

Imaging Microtubules *in vitro* at High Resolution while Preserving their Structure

Camille Cuveillier*, Yasmina Saoudi, Isabelle Arnal and Christian Delphin*

Univ. Grenoble Alpes, Inserm, U1216, CNRS, Grenoble Institut Neurosciences, GIN, France

*For correspondence: camille.cuveillier@univ-grenoble-alpes.fr; christian.delphin@univ-grenoble-alpes.fr

[Abstract] Microtubules (MT) are the most rigid component of the cytoskeleton. Nevertheless, they often appear highly curved in the cellular context and the mechanisms governing their overall shape are poorly understood. Currently, *in vitro* microtubule analysis relies primarily on electron microscopy for its high resolution and Total Internal Reflection Fluorescence (TIRF) microscopy for its ability to image live fluorescently-labelled microtubules and associated proteins. For three-dimensional analyses of microtubules with micrometer curvatures, we have developed an assay in which MTs are polymerized *in vitro* from MT seeds adhered to a glass slide in a manner similar to conventional TIRF microscopy protocols. Free fluorescent molecules are removed and the MTs are fixed by perfusion. The MTs can then be observed using a confocal microscope with an Airyscan module for higher resolution. This protocol allows the imaging of microtubules that have retained their original three-dimensional shape and is compatible with high-resolution immunofluorescence detection.

Keywords: Microtubules, Microtubule-Associated Proteins, TIRF microscopy, High resolution imaging, Airyscan.

[Background] Microtubules (MT) are polymers made by the combination of the heterodimers α - and β -tubulins and are a major component of the cell cytoskeleton. They are involved in fundamental mechanisms of cell function such as mitosis, intracellular transport, cytokinesis and maintenance of cell shape (Akhmanova and Steinmetz, 2015). Although inherently very rigid, MTs often appear curved in cells and few proteins have been described to bend microtubules (Brangwynne *et al.*, 2006; Bechstedt *et al.*, 2014; Leung *et al.*, 2020; Cuveillier *et al.*, 2020). Since the early 1970s (Weisenberg, 1972), the study of MTs *in vitro* has led to a better understanding of the molecular mechanism involved in the formation and dynamics of MTs. However, a detailed analysis of the shape of the microtubules remains technically difficult. Two main approaches are currently used: electron microscopy for the very detailed images obtained (up to a separation limit of a few Angstrom) (Alushin *et al.*, 2014; Harris, 2015), and TIRF microscopy which allows the live observation of dynamic microtubules using fluorescently-labelled molecules (separation limit of about 200 nm) (Al-Bassam, 2014). However, these techniques are not suitable for the complete observation of microtubules with large three-dimensional curvatures such as the helical shape they adopt in the presence of MAP6 (Cuveillier *et al.*, 2020). In order to obtain more clues on the structure of the MTs, we developed an assay that combines the use of fluorescent proteins and high-resolution imaging by Airyscan confocal microscopy (achievable resolution down to 120 nm in XY), while preserving the original shape of the MTs which is very sensitive to manipulation. This protocol

has the advantage of avoiding unusual equipment and material. Moreover, it can be adapted to different super-resolution techniques such as expansion microscopy or stimulated-emission-depletion (STED) microscopy which would allow to increase the resolution up to 10 times (Blom and Brismar, 2014) and reach a separation limit of 50-20 nm.

