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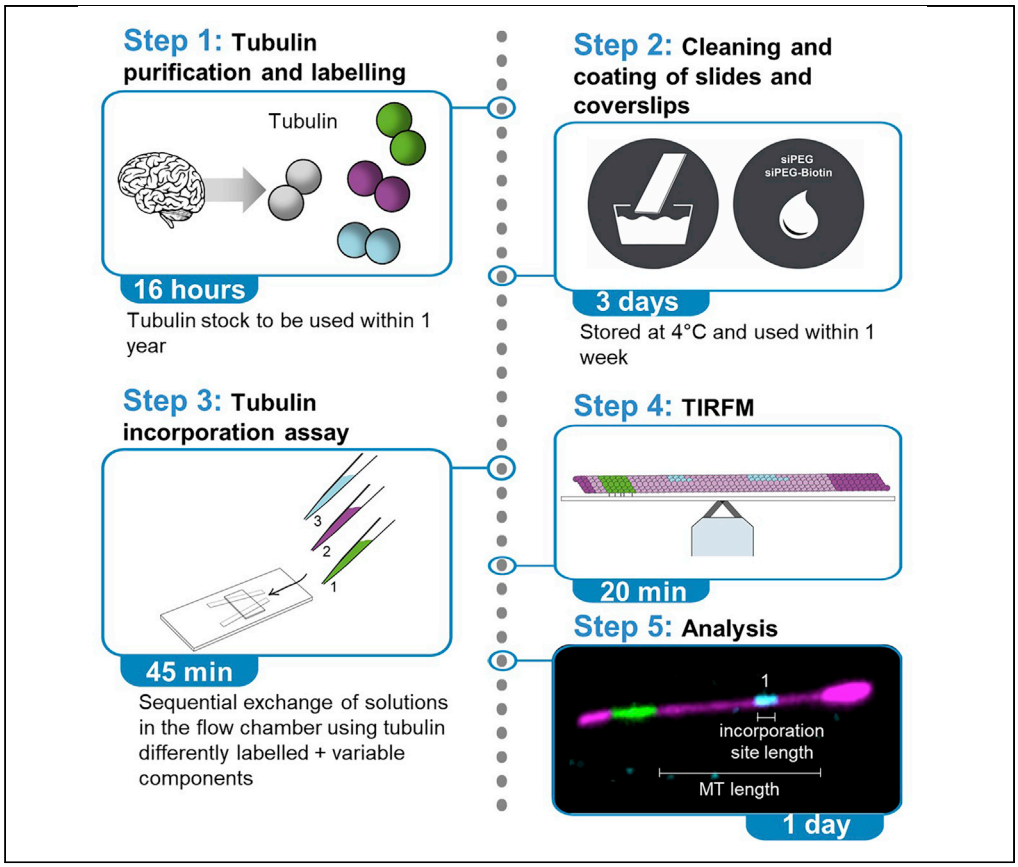
Two-color *in vitro* assay to visualize and quantify microtubule shaft dynamics



Microtubules are dynamic polymers where tubulin exchanges not only at the ends but also all along the microtubule shaft. *In vitro* reconstitutions are a vital approach to study microtubule tip dynamics, while direct observation of shaft dynamics is challenging. Here, we describe a dual-color *in vitro* assay to visualize microtubule shaft dynamics using purified, labeled bovine brain tubulin. With this assay, we can quantitatively address how proteins or small molecules impact the dynamics at the microtubule shaft.

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Highlights

Dual-color *in vitro* assay to visualize microtubule shaft dynamics

Step-by-step guide to purify and label brain tubulin

Assembly of a flow chamber of passivated slides and functionalized coverslips

Steps for analysis of microtubule shaft dynamics

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Two-color *in vitro* assay to visualize and quantify microtubule shaft dynamics

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SUMMARY

Microtubules are dynamic polymers where tubulin exchanges not only at the ends but also all along the microtubule shaft. *In vitro* reconstitutions are a vital approach to study microtubule tip dynamics, while direct observation of shaft dynamics is challenging. Here, we describe a dual-color *in vitro* assay to visualize microtubule shaft dynamics using purified, labeled bovine brain tubulin. With this assay, we can quantitatively address how proteins or small molecules impact the dynamics at the microtubule shaft.

For complete details on the use and execution of this protocol, please refer to Andreu-Carbó et al. (2022).

BEFORE YOU BEGIN

The tubulin incorporation assay below describes how to visualize tubulin dimer exchange along a GDP-microtubule shaft (Figure 1). This assay allows to reconstitute how proteins impact microtubule shaft dynamics (Andreu-Carbó et al., 2022). In addition, you can study how small molecules, temperature, salt concentration and pH impact shaft dynamics. For many in vitro experiments, microtubules are stabilized with Taxol or GMPCPP to simplify the assay. However, stabilization of the microtubule shaft conceals tubulin exchange along the shaft. With the incorporation assay we combine microtubule stabilization approaches with dynamic microtubules. This approach enables to study shaft dynamics of GDP-microtubules, while the ends are protected against depolymerization by stabilizing caps. To observe shaft dynamics in real-time with this assay is not possible, as imaging will be performed at given fixed time points of the reaction. The incorporation assay allows to measure quantitatively the impact of different parameters on shaft dynamics at given time points.

Tubulin purification from bovine brain

Timing: 2–3 days

Tubulin was purified from fresh bovine brain by two cycles of polymerization and depolymerization, with a high-molarity buffer (Castoldi and Popov, 2003). Purified tubulin was labeled with biotin (Hyman et al., 1991), or fluorescent labels: ATTO-488, ATTO-565, or ATTO-647.

Note: Prepare all buffers just before the purification. Pre-warm (37°C) or pre-cool (4°C) all buffers before starting the purification. Prepare working space and pre-warm or pre-cool all equipment the day before the purification.

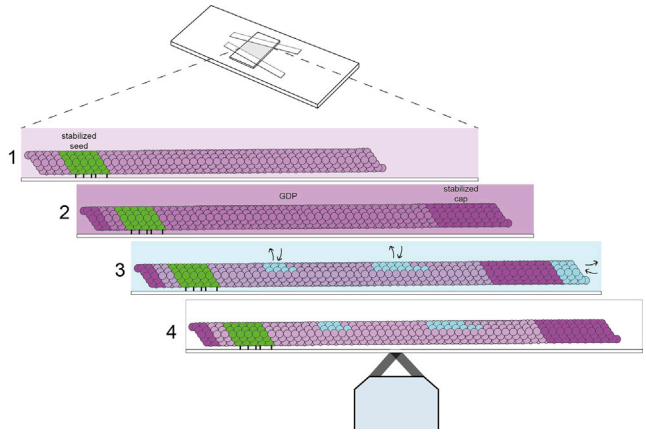

Figure 1. Schematic representation of the successive steps in the tubulin incorporation assay, using a flow chamber.
GDP-Microtubules (light magenta) are grown from seeds (green) and end-stabilized by a GMPCPP-cap (dark magenta) in the flow chamber [1–2]. The solution is exchanged by flowing in tubulin labeled with a different fluorophore (cyan), in the presence or absence of microtubule binding proteins [3]. Washing steps are required to visualize tubulin incorporation along the microtubule shaft with a TIRF microscope (incorporation sites, cyan) [4].

