxol (Paclitaxel; Enzo Life Sciences, Ref: BML-T104-005)
Triton X-100 (EuroMedex, Ref: 2000-C)

Buffers
- PEM: 80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.9
- PEM-G: PEM + 10% glycerol
- Permeabilization buffer: PEM + 10% glycerol + 0.01% Triton X-100
- PEM-T: PEM + 1 μM Taxol
- PEM-C: PEM + 0.2 mg/mL casein

INTRODUCTION

It is well known that microtubules are highly dynamic polymers, which display dynamic instability (Mitchison & Kirschner, 1984). The role of GTP–tubulin in the regulation of microtubule dynamics has long been known, and it was shown that hydrolysis of GTP at the plus ends of growing microtubules is necessary to trigger depolymerization (Hyman, Salser, Drechsel, Unwin, & Mitchison, 1992). Loss of the GTP cap exposes the unstable GDP-bound tubulin core and leads to depolymerization. Many teams have studied the stabilizing microtubule cap, both its nature and size. Among them, Bayley, Schilstra, and Martin (1990) proposed the lateral cap model based on the hypothesis that the addition of a GTP–tubulin at the end causes the hydrolysis of the GTP of the previous tubulin dimer, which becomes incorporated into the microtubule lattice (Bayley et al., 1990). Drechsel and Kirschner (1994) showed that a minimum of 40 subunits is necessary in the GTP cap to stabilize microtubules. In 1996, Caplow and Shanks proposed, measuring the minimum disassembly rates using guanylyl-(alpha, beta)-methylene-diphosphonate (GMPCPP) microtubules, that a cap composed of only 13 or 14 GTP–tubulin subunits is sufficient to stabilize microtubules (Caplow & Shanks, 1996). Panda, Miller, and Wilson (2002) confirmed this idea and proposed that the stabilizing cap is composed of a monolayer of Tubulin-GDP-Pi.

Once the important role of the GTP–tubulin cap was understood, studies linking the presence of a GTP cap to microtubule-associated proteins (MAPs) arose. For example, in an early study (Severin, Sorger, & Hyman, 1997), kinetochores were found to bind
preferentially to GTP microtubules. The authors polymerized chimeric microtubules composed of a GDP segment and GMPCPP ends and found that kinetochores display a higher affinity for microtubules plus ends. This suggested that structural features of the GTP-bound microtubule lattice could be recognizable. In the same study, it was confirmed that Taxol binding to tubulin mimics the GTP-bound conformation of tubulin dimers as previously showed by Arnal and Wade (1995). Indeed, kinetochores bind equally to GMPCPP or GDP portions of microtubules polymerized in the presence of Taxol. In a more recent example, Maurer, Bieling, Cope, Hoenger, and Surrey (2011) showed that EB1, a plus end-tracking protein, and Mal3, its homologue in yeast, bind effectively to GTP– and GTPγS–tubulin, but only weakly to GMPCPP seeds and to the GDP lattice of microtubules polymerized in vitro.

Such biochemical studies of the GTP cap were done in vitro, but in vivo studies have long been missing. To study the GTP cap in cells, we selected a recombinant antibody (MB11) using biotinylated GTPγS–tubulin as an antigen (Dimitrov et al., 2008). Using cosedimentation experiments or immunostaining of in vitro polymerized microtubules, MB11 was shown to recognize almost exclusively GTP-loaded microtubules (Dimitrov et al., 2008). Whether MB11 directly recognizes a particular conformation displayed by GTP–tubulin in the polymer (like EB or CLIP170 proteins do) or whether it recognizes some structural defects is still debated. MB11 efficiently stains the plus end of microtubules that are polymerizing and thus allowed to confirm the existence of the GTP cap at the plus ends of growing microtubules in cells. In addition, it led us to propose that GTP–tubulin is present not only at the plus ends but also in discrete dots along the microtubule lattice. We showed in cells that these GTP–tubulin islands are sites of rescue events. We thus proposed that GTP hydrolysis in microtubules might not always be complete and that, upon microtubule depolymerization, a GTP–tubulin island becomes exposed at the plus end and may behave as a GTP cap to promote a rescue event. The mechanisms of rescue involving GTP islands and the mechanisms of catastrophe are summarized and discussed in a recent review (Gardner, Zanic, & Howard, 2013), which points out the importance of rescues in the regulation of microtubule interactions with the cell cortex or with kinetochores.