Materials and Reagents

1. Tubulin, Atto-565 labelled tubulin, and biotinylated tubulin are prepared as already fully described in Ramirez-Rios *et al.* (2017). Store up to 1 year in liquid nitrogen
2. Cover glasses 26 × 76 mm #1 (VWR, catalog number: 630-2910)
3. Double-face precut tape 70 µm thick, 3 mm wide (LIMA Company, catalog number: 0000P70PC3003)
4. Siligum wax plate (VWR, MODU140013)
5. 1.5 ml Eppendorf tubes (Fisher Scientific, catalog number: 11558232)
6. 0.5 ml Eppendorf tubes (Fisher Scientific, catalog number: 10318661)
7. Petri dishes (Greiner Bio-One, catalog number: 663102)
8. Polycarbonate centrifuge tubes (Beckman, catalog number: 343775)
9. 0.22 µm filters (Merck Millipore, catalog number: SLGP033RS)
10. Silane-PEG (MW 30k) (Creative PEG-Works, catalog number: PSB-2014)
11. Silane-PEG-biotin (MW 3,400) (LaysanBio, catalog number: BIOTIN-PEF-SIL, MW 3,400)
12. NeutrAvidin (ThermoScientific, catalog number: 31000)
13. PLL20K-G35-PEG2K (PLL-g-PEG) (Jenkem, catalog number: 13022755)
14. Pluronic F-127 (Sigma-Aldrich, catalog number: P2443)
15. Bovine Serum Albumin (BSA) (Sigma-Aldrich, catalog number: A7030)
16. Acetone 100% (VWR, catalog number: 20066.321)
17. Ethanol 96% (VWR, catalog number: 20823362)
18. Hellmanex III (Sigma-Aldrich, catalog number: Z805939-1EA)
19. Phosphate buffer saline (PBS) (Sigma-Aldrich, catalog number: P4417)
20. 1,4-Piperazinediethanesulfonic acid (PIPES) (Sigma-Aldrich, catalog number: P6757)
21. Potassium hydroxide (KOH) (Sigma-Aldrich, catalog number: 484016)
22. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541)
23. Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma-Aldrich, catalog number: E3889)
24. Magnesium chloride (MgCl₂) (Sigma-Aldrich, catalog number: 2670)
25. Sodium hydroxide (NaOH) (Carlo Erba, catalog number: 480507)
26. DL-Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: D0632)
27. Guanosine 5'-triphosphate (GTP) (Sigma-Aldrich, catalog number: G8877)
28. Methyl cellulose 1,500 cP (Sigma-Aldrich, catalog number: M0387)
29. GMPCPP (Euromedex, JE-NU-405S)

30. HEPES (Sigma-Aldrich, catalog number: H3375)
31. Glucose (Sigma-Aldrich, catalog number: G8270)
32. Glucose oxidase (Sigma-Aldrich, catalog number: G6766-10KU)
33. Catalase (Sigma-Aldrich, catalog number: C9322-1G)
34. Diamond pencil (Agar Scientific, catalog number: AGT5347)
35. Glutaraldehyde solution 25% (Sigma-Aldrich, catalog number: G5882)
36. Clean nitrogen air flow (Air Liquide)
37. Liquid nitrogen (Air Liquide)
38. 5× BRB80 (see Recipes)
39. BSA 10% (see Recipes)
40. Neutravidine (see Recipes)
41. DTT, 200 mM (see Recipes)
42. KCl, 500 mM (see Recipes)
43. PLL-PEG (see Recipes)
44. Silane-PEG or Silane-PEG-biotin (see Recipes)
45. GTP, 20 mM (see Recipes)
46. Glucose, 450 mg/ml (see Recipes)
47. Deoxymix (catalase and glucose oxydase) (see Recipes)
48. NaOH, 1 M (see Recipes)
49. BSA 1%/BRB80 (see Recipes)
50. Methyl cellulose 1,500 cP (see Recipes)
51. HEPES, 10 mM (see Recipes)
52. Pluronic F27, 10% (see Recipes)
53. PBS (see Recipes)
54. Neutravidin stock solution (see Recipes)
55. Red tubulin mix (see Recipes)
56. Imaging buffer (see Recipes)

Equipment

1. Inverted Eclipse Ti microscope (Nikon) with PSF focus
2. Apochromat 100×/1.49 N.A. oil immersion objective (Nikon) with heated objective (Okolab)
3. Temperature chamber (Technico Plast)
4. Ilas2 TIRF system (Roper Scientific)
5. Cooled Charged-coupled device camera (Evolve 512, Photometrics)
6. Temperature Stage Controller (Linkam Scientific)
7. LSM 710 confocal (Zeiss) Airyscan
8. Plan Apochromat 100×/1.4 N.A. oil immersion objective (Zeiss)
9. Ultracentrifuge (Beckman Coulter Optima, Model Max-XP, catalog number: 393315)