1. Homogenization and 1st Cold Spin:
   a. Harvest two fresh and still warm Bovine brains from a slaughterhouse and transport them in a solution containing 1.5% NaCl on ice.

   Note: Time between slaughter and purification should be kept minimal, stay under 4 h.

   b. In the cold room:
      i. Remove meninges, clots, fat and brain stem by hand. Weigh brains, put them in a blender, add **Depolymerization Buffer (DB)** (100 mL of DB per 100 g of brain) and add 100 µL of ß-Mercaptoethanol per 100 g of brain tissue.

   Note: Wear gloves and coat.

      ii. Homogenize twice for 20 s with a short break in between, so that the blender is not getting warm. Use a conventional kitchen blender at full speed.
   c. To reduce the volume for ultracentrifugation, perform a pre-spin. Fill pre-cooled 500 mL centrifuge bottles with the homogenate solution (expected total volume is around 1.5–2 L) and spin it at 6,693 × *g* for 30 min at 4°C (e.g., Beckman Coulter JLA 10.5).

   Note: If enough ultracentrifuges are available, skip step 1c.

   d. Transfer the supernatant into pre-cooled ultracentrifuge tubes and centrifuge at 160,000 × *g* for 60 min at 4°C (e.g., Beckman Ti45).
   e. In the cold room, decant the supernatant it into a pre-cooled 2 L cylinder. Measure the volume of the supernatant.

2. 1st Polymerization:
   a. Add pre-warmed **High-Molarity PIPES Buffer (HMPB)** and pre-warmed Glycerol to the supernatant at 1:1:1 (v/v) ratio. Supplement the solution with 1.5 mM ATP and 0.5 mM GTP, from a 0.2 M ATP and 0.2 M GTP stock solution.
   b. To rapidly bring the solution to 37°C, close the cylinder with parafilm, transfer it into a sink and rock it slowly with hands under running 50°C water. Transfer the warm solution into a pre-warmed 2 L flask and incubate it in a 37°C water bath for 1 h, to induce polymerization. Swirl the flask every 5 min during the incubation.

Note: During the rapid prewarming of the solution, feel regularly with your hand that the solution is not higher than your body temperature.

⚠ CRITICAL: During polymerization and depolymerization reactions try not to make bubbles in the solution.

c. Transfer the solution containing polymerized microtubules into pre-warmed ultracentrifuge tubes and centrifuge at 160,000 × *g* (max. speed) for 30 min at 37°C using a pre-warmed rotor (e.g., Beckman Ti45).
d. Discard all the supernatant by aspiration with a pipet.

3. 1st Depolymerization, in the cold room:
   a. Harvest the pellet (microtubules) with a spatula into a homogenizer: use a 5 mL pipet to rinse the centrifugation tubes with 5 mL of cold DB. Transfer the microtubule containing solution into a pre-cooled glass homogenizing potter. Rinse again with 5 mL DB. The total volume of combined samples should be < 50 mL.
   b. To induce microtubule depolymerization, homogenize the viscous solution with the potter on ice, by moving the pestle slowly 20 times up and down. Incubate the solution containing depolymerized microtubules for 15 min on ice.
   c. Measure the concentration at the nanodrop (OD_{280}).

Note: The extinction coefficient of tubulin is 115.000 $M^{-1}cm^{-1}$ assuming that the molecular mass of tubulin heterodimer is 100.000 Dalton.

   d. Dilute the solution to a final concentration of 20 mg/mL.
   e. Homogenize again the solution with the potter, and incubate it for 15 min more.
   f. Fill pre-cooled ultracentrifuge tubes with the solution and centrifuge at 160,000 × *g* for 30 min at 4°C (e.g., Beckman Ti60).
   g. Harvest the supernatant, containing tubulin, into a pre-cooled 500 mL cylinder (measure the supernatant volume).
   h. Discard the pellet.

Note: Expect a large pellet from the cold spin.

4. 2nd Polymerization:
   a. Add pre-warmed **High-Molarity PIPES Buffer** (**HMPB**) and pre-warmed Glycerol to the supernatant at 1:1:1 (v/v) ratio. Supplement the solution with 1.5 mM ATP and 0.5 mM GTP.
   b. To rapidly bring the solution to 37°C, close the cylinder with parafilm, transfer it into a sink and rock it slowly with hands under running 50°C water. Incubate it in a 37°C water bath for 1 h to induce polymerization. Swirl the cylinder every 5 min during the incubation.

Note: The volume reduces in the second cycle. Upon microtubule polymerization the solution becomes viscous, air bubbles rise slower.

   c. Centrifuge the solution at 160,000 × *g* for 30 min at 37°C using a pre-warmed rotor (e.g., Beckman Ti60).
   d. Discard the supernatant.

5. 2nd Depolymerization, in the cold room:
   a. Harvest the pellet containing microtubules with a spatula into a pre-cooled homogenizing potter. Rinse the ultracentrifuge tubes with 5–10 mL of BRB80 0.25× (from BRB80 20×) and transfer it into the homogenizer.

Note: Microtubule depolymerize more efficiently in presence of BRB80 0.25× than BRB80 1×.

Note: The volume of buffer used for the resuspension is chosen so that the final tubulin concentration is 20 mg/mL.

b. Homogenize the pellet as in 3b with the potter on ice, to induce the 2nd depolymerization and incubate it for 15 min.
c. Add BRB80 5× to adjust the buffer to BRB80 1×, homogenize it another time and incubate it for 15 min more.

Note: The volume of BRB80 5× to add is 3/16th of the volume of BRB80 0.25× with tubulin.

d. Centrifuge the solution at 105,000 × *g* for 20 min at 4°C (e.g., Beckman TLA100.3).
e. Harvest the supernatant containing tubulin into a Falcon and measure the final tubulin concentration with the nanodrop (OD_{280}). Optimal concentration is between 130 – 200 μM.
f. Aliquot black tubulin over liquid nitrogen, in single-use aliquots (5 μL) or in 1 mL aliquots (for labeling).
g. Snap freeze aliquots and store them in liquid nitrogen.

Note: Tubulin labeling can be performed on the same day (avoiding freezing/thawing steps) or later. Fluorochrome labeling and Biotin labeling can be performed at the same time. Purification plus labeling takes at least 18 h from slaughterhouse to tubulin freezing.