Studying GTP–tubulin conformation in microtubules using the MB11 antibody is a technical challenge because the MB11 antibody does not recognize GTP–tubulin in microtubules after fixation, probably because fixation alters microtubule conformation. In this chapter, we present four protocols to study the GTP cap at microtubule plus ends and the GTP islands along the microtubule lattice using the anti-GTP–tubulin antibody MB11 in permeabilized cells and in in vitro assays.

10.1 IMAGING GTP ISLANDS IN PERMEABILIZED CELLS

MB11 is a conformational antibody. As such, it is very sensitive to antigen denaturation and the best staining results are obtained in permeabilized cells without fixation. To preserve the whole microtubule network, cells should be permeabilized
using a nonionic detergent in an effective microtubule-stabilizing buffer. Using only a PIPES-based buffer is not sufficient to preserve a large proportion of cellular microtubules for more than a few minutes, thus glycerol and optionally Taxol are also added throughout the labeling operations (Fig. 10.1). To allow multiple labeling, cell fixation can be achieved as soon as incubation with MB11 and consecutive rinses have been performed. GTP–tubulin staining with MB11 is also compatible with the expression of GFP–tubulin (Fig. 10.1).

10.1.1 Permeabilization

Cells are grown in culture medium at 37 °C, 5% CO₂. Replace the culture medium by warm permeabilization buffer. Incubate exactly 3 min at 37 °C. Wash twice 1 s with caution in PEM-G, holding the coverslip. Optionally, 1 μM Taxol can be added to the extraction, incubation, and washing solutions, especially if labeling is to be correlated with prior in vivo imaging. Note that, in contrast with what happens when Taxol is added during microtubule assembly or when using it at high concentration (Dimitrov et al., 2008), low doses of Taxol do not alter the number and length of GTP–tubulin islands \textit{a posteriori}. Put the coverslip directly on the drop of MB11 antibody.
Tips
Depending on the cell type, adjust the amount of Triton X-100 in the permeabilization buffer, between 0.03% and 1% so that cells remain attached to their substratum. Cells indeed tend to detach very easily after Triton permeabilization. All the steps performed prior to fixation must be made cautiously. As much as possible, do not aspirate or flow medium over the cells. Prefer holding the coverslips between tweezers and process by short immersions.

10.1.2 MB11 staining
Immunofluorescence of GTP–tubulin should be performed in a wet chamber at 37 °C. Drops of antibodies are deposited on a sheet of Parafilm in a closed and humid chamber.
Both MB11 and the antihuman secondary antibody are diluted in PEM-G supplemented with 2 g/L BSA.
After permeabilization, coverslips are put on a drop of MB11 antibody and incubated for 15 min at 37 °C. After incubation, prior to washes, gently add warm PEM-G under the coverslips before picking them up to prevent cells from detaching.
Wash out the primary antibody (3 times, 1 s) in a warm PEM-G bath.
Put the coverslips directly on the secondary antibody drop, on Parafilm, in the humid chamber. Incubate 15 min at 37 °C. Once again, lift slowly the coverslips using PEM-G, wash with caution in PEM-G, and fix the cells.

10.1.3 Cell fixation and colabeling
Cells can then be fixed either with methanol or with paraformaldehyde (PFA) depending on the needs of the experiments.
Fix the cells with cold methanol (−20 °C) for 4 min at −20 °C or with 3–4% PFA for 15 min at room temperature. Wash twice with PBS. Once the cells are fixed, a classical immunofluorescence can be performed for colabeling GTP islands with another staining. Note that cell permeabilization preserves the conformation of microtubules and of tubulin, but plus ends-tracking proteins (+TIPs) are lost from microtubule plus ends.