10. Rotor (Beckman, model: TLA100-1, catalog number: 343837)
11. Plasma cleaner FEMTO Diener Electric (Germany) coupled to a vacuum pump Trivac D2.5E Oerliken (Germany)
12. Sonicator (Elmasonic S30, Elma, Germany)
13. Glass staining dishes and tray (VWR, catalog number: MARI4200004)
14. Plastic box for glass microscopy (any brand)
15. Diamond Pencil (Oxford Instruments, catalog number: T5347)
16. Tweezers (Dutscher, catalog numbers: 076100 and 005093)
17. Gloves powder-free (any brand)
18. Water-bath (any brand)

Software

1. MetaMorph 7.8.5 software (Molecular Devices, <https://www.moleculardevices.com>)
2. Zen Black 2.1 (Zeiss)

Procedure

A. Flow chamber preparation

Note: This procedure is adapted from Portran et al. (2013) and Leslie et al. (2013). Cover glasses are used to make both sides of the perfusion chambers. Cover glasses are handled with tweezers or by hand with non-powdered gloves on the edges when drying with airflow so as not to damage their surface by more than 1 cm.

1. Glass cleaning
 - a. Immerge the cover glasses in a clean glass staining dish (105 × 85 × 70 mm) filled with 200-250 ml acetone and sonicate for 30 min in sweep mode.
Note: 6 cover glasses are required to make 10 flow chambers, but some glass breakage may occur during the procedure, so it is advisable to start with a little more cover glasses.
 - b. Replace acetone and incubate for an additional 30 min with orbital shaking (80 rpm).
Note: Acetone from the second bath can be recycled for the first bath of the next cleaning procedure.
 - c. Incubate 15 min with ethanol and rinse 10 times with deionized filtered water.
 - d. Incubate 2 h in prewarmed (60 °C) 2% (v/v) Hellmanex on an orbital shaker (80 rpm), then rinse 10 times with deionized filtered water.
 - e. Sonicate 15 min in 1 M NaOH solution.
 - f. Rinse 10 times with deionized filtered water.
 - g. Sonicate 15 min in ethanol and rinse each coverslip 10 times in a large volume (2 × 2 L) of deionized filtered water.
 - h. Dry the cover glasses with a clean air flow (nitrogen gas).

- i. Dispense the glasses into two clean, dry glass staining tray for activation and silanization (see the next step below).

Note: 5 silane-PEG-biotin-coated glasses and 1 silane-PEG-coated glass are used to prepare 10 perfusion chambers (see below paragraph 3).

2. Glasses activation and silanization

- a. Plasma activate the glasses for 3 min at 80% of maximum power at a pressure of 0.7 mbar.
- b. Rapidly immerse the glasses in either silane-PEG-biotin or silane-PEG solutions and incubate overnight on an orbital shaker.

Note: Identify the glass staining dishes containing either silane-PEG-biotin or silane-PEG to avoid mistakes.

- c. Recover the coating solutions (they can be reused for several months as long as they remain clean) and replace them with ethanol (Figure 1A).
- d. Wash each coating slide 10 times in a beaker containing ethanol (Figure 1B) and 10 times in a large volume (2 × 2 L) of water (Figure 1C, 1D).

Note: We recommend to wash first the silane-PEG slides then the silane-PEG-biotin ones to avoid potential silane-PEG-biotin binding to silane-PEG slides.



Figure 1. Washing of coated slides. In A we have coated slides in ethanol. B contains ethanol. C and D contain deionized filtered water. E containing deionized filtered water is used to keep rinsed slides before drying.

- e. Dry the coverglasses with a stream of nitrogen.
 - f. Store the silanized lamellae at 4 °C in a plastic box sealed with plastic film for a maximum of one week.
- ## 3. Construction of the flow chamber

Note: When handling the silanized cover glasses, make sure that the face inside the chamber remains clean and un-touched.