Note: If labeling is performed at the day of the purification, microtubules are taken at the end of the second polymerization cycle (before step 5) and directly used in step 6c (Fluorochrome-Labeled Tubulin) or 12 (Biotin-Labeled Tubulin).

Pause point: Once snap freeze, tubulin aliquots can be stored up to one year.

6. Fluorochrome-Labeled Tubulin, 1st Polymerization:
   a. Thaw purified tubulin on ice (60–100 mg per labeling) and dilute it with pre-warmed **Glycerol PB** to have a final concentration of 30 μM tubulin.
   b. Incubate tubulin solution in a water bath for 30 min at 37°C to induce polymerization.
   c. Layer carefully microtubule solution with a 5 mL pipet onto pre-warmed cushions of 0.1 M NaHEPES at pH 8.6, 1 mM $MgCl_2$, 1 mM EGTA and 60% (v/v) Glycerol.

Note: The volume of the cushion has to be 60% of the total volume of the ultracentrifuge tube (e.g., 12 mL of cushion for Beckman Ti60 ultracentrifuge tubes).

Note: Fill first the ultracentrifuge tubes with the pre-warmed cushion and then layer the microtubule solution on it.

⚠ CRITICAL: Avoid unnecessary pipetting of the solution containing microtubules to prevent depolymerization.

   d. Centrifuge microtubule solution at 161,000 × *g* for 30 min at 37°C, using a pre-warmed rotor (e.g., Beckman Ti60).

Note: For the next steps work in a 37°C water bath to reduce microtubule depolymerization as much as possible.

e. Aspirate the supernatant, and wash the cushion 2 times (~ 2 mL) with pre-warmed **Resuspension buffer (RB).**

Note: Always aspirate from the top of the solution to minimize mixing of different buffers.

f. Resuspend microtubule pellet in minimal volume (100–200 µL per microtubule pellet, do not use more than 1 mL in total) with pre-warmed **RB** by pipetting up and down with a cut-off yellow pipette tip.

⚠ CRITICAL: Pipet carefully while keeping the solution warm in a water bath, to avoid depolymerization of microtubules.

Note: Adjust the RB volume so that the tubulin concentration is around 50 µM.

7. Fluorochrome-Labeled Tubulin, Labeling:
   a. Prepare dyes (e.g., ATTO-488, 565, 647 or equivalent) just immediately before usage.
   b. Bring dyes to 24°C.
   c. Resuspend them with anhydrous DMSO to 100 mM NHS-fluorochrome (e.g., 5 mg of fluorophore into 100 µL).
   d. Add fluorochrome solution 1/10 (v/v) to the solution containing resuspended microtubules, while pipetting to ensure rapid and complete mixing.
   e. Incubate for 10 min at 37°C.
   f. Mix the solution by pipetting with a cut-off yellow pipette tip every 2 min.
   g. Stop the reaction by decreasing the pH, by adding 1:1 (v/v) of BRB80 2× containing 100 mM potassium glutamate and 40% (v/v) glycerol.
   h. Mix it properly.
   i. Layer carefully the solution with a pipet onto pre-warmed BRB80 cushions (BRB80 1×, 60% (v/v) Glycerol).

Note: The volume of the cushion has to be 60% of the total volume of the ultracentrifuge tube (e.g., 2.1 mL of cushion for Beckman TLA100.3 ultracentrifuge tubes).

   j. Centrifuge it at 265,000 × *g* for 30 min at 37°C using a pre-warmed rotor (e.g., Beckman TLA100.3).
   k. Aspirate the supernatant and wash the cushion 2 times with 500 µL ddH_2O.

8. Fluorochrome-Labeled Tubulin, 1st Depolymerization:
   a. Aspirate carefully the cushion and resuspend the pellet with cold BRB80 0.25× on ice. Pipet up and down to induce microtubule depolymerization.

Note: The resuspension volume was chosen to have a concentration between 20-30 mg/mL.

   b. Incubate resuspended solution on ice for 15 min to induce depolymerization.
   c. Measure the concentration with the nanodrop (OD_{280}).

Note: The extinction coefficient of tubulin is 115,000 $M^{-1}cm^{-1}$ assuming that the molecular mass of tubulin heterodimer is 100,000 Dalton. The OD of the fluorochrome was also measured with a specific extinction coefficient in function of the dye.

   d. Add BRB80 5× to adjust the buffer to BRB80 1×. Incubate for 15 min on ice.
   e. Centrifuge the tubulin solution at 160,000 × *g* for 15 min at 4°C (e.g., Beckman TLA100.3).

Note: At this step of depolymerization about $^1/_2$ of the initial tubulin is lost.

9. Fluorochrome-Labeled Tubulin, 2nd Polymerization:
   a. Dilute the supernatant containing tubulin with pre-warmed **Glycerol PB** to have a final concentration of 30 μM tubulin.
   b. Incubate the solution in a water bath at 37°C for 30 min.
   c. Layer carefully the solution with a pipet onto pre-warmed BRB80 cushions (BRB80 1×, 60% (v/v) Glycerol).
   d. Centrifuge it at 265,000 × *g* for 30 min at 37°C using a pre-warmed rotor (e.g., Beckman TLA100.3).
   e. Aspirate the supernatant and wash the cushion 2 times with 500 μL ddH_2O.

10. Fluorochrome-Labeled Tubulin, 2nd Depolymerization:
   a. Aspirate carefully the cushion and resuspend the pellet with cold BRB80 0.25× on ice.

Note: The volume of the buffer is chosen to have a tubulin concentration of 10–30 mg/mL.

   b. Incubate resuspended solution on ice for 15 min to induce depolymerization.
   c. Add BRB80 5× to adjust the buffer to BRB80 1×.
   d. Centrifuge the tubulin solution at 160,000 × *g* for 15 min at 4°C (e.g., Beckman TLA100.3).
   e. Measure the concentration with the nanodrop (OD_{280}). Aliquot tubulin at a concentration between 80 – 150 μM.
   f. Calculate tubulin concentration:

$$\textit{Tubulin concentration}\ (\mathrm{M}) = \frac{A_{280} - (A^* \times CF^*_{280})}{\epsilon_{\mathrm{Tubulin}}} \times \text{dilution factor}$$

Calculate degree of labeling:

$$\textit{Moles dye per mole protein} = \frac{A^* \times \text{dilution factor}}{\epsilon^* \times \textit{tubulin conenctration}\ (M)}$$

$\epsilon_{tubulin}$ = protein molar extinction coefficient (115,000 $M^{-1}cm^{-1}$ assuming that the molecular mass of tubulin heterodimer is 100,000 Dalton).
CF = Correction factor.

Note: A*, CF*, and ϵ* are dye specific values which can be found on the datasheet supplied by the manufacturer (ATTO-488, ATTO-565, ATTO-647).