Tips
– After secondary antibody incubation and washes in PEM-G, put the coverslip on a sheet of Whatman paper to eliminate residual PEM-G. Directly immerse the coverslip in cold methanol.
– After the entire methanol is removed, quickly add PBS in one step. This step is very important to keep cells in good shape and image individual microtubules spread out in the cell.
10.2 IMAGING GTP CAPS AND GTP ISLANDS USING CENTROSOME-BASED MICROTUBULE ASSEMBLY OR ENDOGENOUS MICROTUBULE ELONGATION IN PERMEABILIZED CELLS

After microtubule depolymerization, centrosomes retain their capability to nucleate new microtubules in the presence of free GTP–tubulin once cells are permeabilized and cytosol proteins are extracted. This procedure was adapted from that used by Brinkley et al. (1981). Here, permeabilized cells are used to nucleate oriented microtubules and to control their regime of growth. A similar procedure can be undertaken using permeabilized cells without prior microtubule depolymerization to elongate microtubules in the pericellular region and observe their GTP-bound tubulin domains.

10.2.1 Cell plating, culture, and treatment

10.2.1.1 Prior to assembly of centrosome-nucleated microtubules

HeLa or RPE-1 cells are plated on glass coverslips coated with gelatin and cultured until they reach 50% confluence. Cellular microtubules are depolymerized in two steps: first cells are incubated at 37 °C for 1 h 30 min in 10 mM nocodazole and then put on ice for 2 h in the presence of nocodazole. The drug is then washed out by three rinses in ice-cold culture medium and immediately extracted on ice using PEM supplemented with 0.1% Triton X-100 (three extractions of 1 min). Cells are rinsed twice on ice with PEM to remove Triton.

10.2.1.2 Prior to extracellular microtubule elongation

Cells plated on gelatin and cultured as described above are permeabilized in the permeabilization buffer and rinsed twice in PEM-G without Triton.

10.2.2 Microtubule growth

For both assays, microtubule growth is performed using purified porcine brain tubulin (see Section 3.2.1) diluted at 0.25 g/L in PEM supplemented with 1 mM GTP. To elongate extracellularly endogenous microtubules, the addition of 200–500 μM adenosine triphosphate (ATP) greatly enhances the number of polymerization-competent microtubules (Infante, Stein, Zhai, Borisy, & Gundersen, 2000). For centrosome-nucleated microtubules, extracted cells are kept on ice before polymerization; tubulin assembly starts upon shifting the temperature to 37 °C.

In both assays, assembly is performed for 5–30 min. At the end of the assembly period, soluble tubulin is removed by gently washing coverslips with warm PEM-G and optionally with 1 μM Taxol prior to MB11 labeling, fixation, and colabeling if required. GTP–tubulin labeling is performed as described previously.
Tips
- Cell density should be kept below 50% of confluence to prevent massive cell detachment during the incubations, which is greatly enhanced by the incubation with tubulin.
- Gelatin coating not only helps keeping cell attachment to coverslips but also prevents excessive tubulin adsorption to the substratum and keeps tubulin staining background to reasonable levels.
- Microtubule number and length are limited by the concentration of tubulin added during the elongation phase. Tubulin concentration should be kept lower than 0.5 g/L to prevent excessive protein adsorption on cellular ghosts. To visualize internal GTP islands, growing microtubules to steady state is sufficient. To visualize GTP caps, labeling with MB11 should be performed while microtubules are still in a growth phase. This is usually obtained within the first 10–15 min of incubation, yielding an important amount of short microtubules. Note that some microtubules may also detach (spontaneously or mechanically during incubations and washes) and be observed in various cellular or even extracellular locations (Fig. 10.2).

**FIGURE 10.2**
GTP–tubulin labeling in microtubules nucleated from cellular centrosomes or elongated from cellular microtubules. (A) After complete microtubule disassembly with nocodazole and cold, RPE-1 cells were permeabilized and microtubules were grown from cellular centrosomes with GTP. Note the quite high tubulin staining in cell ghosts and the presence of detached microtubules (inset). Images were acquired on a Leica DMLB microscope. (B) HeLa cells were permeabilized and treated with ATP prior to extracellular microtubule elongation using purified tubulin and GTP. Images are confocal planes acquired using a Zeiss LSM510 microscope. Note that the cell from the right panel detached after fixation, but extracellularly elongated microtubules remained visible. Microtubules were stained for GTP–tubulin using MB11 (red) and total tubulin (green) using anti alpha-tubulin antibody (clone DM1A). GTP caps and internal GTP islands are indicated with arrows and arrowheads, respectively. Scale bar = 10 μm. Signals have been artificially increased for better visualization in the black and white version of the figure.
10.3 IMAGING GTP ISLANDS IN MICROTUBULES ASSEMBLED IN VITRO