- a. Transfer 1 silane-PEG-biotin-coated slide to a clean Petri dish and, using the diamond pencil, cut the glass into four parts (approx. 19/26 mm) (Figures 2A, 2B).

Note: When cutting with the diamond pencil, the Petri dish bottom bends leading to slide breaking. To avoid this, we use a thin plastic that we put under the Petri dish (Figure 2A).

- b. Similarly, transfer the silane-PEG-coated slide to another Petri dish and cut them into 7 parts of about 11 mm width. Discard the two end parts and cut the others in half to make fourteen 11 × 13 mm pieces (Figures 2C, 2D).

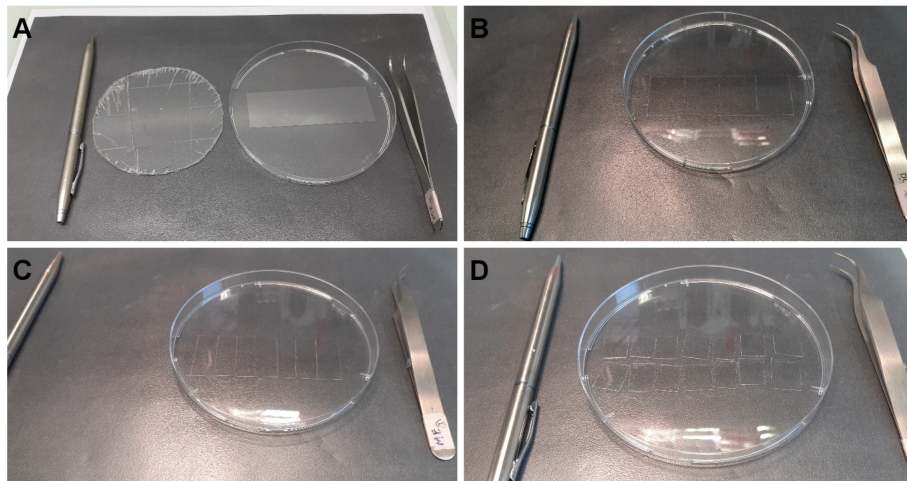


Figure 2. Cutting coated slides. A. From left to right, diamond pencil, plastic to avoid breaking, Petri dish containing silane-PEG-biotin slide, tweezers. B. The slide is cut in four equal parts. C. A silane-PEG slide is cut in 7 parts. D. Each part is cut in half.

- c. Place two pieces of double-sided adhesive tape, 5 mm apart, in the center of a piece of silane-PEG-biotin coated coverslips (Figure 3A).
- d. Tape one piece of PEG-silane-coated strips above the two pieces of tape (the volume of the chamber is about 5 μ l) (Figure 3B).

Note: Carefully press the silane-PEG-coated strip on the tape to ensure correct sealing. You might gently remove excess tape (Figure 3D).