Note: Expected labeling efficiency is around 90%

   g. Aliquot fluorochrome labeled tubulin over liquid nitrogen in single-use aliquots (5 μL) and store them in liquid nitrogen.

11. Biotin-Labeled Tubulin, 1st Polymerization:
   a. Thaw purified tubulin (60–100 mg per labeling) and dilute it with pre-warmed **Glycerol PB** to have a final concentration of 30 μM tubulin.
   b. Incubate tubulin solution in a water bath for 30 min at 37°C to induce polymerization.
12. Biotin-Labeled Tubulin, Biotinylation:
   a. Dissolve Biotin reagent at 0.1–0.2 M in anhydrous DMSO.
   b. Add Biotin solution to polymerized microtubules, to a final concentration of 2 mM, while pipetting to mix rapidly.
   c. Incubate for 20 min at 37°C, while mixing occasionally the solution with a cut-off yellow pipette tip.
   d. Stop the reaction by adding 100 mM potassium glutamate.
   e. Layer carefully the solution onto pre-warmed BRB80 cushions (BRB80 1×, 60% (v/v) Glycerol).

f. Centrifuge it at 265,000 × *g* for 30 min at 37°C using a pre-warmed rotor (e.g., Beckman TLA100.3).
g. Aspirate the supernatant and wash the cushion 2 times with 500 μL ddH_2O.

13. Biotin-Labeled Tubulin, 1st Depolymerization:
    a. Repeat step 8.
14. Biotin-Labeled Tubulin, 2nd Polymerization:
    a. Repeat step 9.
15. Biotin-Labeled Tubulin, 2nd Polymerization:
    a. Repeat steps 10a–10e.
    b. Adjust Biotin labeled tubulin concentration around 100–200 μM.
    c. Aliquot biotin labeled tubulin over liquid nitrogen in single-use aliquots (5–10 μL) and store them in liquid nitrogen.

Pause point: Once snap freeze, tubulin aliquots can be stored up to one year.

Cleaning and silanization of glass slides and coverslips

Timing: 3 days

In this step we describe the preparation of clean slides and coverslips with subsequent siPEG surface coating, to block non-specific protein binding to glass surfaces and to functionalize glass surfaces.

Note: Ensure that slides and coverslips do not contact each other, to allow full exposure to solutions.

Note: Work in a clean environment. To prevent dust accumulation, close the containers in all of the incubation steps. Wear gloves to avoid fingerprint on the glass.

16. Place slides and coverslips (76 × 26 mm) in glass spacers (e.g., glass staining trays for 10 slides of approx. 76 × 26 mm; Assistent) with the help of tweezers.
17. Transfer the trays into glass beakers (e.g., glass staining jars; Assistent) and fill them with 1 M NaOH until slides and coverslips are fully immersed.
18. Transfer glass jars containing slides and coverslips into a sonicator bath (e.g., Bandelin Sonorex RK514 225 W).
19. Sonicate for 40 min.
20. Wash the glass trays containing slides and coverslips through three sequentially bidistilled water (ddH_2O) baths:
    a. With the help of a wire handle, submerge and remove the tray in the first ddH_2O bath sequentially for at least 5 times.

Note: Taking care that most of the water dripped off from the glass surfaces before submerging it again.

    b. Repeat these washing steps in the other two ddH_2O baths.

21. Dry slides and coverslips using compressed air.
22. Transfer racks to a new glass jar and fill it with 96% Ethanol until the slides and coverslips are fully immersed.
23. Repeat steps 18–20.
24. Dry slides and coverslips completely with compressed air.

⚠ CRITICAL: Slides and coverslips need to be completely dry before step 25.

Note: Alternative methods to clean glass surfaces could be used, such as Piranha solution (Altavilla et al., 2008).

25. Place slides and coverslips in a plasma cleaner (e.g., Electronic Diener, Plasma surface technology) for 3:30 min at 80% power.
26. Place the slides into fresh glass beaker (e.g., staining jar; Assistent) and fill it with a coating solution, tri-ethoxy-silane-PEG (siPEG; 1 mg/mL in 96% ethanol and 0.02% HCl).
27. Place the coverslips into another glass beaker (e.g., staining jar; Assistent) and fill it with a premixed coating solution of 20% tri-ethoxy-silane-PEG-biotin and 80% tri-ethoxy-silane (from now and on siPEG-biotin; 1 mg/mL in 96% ethanol and 0.02% HCl).
28. Incubate slides and coverslips submerged in coating solutions for 48 h on a rocking platform, at 24°C and protected from light.

Note: Seal the glass beakers with Parafilm and aluminum foil, to avoid evaporation of the coating solutions.

Note: siPEG and siPEG-biotin coating solutions can be reused for about 4 months. Store glass bottles at 24°C and protected from light.

29. After ~2 days of incubation, wash the silanized slides and coverslips:

Note: Slides and coverslips will be washed individually. Use tweezers to transfer and wash them.

a. Submerge slides and coverslips in a glass beaker filled with 96% ethanol for few seconds.
b. Transfer them into the first ddH_2O bath.
c. Submerge and remove slides and coverslips in first ddH_2O bath sequentially for at least 5 times.

Note: Remove slowly the slides and coverslips from the water taking care that most of the water dripped off from the glass surfaces before submerging it again.

d. Repeat these washing steps in the other two ddH_2O baths.

30. Dry carefully slides and coverslips completely with compressed air.

⚠ CRITICAL: Dry glass surfaces gently with compressed air, parallel to surfaces to prevent coating detachment.

31. Store slides and coverslips in clean containers sealed with Parafilm at 4°C.

Pause point: Silanized slides and coverslips can be stored up to 1 week. The antifouling properties of the coating decreases over time.

Preparation of seeds using GMPCPP-tubulin

Timing: 2 h

In this step we prepare short, stabilized microtubules (hereafter called seeds) by polymerizing tubulin in the presence of GMPCPP, a slowly hydrolysable GTP analog (Hyman et al., 1991) followed by Taxol incubation to enhance seeds stability. By using a biotinylated tubulin mixture, the seeds will bind to neutravidin-coated siPEG-biotin coverslips and serve as nucleators from which microtubules can grow.

32. Thaw tubulin aliquots on ice.

Note: To mix the solutions containing tubulin pipet or tap the bottom of the tube, never vortex proteins.

33. Incubate 10 μM tubulin composed of 30% labeled tubulin (depending on the experiment, different fluorochrome labeled tubulin can be used), 70% biotinylated tubulin, 0.5 mM GMPCPP in BRB80 1× at 37°C for 45 min.
34. Add 1 μM Taxol and incubate the solution for another 30 min at 37°C or 24°C.