In vitro techniques may also be used to visualize GTP–tubulin regions in microtubules. These approaches allow GTP island formation in the absence of MAPs or of insoluble cellular structures after detergent extraction. One of the following protocols also makes use of the GTP analogue GMPCPP, which locks the conformation of tubulin in a GTP-like state. GMPCPP is a slowly hydrolyzable molecule used formerly by Hyman and colleagues (Hyman et al., 1992) who showed that GTP hydrolysis is essential for depolymerization of microtubules and thus for dynamic instability.

10.3.1 Microtubules assembled in suspension with GTP or GMPCPP

10.3.1.1 Separate preparation of GMPCPP and GTP microtubules

Unlabeled and fluorescent tubulins (see Section 3.2) are used to polymerize GMPCPP and GTP microtubules separately. The ratio between fluorescent tubulin and nonfluorescent tubulin is 1:10. Tubulin diluted in PEM supplemented with 10 mM DTT is used at 2.5 g/L (final concentration) and incubated on ice with 1 mM GTP or 1 mM GMPCPP for 5 min. Both mixes are polymerized for 30–45 min at 37 °C. After polymerization, microtubules are diluted at least 50- to 100-fold in warm PEM-T.

10.3.1.2 Mixed GMPCPP and GTP microtubules and staining with MB11 antibody

Microtubules polymerized separately in the presence of each nucleotide are spun down at 30 °C for 10 min at 20,000 × g to remove unpolymerized tubulin. Both pellets are resuspended separately in a small volume of PEM-T and then one volume of GMPCPP microtubules is mixed with one volume of GTP microtubules. The MB11 antibody is added to the mixture, which is incubated for 15 min at 37 °C. Microtubules are centrifuged for 15 min at 20,000 × g at 30 °C and washed once in PEM-T. They are spun down again; the pellet is resuspended in PEM-T containing the secondary antibody and incubated 15 min at 37 °C.

After final pelleting for 10 min at 20,000 × g and resuspension in PEM-T supplemented with antifading agents, a drop of the microtubule suspension is directly observed without fixation at room temperature, with a fluorescence microscope.

Tips

– To pipet polymerized microtubules, use large pipette tips or cut the extremity of the tip so that pipetting does not break polymerized microtubules.
– A small amount of Taxol (1 μM) allows stabilization of the microtubules without changing their conformation as described above. Adding large amounts of Taxol will induce changes in microtubule conformation and such microtubules will be stained all along the lattice by the MB11 antibody.
– The supernatants over pelleted microtubules must be taken off with great care to avoid losing microtubules at each cycle of centrifugation between labelings and washes.

10.3.2 Microtubules elongated from Taxol seeds immobilized on kinesin-coated glass

10.3.2.1 Preparation of MAP-free tubulin
MAP-free tubulin is purified from porcine brain as described previously by Walker et al. (1988). MAPs are removed by phosphocellulose chromatography and tubulin aliquots are frozen in liquid nitrogen and stored at $-80 \, ^\circ$C. For each experiment, an aliquot of purified tubulin is thawed on ice and centrifuged at 20,000 $\times$ g for 20 min to sediment aggregated tubulin, which is discarded.

10.3.2.2 Fluorescent–tubulin labeling
Tubulin is labeled with Cy3 or Cy5 monoreactive NHS ester dyes using a protocol adapted from Peloquin, Komarova, and Borisy (2005). For one labeling, 500 $\mu$g of MAP-free tubulin (10 $\mu$g/µL) is polymerized with 1 mM GTP for 30 min at 37 $\, ^\circ$C. One vial of Cy3 or Cy5 monoreactive dye is solubilized in 20 $\mu$L DMSO and polymerized microtubules are incubated for 15 min at 37 $\, ^\circ$C with 5 $\mu$L of this solution. Microtubules are then depolymerized for 10 min on ice and labeled tubulin is separated from the excess of unconjugated dye by two cycles of assembly–disassembly. Tubulin-labeled aliquots are frozen in liquid nitrogen and stored at $-80 \, ^\circ$C.