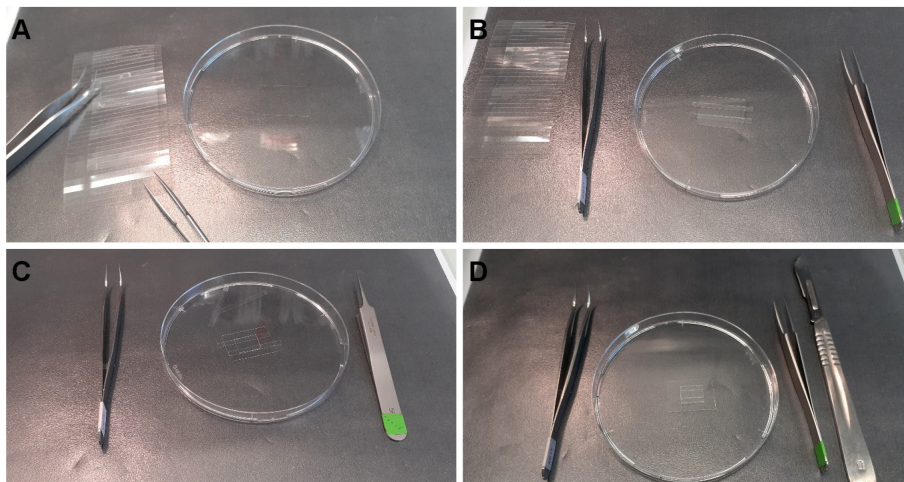


Figure 3. Construction of the flow chamber. A. One piece of silane-PEG-biotin is put in a Petri dish. B. Adhesive tape is stuck to the slide. C. Protective plastic above the tape is removed and one piece of silane-PEG slide is placed onto the tape. D. Excessive tape is cut and removed.

B. Preparation of GMPCPP seeds

Note: GMPCPP seeds tend to depolymerize at low temperatures, try to keep them warm all along the procedure.

1. Prepare a mix of freshly thawed ATTO-565 tubulin and biotinylated tubulin at a 1:1 molar ratio for a final concentration of 10 μM tubulin in BRB80 supplemented with 0.5 mM GMPCPP.

Note: Usually, 300 μl of seeds are necessary for at least 500 experiments.

2. The mix is placed at 35 $^{\circ}\text{C}$ in a water-bath during 1 h to allow polymerization.
3. Spin down the GMPCPP seeds at 130,000 $\times g$ using a TLA 100 rotor during 5 min at 35 $^{\circ}\text{C}$.
4. Discard supernatant and gently rinse with 2 \times 100 μl of prewarmed BRB80.

Note: After the centrifugation, the red seeds are visible in the pellet. Avoid resuspending the pellet during washing steps.

5. Gently resuspend the pellet in prewarmed BRB80 containing 1 mM GMPCPP.

Note: Resuspending might take some time. For 300 μl of polymerization solution, resuspend the pellet in 500 μl .

6. Aliquot by 1-2 μl in 0.5 ml Eppendorf and quickly freeze in liquid nitrogen.
7. Store in liquid nitrogen up to one year.

C. Microtubule polymerization and fixation

Note: The perfusion is performed by loading the solution into one side of the chamber while wiping the other side with a piece of paper (Figure 4).

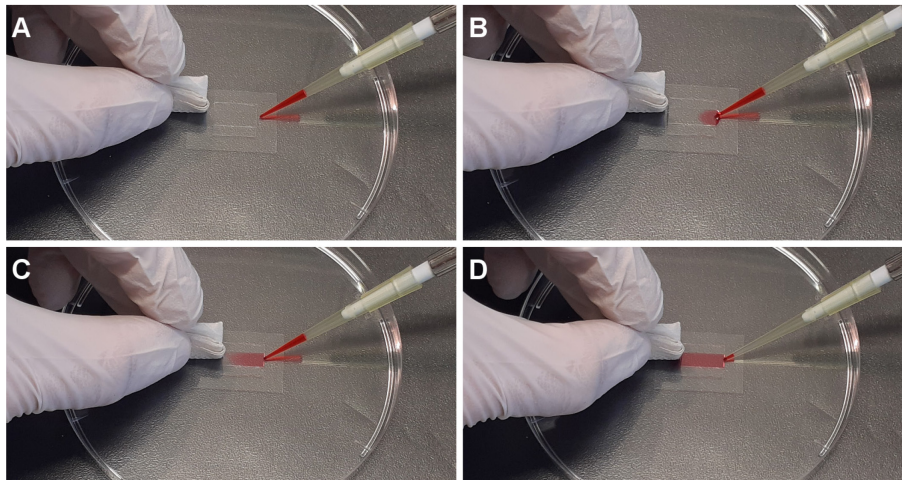


Figure 4. Perfusion in flow chamber. From A to D are sequential images of perfusion of the desired solution into the flow chamber.