⚠ CRITICAL: Once tubulin polymerization has started, mix the solution by tapping the bottom of the tube. Use a cut-off yellow-pipette tip to transfer the solution containing polymerized microtubules, to avoid shearing of microtubules. Prepare the 1 μM Taxol solution fresh from a 25 mM stock solution, by diluting with DMSO.

35. Transfer the solution to a pre-warmed ultracentrifuge tube (e.g., 500 μL thick-wall polycarbonate tubes from Beckman Coulter).
36. Ultracentrifuge the solution at 109,000 × *g* 15 min at 25°C using a prewarmed rotor (e.g., Beckman TLA 100.1).
37. Discard the supernatant.
38. Resuspend the pellet in an appropriate volume of prewarmed BRB80 1× containing 0.5 mM GMPCPP and 1 μM Taxol, using a cut-off pipette tip to avoid seeds breaking into small pieces.
39. Aliquot into 6 μL aliquots using a cut-off pipette tip and snap freeze in liquid nitrogen.

Pause point: Seed aliquots are stored in liquid nitrogen and can be used for experiments up to 2 weeks.

Assembly of flow chambers

Timing: 5 min

These steps describe how to assemble a simple flow chamber, by fixing a siPEG treated slide to a siPEG-biotin treated coverslip using double-sided adhesive tape (Figure 2). The flow chamber enables sequential exchange of solutions required for this assay.

40. Take a siPEG treated slide and a siPEG-biotin treated coverslip with the help of tweezers.
41. Use a diamond knife to cut the siPEG-biotin coverslip into the desired size (Figure 2A). A small flow channel reduces the volume of the reaction mixtures needed.
42. Attach 2 strips of double-sided adhesive tape to the slide in an almost-parallel-V shape (Figure 2B).

Note: Parallel or V-shape chambers can be used. The V-shape has a larger opening for absorption which allows faster solution exchange.

43. Place the siPEG-biotin coverslip upside down onto the tape. Make sure that the side facing the slide was not in contact with any surfaces. Attach the siPEG-biotin glass to the slide, by gently stroking over the coverside with the help of tweezers (Figure 2C).

Note: It is recommended to press gently the coverslip on double-sided tape to make sure that it is well assembled and not detaching during the experiment (Figure 2D).

Pause point: Assembled flow chambers can be used within the same day.

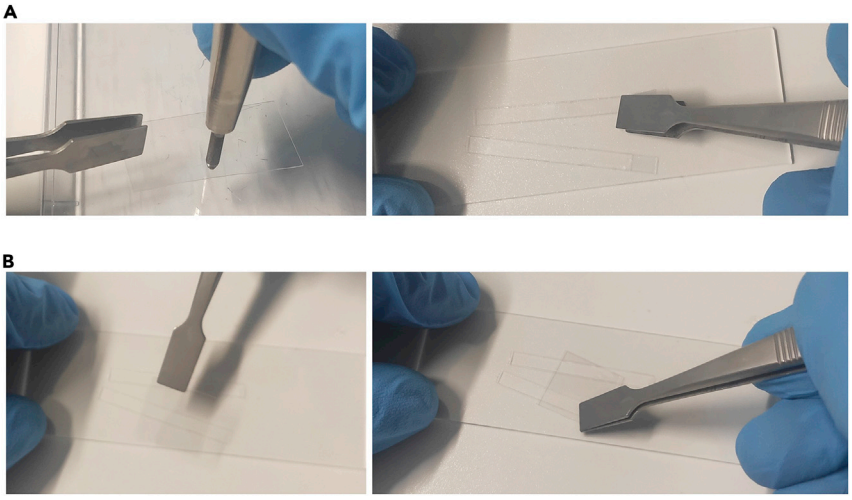

Figure 2. Illustration of a flow chamber assembly

(A) (Left) The size of the siPEG-biotin coated coverslip can be adjusted by cutting the glass with a diamond knife.
(A) (Right) Adhere 2 strips of double-sided adhesive tape in a V-shape shape on the siPEG coated slide with the help of tweezers.
(B) (Left) Assembly of the flow chamber by placing the coverslip (turned upside down) onto the tape.
(B) (Right) Press the coverslip gently onto the adhesive tape.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Bovine Brain	Slaughterhouse	N/A
Chemicals, peptides, and recombinant proteins		
ATTO-488	ATTO-TEC GmbH	Cat#AD 488
ATTO-565	ATTO-TEC GmbH	Cat#AD 565
ATTO-647	ATTO-TEC GmbH	Cat#AD 647
EZ-Link™ Sulfo-NHS-LC-LC-Biotin	Thermo Fisher Scientific	Cat#21338
Tri-ethoxy-silane-PEG (siPEG)	Creative PEGWorks	Cat#PSB-3385
Tri-ethoxy-silane-PEG-biotin (siPEG-biotin)	Creative PEGWorks	Cat#PJK-1915
GMPCPP	Jena Bioscience	Cat#NU-405L
Paclitaxel	Sigma-Aldrich	Cat#T1912-5MG
NeutrAvidin	Thermo Fisher Scientific	Cat#31002
Methyl cellulose (1500 cP)	Sigma-Aldrich	Cat#M0387-500G
Catalase	Sigma-Aldrich	Cat#C9322
Glucose-oxidase	Sigma-Aldrich	Cat#G2133
D-Glucose	Merck	Cat#4074
DTT	Sigma-Aldrich	Cat#D9779
GTP	Sigma-Aldrich	Cat#G5884
ATP	Sigma-Aldrich	Cat#A3377
ß-Mercaptoethanol	Sigma-Aldrich	Cat#M3148
Software and algorithms		
SlideBook 6	3i - Intelligent Imaging Innovations	https://www.intelligent-imaging.com
ImageJ	National Institutes of Health (NIH)	https://imagej.nih.gov
GraphPad Prism	GraphPad	https://www.graphpad.com
Other		
Plasma cleaner	Electronic Diener	N/A
Ultrasonic bath	BANDELIN	N/A
76 × 26 mm slides	Hecht Assistent	Cat#42406020

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
76 × 26 mm (thickness 1) coverslips	Hecht Assistent	N/A
Staining jars with cover	Hecht Assistent	Cat#42480010
Staining jars with cover	Hecht Assistent	Cat#42470110
Staining trays	Hecht Assistent	Cat#42481110
Wire handles	Hecht Assistent	Cat#42482010
Double-sided adhesive tape (70 μm height; 3 × 30 mm)	LiMA Adhésif	Cat#0000P70PC3003
Glass-writing diamond pen	Hecht Assistent	Cat#41182010
Hand Homogenizer	Thomas Scientific	Cat#885300

MATERIALS AND EQUIPMENT

Note: Use ddH_2O as solvent for all buffers. Volumes can be adapted. Measure and equilibrate the pH before completing the final volume, and before using the solution. Some reagents have to be added into solutions before usage.