The advantage of this protocol is that tubulin is labeled in polymerized microtubules and dyes cannot react with primary amines involved in the interaction between tubulin heterodimers during microtubule polymerization. Thus, labeled tubulin is not excluded from microtubules when combined with unlabeled tubulin in the polymerization protocols described above. We could not exclude however that tubulin is labeled on sites important for MAP interactions. In polymerization studies, we used less than 25% of labeled tubulin to avoid the inhibition of MAP interactions with microtubules due to the presence of the fluorochrome.

10.3.2.3 Recombinant KHC reconstitution
In these experiments, microtubules are specifically bound on glass coverslips using the interaction with recombinant, active KHC. We use a protocol described for microtubule gliding motility assays (Howard, Hunt, & Baek, 1993), with minor modifications. Classical gliding assay using ATP is first used to determine the polarity of microtubule seeds. Then, microtubule growth is monitored in the absence of movement because of kinesin loading with the nonhydrolyzable ATP analogue, AMPPNP, which allows microtubule/kinesin interaction but prevents nucleotide hydrolysis necessary for microtubule displacement.
Lyophilized KHC is resuspended at 5 μg/μL in kinesin reconstitution buffer composed of 100 mM PIPES pH 7.0, 200 mM KCl, 2 mM MgCl₂, 1 mM DTT, 20 μM ATP. 0.5 μL Aliquots are frozen in liquid nitrogen and stored at −80 °C.

For use, one aliquot of kinesin is diluted at 0.5 mg/mL in PEM buffer containing 0.2 mg/mL casein (PEM-C) and 20 μM ATP. Then kinesin is diluted at 10 μg/mL in PEM-C containing 100 μM ATP.

Tips
Casein is used to avoid nonspecific interactions between microtubule seeds or free tubulin with glass coverslips. It is poorly soluble at neutral pH, but it can be rapidly dissolved after effective alkalinization with 10N KOH. After complete solubilization, pH is set back to 6.9 using HCl.

10.3.2.4 Taxol-stabilized microtubule seeds polymerization
To prepare stabilized microtubule seeds as templates to nucleate microtubule growth, we use an alternative method to GMPCPP-stabilized microtubule seeds. Seeds are stabilized using Taxol for easiness and swiftness.

A 4 g/L mix of Cy5-labeled tubulin and nonlabeled tubulin (with a 1:10 ratio of labeled to unlabeled tubulin) is incubated for 15 min at 37 °C with 1 mM GTP and 10% DMSO. These conditions allow the spontaneous polymerization of microtubule seeds. After incubation, microtubule seeds are diluted to 1:100 in PEM containing 10 μM Taxol. These seeds can be stored for 2 days at room temperature.

10.3.2.5 Preparation of the incubation chambers
Sample chambers are prepared using standard glass microscopy coverslips. Coverslips are washed consecutively in 10N HCl, Nanopure water, and 90% ethanol and air-dried. A 2- to 3-mm-width channel is delimited by two strips of adhesive tape on a 25-mm-diameter glass coverslip. A 12-mm-diameter coverslip is clamped on the first one with two additional strips of tape. The solutions are perfused in the incubation chamber using a pipette and a filter paper to aspirate the solutions at the opposite end of the channel. The incubation chamber is then placed in an Attofluor chamber for observation under the microscope.

10.3.2.6 Binding of microtubule seeds and microtubule elongation

10.3.2.6.1 Nonspecific kinesin immobilization
The 10 μg/mL kinesin solution is incubated for 5 min at room temperature in the chamber to allow the adsorption of motor protein on glass. The chamber is then washed three times with PEM containing 0.5 mg/mL casein, and this solution is incubated for 5 min at room temperature to block the nonspecific adsorption of microtubule seeds and of free tubulin added in the experiments. It also prevents kinesin-1 from denaturation (Ozeki et al., 2009).
10.3.2.6.2 Attachment of microtubule seeds and elongation

Microtubule seed suspension is diluted 10 times in PEM containing 10 μM Taxol and 100 μM ATP. Once microtubule seeds are loaded, the chamber is placed at 37 °C on a Zeiss LSM510 confocal microscope (Heidelberg Germany). Microtubule seed attachment and movement are monitored by acquisition of the Cy5 fluorescent signal (63 × 1.4 NA objective).