1. GMPCPP seeds immobilization in the flow chamber
 - a. Perfuse 10 μ l of Neutravidin (25 μ g/ml in 1% (w/v) BSA/BRB80) in the flow chamber and wait for 2 min.
 - b. Perfuse 30 μ l of PLL-PEG (0.1 mg/ml in 10 mM HEPES, pH 7.4) and wait for 30 s.
 - c. Wash by perfusing 60 μ l of 1% (w/v) pluronic in 1% (w/v) BSA/BRB80.
 - d. Wash by perfusing 2 times with 70 μ l 1% (w/v) BSA/BRB80.
 - e. Quickly thaw GMPCPP seeds and dilute in 1% (w/v) BSA/BRB80.
Note: Adjust seeds dilution if needed, usually start with a 1/100 to 1/500 dilution.
 - f. Perfuse 30 μ l of diluted GMPCPP seeds and let them adhere for 5 min at RT.
 - g. Wash unbound seeds 3 times with 70 μ l 1% (w/v) BSA/BRB80.
2. Microtubule polymerization and fixation
 - a. Perfuse freshly prepared polymerization mix (Recipe 19).
Note: Adjust tubulin/associated proteins concentration if needed. It depends on the affinity and effects of the associated proteins of interest. For tubulin, a concentration of 5 to 20 μ M is a good start and for associated proteins from 5 nM to 500 nM should be informative.
 - b. Put the flow chamber in a warm (32-35 $^{\circ}$ C) place with a water-saturated atmosphere to avoid desiccation for MT polymerization (usually at least 30 min).
 - c. After polymerization, fix MTs by gently perfusing approximately 30 μ l of 0.1% Methyl cellulose 1,500 cP, 0.5% (v/v) Glutaraldehyde/BRB80.
Note: Critical step, fast perfusion will alter MT structure. Put the chamber inside a Petri dish (you might use tape to prevent it from moving). Then, slightly tilt the Petri dish and add drop by drop the fixing buffer at the top of the chamber. The solution will slowly flow down the chamber. You can use blotting paper to clean excess liquid. The fixation buffer should be at least 3 min inside the chamber for proper fixation. Even if the fixation occurs rapidly, dilution might induce MT depolymerization. To overcome this issue, use MT stabilizing compounds

such as taxol (taxol affects MT persistence length) or grow MT longer.

- d. Wash fixation buffer with 50 μ l of 0.1% (v/v) Methyl cellulose 1,500 cP/BRB80.
- e. Perfuse imaging buffer (BRB80 supplemented with 0.1% Methyl cellulose 1,500 cP, 2 mg/ml glucose, 1mg/ml glucose oxidase and 150 μ g/ml catalase).
- f. Seal the flow chamber with the siligum wax.

Note: Imaging of the chambers should be done within one week.

D. Imaging of the microtubules

1. Control fixation: TIRF Microscopy

Note: To estimate the effect of the fixation process on the sample, it is necessary to compare the sample before/after fixation.

- a. Place the flow chamber on the inverted microscope.

Note: For imaging before fixation, set the temperature of the stage at 35 °C to avoid MT depolymerization.

- b. Adjust laser intensity, exposure and focus in order to observe the microtubules.
- c. Acquire images both before (Figure 5A) and after fixation (Figure 5B) of the sample.

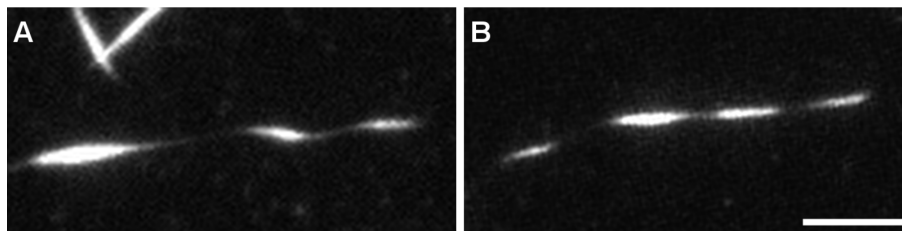


Figure 5. Structure of helical microtubules is not altered by the fixation process. TIRFM image of a helical MT before (A) and after (B) fixation. MTs were grown from GMPCPP seeds with 12 μ M tubulin (9:1 non-labeled tubulin:ATTO-565 labelled tubulin) with 200 nM MAP6-N-GFP. At this concentration, MAP6-N-GFP induces microtubule coiling. Scale bar: 5 μ m.