Depolymerization Buffer (DB), pH 6.6 [700 mL]		
Reagent	Final concentration	Amount
MES-HCl	50 mM	6.83 g
$CaCl_2$ (2 M)	1 mM	0.35 mL
Store at 4°C up to one month.		

- Add 0.1% (v/v) ß-Mercaptoethanol before usage.

Note: PIPES in solution starts to dissolve at physiological pH.

High-Molarity PIPES Buffer (HMPB), pH 6.9 [700 mL]		
Reagent	Final concentration	Amount
PIPES-KOH	1 M	211.66 g
$MgCl_2$ (2 M)	10 mM	3.5 mL
EGTA (0.5 M)	20 mM	2.8 mL
Store at 4°C up to one week.		

20× BRB80, pH 6.8 [50 mL]		
Reagent	Final concentration	Amount
PIPES-KOH	1.6 M	24.19 g
$MgCl_2$ (2 M)	20 mM	0.5 mL
EGTA (0.5 M)	20 mM	2 mL
Store at −20°C for several months.		

Glycerol Polymerization Buffer (Glycerol PB), pH 6.8 [100 mL]		
Reagent	Final concentration	Amount
PIPES-KOH	80 mM	2.42 g
$MgCl_2$	5 mM	0.25 mL
EGTA	1 mM	0.2 mL
Glycerol	33% (v/v)	33 mL
Store at 4°C up to one week.		

- Add 1 mM GTP before usage.

Resuspension Buffer (RB), pH 8.6 [100 mL]		
Reagent	Final concentration	Amount
Na-HEPES	100 mM	2.38 g
$MgCl_2$ (2 M)	1 mM	0.05 mL
EGTA (0.5 M)	1 mM	0.2 mL
Glycerol	40% (v/v)	40 mL
Store at 4°C up to one week.		

Na-HEPES Cushion, pH 8.6 [100 mL]		
Reagent	Final concentration	Amount
Na-HEPES	100 mM	2.38 g
$MgCl_2$ (2 M)	1 mM	0.05 mL
EGTA (0.5 M)	1 mM	0.2 mL
Glycerol	60% (v/v)	60 mL
Store at 4°C up to one week.		

Anti-bleaching buffer 3× stock [125 μL]		
Reagent	Final concentration	Amount
H2O	/	65 μL
Methyl cellulose	0.375%	23.5 μL
BRB80 20×	BRB80 1×	6.25 μL
D-glucose (15 mg/mL)	0.9 mg/mL	7.5 μL
Glucose oxidase (5 mg/mL)	0.3 mg/mL	7.5 μL
Catalase (1 mg/mL)	0.06 mg/mL	7.5 μL
DTT (1 M)	30 mM	3.75 μL
GTP (0.1 M)	3 mM	4 μL
Store on ice for 4 h.		

Note: In order to increase the lifetime of the fluorophores and to decrease bleaching or photo-induced damage of microtubules, we use an oxygen scavenger cocktail composed of D-glucose and glucose oxidase and catalase enzymes. DTT will help to maintain a reducing environment. Aliquot separately stock solutions of all components and store them at −20°C. Thaw them and place them on ice right before preparing the anti-bleaching buffer. Use the anti-bleaching buffer for a maximum of 4 h.

Note: Methyl cellulose is a crowding agent that will decrease microtubule fluctuations. Prepare a 2% methyl cellulose stock solution that can be used up to 1 week. Due to its viscosity, cut the tip of the yellow tip (to increase the diameter of the opening of the tip) to take the precise volume.

STEP-BY-STEP METHOD DETAILS

Tubulin incorporation assay

Timing: 45 min for preparation, 20 min for imaging

In this assay, tubulin dimer exchange along the GDP-microtubule shaft can be visualized, by sequential exchange of tubulin labeled with different fluorophores (Figure 3). The impact of proteins, small molecules, buffer conditions, or temperature on microtubule shaft dynamics can be studied with this assay.

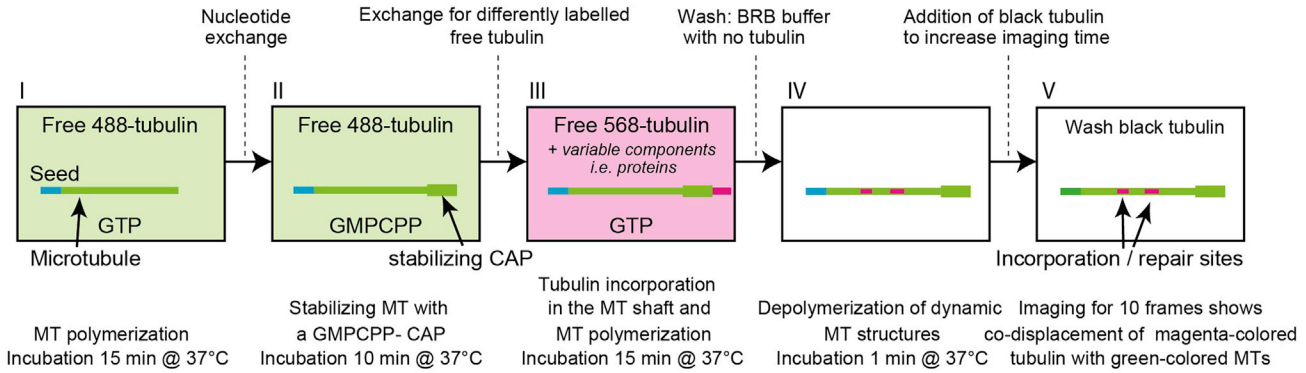

Figure 3. Experimental set up of the incorporation assay:
I) Microtubules polymerize form double stabilized GMPCPP/Taxol- seeds in the presence of tubulin 8 μM (20% labeled); II) Stabilizing dynamic GDP-microtubules with a GMPCPP-Cap. Exchange of nucleotide from GTP to GMPCPP and free tubulin 5 μM (70% labeled); III) Incorporation condition with 9 μM tubulin (100% labeled different color). At this step proteins, small molecules, temperature, ion strength, pH… can be varied to study their effect on shaft dynamics; V) Depolymerization of all dynamic structures grown on top of the microtubule in BRB with no tubulin; V) Stabilizing the microtubule for imaging with 8 μM black tubulin.

1. Assemble a flow chamber as described above.
2. Prepare anti-bleaching buffer 3× stock (30 mM DTT, 0.9 mg/mL D-glucose, 0.3 mg/mL glucose oxidase, 0.06 mg/mL catalase, 0.375% methyl cellulose) in BRB80 1× (100–200 μL) and keep it on ice.
3. Thaw on ice: 1 aliquot of ATTO-488-labeled tubulin, 1 aliquot of ATTO-565-labeled tubulin and 1 aliquot of black tubulin.