Seed gliding on kinesin-coated glass allows the unambiguous identification of microtubule plus and minus ends: because immobilized kinesin walks toward the plus ends, the seeds move with minus ends in front. To stop seeds from gliding prior to elongation, the chamber is washed twice with PEM-C supplemented with 10 μM Taxol and 1 mM AMPPNP.

To elongate microtubule seeds, a 1 g/L mix of unlabeled and Cy3-labeled tubulin (with a 1:4 ratio of labeled to unlabeled tubulin) in PEM supplemented with 1 mM GTP is perfused twice in the chamber to eliminate Taxol and to avoid stabilization of growing microtubules. To monitor microtubule elongation, time-lapse sequences are acquired during 20 min. Microtubules that elongate at the plus and minus ends of the seeds interact with the kinesin adsorbed on glass and polymerize parallel to the confocal plane. At the end of microtubule growth, free tubulin is removed by washing the chamber with PEM-G containing 1 μM Taxol.

Tips

- Imaging of microtubule polymerization is done at 37 °C. Placing a humid lid over the incubation chamber so that liquid in the channel does not evaporate from its extremities is strongly recommended.
- We also recommend using an oxygen scavenger cocktail (20 mM D-glucose, 0.02 mg/mL glucose oxidase, 0.08 mg/mL catalase, and 0.5% β-mercaptoethanol). Oxygen scavenging protects dyes from photobleaching and prevents tubulin oxidation, which can cause the spontaneous breaking and depolymerization of microtubules. This cocktail is added to every solution injected in the chamber before imaging. It has to be used within 1 h after preparation.

To label GTP–tubulin, we use the protocol described above (see Section 1.2). After labeling, microtubules can be imaged directly or fixed with methanol for further analysis (Fig. 10.3).

10.4 DISCUSSION AND FUTURE PROSPECTS

The MB11 conformational antibody specifically binds to GTP–tubulin. It is a unique tool to study the distribution of GTP–tubulin islands along microtubules and their implication in microtubule dynamics. Although the exact structure that the antibody recognizes in tubulin is not yet known, MB11 may prove to be a powerful tool to study modifications in the microtubule network depending on changes in the cell status in pathophysiological conditions (e.g., differentiation, division, migration).
Certain microtubule-stabilizing MAPs, like the Von Hippel–Lindau antioncogene (Thoma et al., 2010), may modulate the occurrence of microtubule internal GTP islands. It suggests that cells may finely regulate rescue events by controlling the local density of GTP islands. Understanding how GTP islands occur in microtubules and if other MAPs facilitate their persistence is an important challenge. Conversely, understanding whether GTP-bound tubulin in the internal regions of microtubules or in the GTP cap of growing microtubules can be readily recognized by +TIPs to control microtubule elongation and rescues will be of great interest to get a more comprehensive view of rescue events. However, colabelings of GTP–tubulin and +TIPs in living cells are still an open challenge. Gaining access to such information will be valuable, for example, to measure the actual size of the GTP cap or to evaluate whether GTP–tubulin conformation is immobile or can propagate along microtubules.

Acknowledgments

We thank A. Dimitrov (Institut Curie, Paris) who contributed to the setup of some of the protocols described here. We thank L. Sengmanivong for support in SIM microscopy and
acknowledge the Nikon Imaging Centre at Institut Curie. The authors also thank V. Nicolas (Institut Fédératif de Recherche IPSIT, Univ. Paris-Sud 11) for helpful advice in imaging in vitro assembled microtubules on the LSM510 microscope. F. P. team is supported by the Centre National de la Recherche Scientifique and by the Institut Curie and by grants from the Agence National de la Recherche, the Fondation pour la Recherche Médicale, and the Institut National du Cancer. H. F. is supported by the Ministère de l’Enseignement Supérieur et de la Recherche and the Fondation ARC pour la Recherche sur le Cancer.

References