2. Confocal imaging with Airyscan processing

- a. Place the chamber above the objective.
- b. Adjust focus and laser intensity in order to observe MTs.
- c. Perform acquisition (z-stack series using 220 nm step-size).
- d. Use Zen built-in Airyscan processing. See Figure 6 for TIRF versus confocal imaging with Airyscan processing of helical MT. Figure 7 shows the advantage of performing z-stacks on MTs with a particular shape.
- e. Analyse images using Zen Blue.

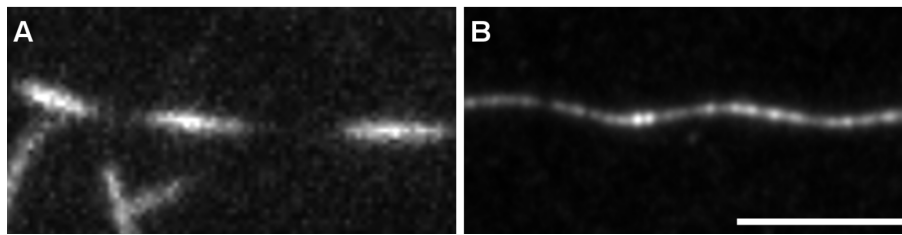


Figure 6. Observation of helical MTs with TIRFM or confocal Airyscan. MTs were grown as in Figure 5 and fixed using the described protocol. A. Fixed helical MTs observed using TIRF microscopy. B. z-projection of a fixed helical MT observed using confocal microscope followed by Airyscan processing. Scale bar: 5 μ m.

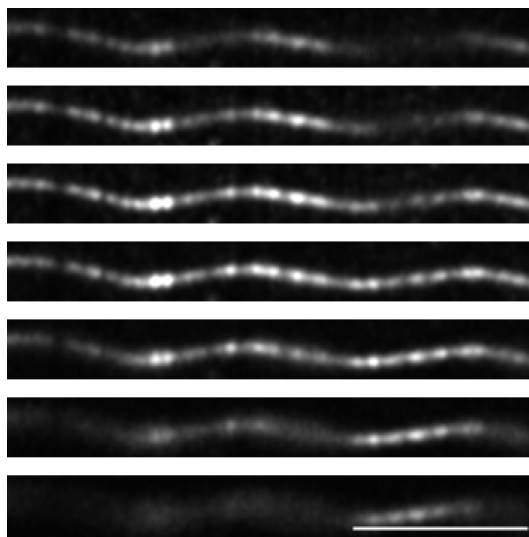


Figure 7. z-stack of a helical MT observed using confocal imaging with Airyscan processing. Images of the z-stack of the same MT as in Figure 6B, with a step of 220 nm between each plane. Scale bar: 5 μ m.

Recipes

All solutions were conserved at indicated temperature without any observable deterioration along time unless stated otherwise. Most solutions were aliquoted to avoid freezing-thawing cycles.

1. 5 \times BRB80

36.28 g PIPES

1.5 ml of MgCl_2 (1 M)

570.6 mg EGTA

Dissolve in 250 ml of deionized water and adjust pH to 6.85 with KOH

Add deionized water to 300 ml, filtrate, aliquot (1 ml and 50 ml) and store at -20 $^{\circ}\text{C}$

Note: The 5 \times BRB80 is used to make the BRB80 solution and to adjust the final concentration of the reaction to 1 \times BRB80. Upon 5 \times dilution the pH drops to 6.75.