   Note: Once on ice, tubulin can be used for several experiments, but needs to be used within 2 h after thawing.

4. Thaw seeds at 37°C for 2 min. Transfer to 24°C. Seeds can be kept 24°C and used for 4 h.

   Note: In order to mix tubulin and seeds aliquots it is recommended to tap the bottom of the cryotube, or pipet, never vortex.

5. Flow in 100 μL of 50 μg/mL neutravidin in BRB80 1× into the flow chamber. The neutravidin will bind the biotin present on the coverslip surface. Through the binding to neutravidin, biotinylated seeds will attach to siPEG-biotin.

   Note: Use a yellow tip to flow solutions into one side of the channel and kitchen paper on the opposite site to perfuse the solutions into the chamber.

   ⚠ CRITICAL: Avoid introducing air bubbles to the flow chamber. Air bubbles can detach components bound to the glass, disrupt the flow of the introduced solutions and cause oxidation. Microtubules will not polymerize where an air bubble passed.

   Note: To avoid drying out of the reaction chamber, leave few drops of the reaction mix at both sites of the flow chamber in all of the following incubation steps.

6. Incubate for 3 min at 24°C.

   Note: For seed dilution and washing steps, store an aliquot of BRB80 1× at 24°C.

7. Wash out neutravidin with 1 × 100 μL BRB80 1× (at RT).
8. Flow in 100 μL of GMPCPP-seeds diluted in BRB80 1× (e.g., dilute seeds 100-200-fold).

⚠ CRITICAL: To pipet seeds, cute the tip of the yellow tip, to minimize shearing microtubules into smaller fragments.

9. Wash out non-attached seeds with 3 × 100 µL BRB80 1× containing 0.2% BSA.

Note: For all the following steps to avoid dilution, dry the entry of the flow chamber with paper before you flow in the new buffer.

10. Prepare 30 µL of **Elongation buffer** with 8 µM tubulin (20% ATTO-488-labeled, 80% black), 10 µL of anti-bleaching buffer and 1 mM GTP in BRB80 1× 0.2% BSA and flow it through the chamber.
11. Incubate for 15 min at 37°C in a closed and humidified chamber (e.g., square Petri Dish with wet tissue) to allow microtubule to polymerize.

Note: In our hands the optimal condition to get microtubules with an average length of 10 µm was 15 min of polymerization reaction at 37°C using 8 µM tubulin. To reach the desired microtubule length, incubation time and tubulin concentration can be adapted.

12. To cap microtubules, flow in 30 µL of pre-warmed **Capping buffer** containing 5 µM tubulin (70% ATTO-488-labeled, 30% black) in BRB80 1× supplemented with 0.5 mM GMPCPP.

Note: Stabilizing the dynamic microtubule tip with a GMPCPP-microtubule cap enables to study GDP-shaft for at least 45 min, while the microtubule is protected from depolymerization. We use 70% labeled tubulin to be able to differentiate under TIRFM the GDP-microtubule (20% labeled) from the GMPCPP-cap.

⚠ CRITICAL: Pre-warm the capping solution to 37°C for 30 s. Keep the flow chamber at 37°C, while perfusing the mixture to prevent microtubule depolymerization.

13. Incubate for 12 min at 37°C.
14. Wash one time with 100 µL of BRB80 1× + 0.2% BSA that is pre-warmed to 37°C.
15. Flow in 30 µL of **Incorporation buffer** containing 9 µM tubulin (100% ATTO-565-labeled) in BRB80 1× supplemented with 0.2% BSA, anti-bleaching buffer and 1 mM GTP.

Note: To study which parameters impact shaft dynamics, you can add your protein or molecule of interest at this step. You can incubate between 1 – 45 min depending on your research question. To study how kinesin-1 impacts shaft dynamics, we incubated for 15 min.

⚠ CRITICAL: Pre-warm the incorporation solution at 37°C for 30 s. Keep the flow chamber at 37°C, while perfusing the mixture to prevent microtubule depolymerization.

16. Incubate for 1–45 min at 37°C.
17. Wash one time with 200 µL BRB80 1× + 0.2% BSA in the absence of tubulin and incubated for 1 min at 37°C. This incubation time is sufficient to depolymerize all dynamic structures beyond the GMPCPP-cap.
18. Flow in 30 µL of **Imaging buffer** containing 6 µM tubulin (100% black) in BRB80 1× supplemented with 0.2% BSA, anti-bleaching buffer and 1 mM GTP.

Note: Addition of 6 µM tubulin protects microtubule from breaking and elongates the imaging time.

19. Seal both openings of the chamber with sealing wax (e.g., haematocrit sealing wax from Hecht Assistant).

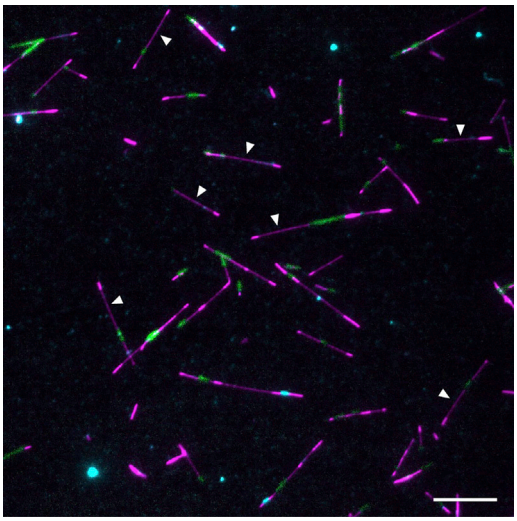

Figure 4. Whole field of view of a representative position imaged

Only isolated microtubules grown from a seed (green) and capped by GMPCPP (magenta) were analyzed (white arrowhead). Scale bar 10 μm.

20. Image with TIRFM (e.g., Axio Observer Inverted TIRF microscope (Zeiss, 3i) and a Prime BSI (Photometrics)):
    a. Warm the 100× objective before starting the imaging (e.g., Chamlide Live Cell Instrument incubator to keep the temperature constant during the experiment).
    b. Verify that the flow chamber is well sealed, and clean the coverslip with ethanol and a wipe.
    c. Place the chamber in the stage, so that the coverslip faces the objective.
    d. Set several positions. Choose positions where microtubules are grown from seeds, do not contact each other, and are capped with a GMPCPP-cap (Figure 4). We take between 10 – 20 positions.
    e. Record each position over 5–10 frames. Within this time frame microtubules should fluctuate (Figure 5). Only analyze fluctuating microtubules, this decreases the possibility that microtubule - surface interactions impact shaft dynamics in your experiments.

Note: The imaging frame rate depends on how many positions are imaged, but should not exceed 2 min.

⚠ CRITICAL: Imaging for several frames enables to differentiate whether the measured signal is an incorporation site moving with the microtubule, or an aggregate on the surface. Microtubules sticking to the surface of the coverslip are excluded from analysis.

EXPECTED OUTCOMES

A representative single frame image made with TIRF microscopy is shown in Figure 4. The GDP-microtubules (magenta; 20%) are grown from seeds (green) and capped by GMPCPP-tubulin (magenta; 70%). The fluorescent intensity of incorporation sites (blue) can be in the range of a single protofilament. The fluorescent intensity further can depend on the protein added to the assay. To distinguish between incorporations sites and aggregates on the coverslip it is important to image each position for at least 5 frames (Figure 5). An incorporation site moves together with the fluctuating microtubule, while aggregates at the surface are stationary (Figure 5).

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Open a position in Fiji and choose the microtubule channel. Select fluctuating microtubules for the analysis. Microtubules sticking to the surface will be excluded from the analysis.

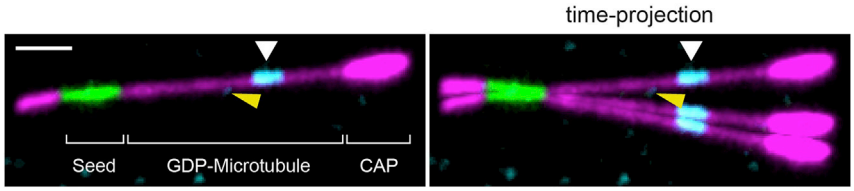

Figure 5. Representative tubulin incorporation site (cyan) along a GDP-microtubule (magenta; 20% labeled) grown from a seed (green) and capped by a GMPCPP-microtubule (magenta; 70% labeled).
White arrowhead shows an incorporation site, yellow arrowhead shows an aggregate on the coverslip. Right: time projection (max intensity) of 3 subsequent images of a single fluctuating microtubule. Scale bar 2 μm.

Note: Work blinded, do not look at the incorporation channel before you chose microtubules for analysis.

2. To be able to trace back which microtubules were analyzed draw a line along selected microtubules using the line tool and save the region of interest (ROIs).
   a. Microtubule channel: measure microtubule length (distance in between the end of the microtubule seed and the beginning of the GMPCPP cap) using the line tool.

Note: Select microtubules with a minimum length of 3 μm.

   b. Incorporation sites channel:
      i. Distinguish between an incorporation site or an aggregate bound to the surface.

Note: An incorporation site moves together with the microtubule (Figure 5).

Note: To measure incorporation sites, we first calculated the fluorescence intensity of one protofilament. We therefore performed a line-scan (3-pixel wide line) along microtubules grown beyond the GMPCPP-cap, these microtubules are polymerized from 100% ATTO-565 labeled tubulin. We subtracted the background from the signal and divided it by 14, the majority of microtubules grown from GMPCPP have 14 protofilaments (Vemu et al., 2017). We set the fluorescence intensity of one protofilament as a threshold to count a signal as an incorporation site. We defined an incorporation site as a fluorescence signal equal to one or more protofilaments. We counted incorporations as two sites where the signal dropped below one protofilament along the incorporation signal.

      ii. Measure the length of incorporation sites using the line tool.
      iii. Count the number of incorporation sites.
      iv. Measure the distance between seed - incorporation site, incorporation site – cap and between two incorporation sites.

⚠ CRITICAL: Exclude incorporation sites in proximity of seeds or GMPCPP-caps. Tubulin dimer incorporation in those areas might reflect protofilament number change in the transition from GMPCPP-microtubules (seed and cap) to the GDP-microtubule lattice rather than the stochastic process of tubulin dimer exchange along the shaft.

3. Analysis:
   a. **Fraction of replaced microtubule shaft**: total length of all incorporation sites / total microtubule length.
   b. **Density of incorporation sites**: total number of incorporation sites / total microtubule length.
   c. **Integrated fluorescence intensity of incorporation sites**:
      i. Draw a line (3-pixel wide line) along incorporation sites, and perform a line-scan.

Measure the background fluorescence intensity at the exact same position with a second line-scan. For this choose a time frame where the microtubule with the incorporation site fluctuated out of the field of measurement.

ii. Subtract the background raw intensity from the incorporation raw intensity.

LIMITATIONS

The number of tubulin incorporation sites might be underestimated. It is possible that single incorporation sites have a distance between them below the limit of optical resolution (200 nm).

TROUBLESHOOTING

Problem 1

The surface of the coverslip is dirty with excessive background signal that compromises the identification of incorporation sites. Cleaning and silanization of glass slides and coverslips.

Potential solution

The quality of the clean and coated glass surfaces decreases over time. Even though slides and coverslips are stored at 4°C, in a close container dust accumulates and the coating becomes less homogenous over time. Ideally, cleaning and silanization of glass surfaces should be performed freshly for the week of the experiment.

Alternatively, siPEG and siPEG-biotin coating solutions might be too old. The plasma treatment to activate the surface could be also increased.

Problem 2

Microtubules are not fluctuating and stick to the glass surface. Cleaning and silanization of glass slides and coverslips.

Potential solution

Same potential solution as for problem 1.

Problem 3

No, or only too short microtubules are present in the flow chamber. Tubulin incorporation assay step 11.

Potential solution

Make sure that all the Capping buffer was well pre-warm before the injection. Verify if the temperature during polymerization is stable at 37°C and that the chamber is not drying out. Increasing the incubation time and/or tubulin concentration can increase microtubule length.

Problem 4

Unattached, freely floating GMPCPP-microtubules are present in the flow chamber. Tubulin incorporation assay step 14.

Potential solution

At 5 μM GMPCCP-microtubules nucleate in the chamber. The short GMPCCP-microtubules can be removed from the flow chamber by increasing the volume of the washing buffer in step 16.

Problem 5

Microtubules are not sufficiently stabilized by the GMPCPP-cap, e.g., Microtubules depolymerize quickly from the GMPCPP-cap while imaging. Tubulin incorporation assay steps 12 and 13.

Potential solution

Increasing the incubation time during the capping process for 2–5 min can help. Do not increase the tubulin concentration as this would cause more nucleation.

Problem 6

Tubulin labeling ratio is too low. Fluorochrome-Labeled Tubulin steps 6e and 6f.

Potential solution

Dissolve the dye just before usage. Use only anhydrous DMSO. Make sure you washed the cushion, to prevent low pH buffer to contaminate the **Resuspension buffer** (RB).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Aumeier Charlotte (charlotte.aumeier@unige.ch).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS

M.A.C. performed the experiments with the help of S.F. M.A.C. and C.A. designed the experiments. M.A.C., S.F., and C.A. analyzed data. M.A.C. conceptualized the manuscript. M.A.C. and S.F. wrote the original draft. C.A. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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