2. BSA 10%
Dissolve 2 g of BSA in 20 ml PBS
Filter and store at -20 °C up to 1 year
3. Neutravidine
Dissolve 10 mg Neutravidine in 10 ml H₂O, aliquot (5 and 100 µl) and store at -20 °C
Before the experiment, predilute 5 µl with 195 µl of 1% BSA/BRB80 (final concentration 25 µg/ml)
4. DTT, 200 mM
Dissolve 617 mg DTT powder in 4 ml deionized H₂O, filtrate, aliquot and store at -20 °C
The day of use, dilute 1/5 in H₂O or BRB80. Discard aliquot after each day of use
5. KCl, 500 mM
Dissolve 1 g KCl into 50 ml deionized H₂O (-20 °C) filtrate, aliquot and store at -20 °C
6. PLL-PEG
 - a. Make aliquot of the powder of known weight (20-30 mg) under argon gas and store at -20 °C.
 - b. To make stock solution, dissolve the powder in 10 mM HEPES (pH 7.4) at 1 mg/ml, make 50 µl aliquots and store at -20 °C.
 - c. The day of use, dilute one 50 µl aliquot with 450 µl of 10 mM HEPES (pH 7.4). Diluted PLL-PEG can be stored at 4 °C for one week.
7. Silane-PEG or Silane-PEG-biotin
Notes:
 - i. *Silane-PEG or Silane-PEG-biotin powders are weighted and aliquoted in 200 mg per eppendorf tube under argon gas. The tubes are tightly sealed with parafilm and stored at -20 °C.*
 - ii. *Silane-PEG and Silane-PEG-biotin solutions must be kept anhydrous and in the dark.*
 - a. Dissolve 200 mg powder in 200 ml of 96% ethanol plus 0.4 ml of 37% HCl.
 - b. To solubilize the Silane-PEG solution, heat to 50 °C in a water-bath. Keep the solution in a glass bottle.
 - c. Silane-PEG and silane-PEG-biotin solution are stored at room temperature in the dark for up to 4 months or around 15 coating. If experiments look dirty in the background, use freshly prepared coating solutions.
8. GTP 20 mM
Dissolve 250 mg GTP in 5 ml H₂O
Make aliquots and store at -20 °C
Before use, dilute the stock solution to 20 mM in H₂O or BRB80
9. Glucose 450 mg/ml
Dissolve 2.250 g of glucose in 5 ml of BRB80
Filtrate, aliquot and store at -80 °C
The day of use pre-dilute 1/10 in BRB80 and keep on ice
10. Deoxymix (catalase and glucose oxydase)

Dissolve 35 mg of catalase plus 250 mg of glucose-oxylase in 10 ml of BRB80

Filtrate, make 25 µl aliquots, freeze in liquid nitrogen and store at -80 °C

Discard thawed aliquot after each day of use

11. NaOH, 1 M

Dissolve 8 g of NaOH in 200 ml deionized water

Discard after each use

12. BSA 1%/BRB80

Dilute 500 µl of 10% BSA with 1 ml of BRB80 5× in 3.5 ml of filtrated deionized water

13. Methyl cellulose 1,500 cP

Dissolve 100 mg of methyl cellulose 1,500 cP in 10 ml of prewarm (60 °C) deionized water

Gently shake on rotating wheel for 30 min

Store at 4 °C for two weeks

14. HEPES, 10 mM

Dissolve 477 mg in 200 ml deionized water

Adjust pH to 7.4 with KOH

Store in aliquots at -20 °C

15. Pluronic F27, 10%

Dissolve 100 mg in 1 ml of deionized water

Store at 4 °C up to 2 months

16. PBS

Dissolve 1 tablet in 200 ml of deionized water

Store at -20 °C

17. Neutravidin stock solution

Dissolve Neutravidin in deionized water at a final concentration of 1 mg/ml

Store at -20 °C

18. Red tubulin mix

Dilute purified non-labelled and ATTO-565 tubulin at a 9:1 molar ratio in BRB80

Spin down aggregates at 100,000 × g, 4 °C using TLA100 rotor

Estimate tubulin concentration by measuring the OD_{280nm} (1OD_{280nm} = 1 mg/ml = 10 µM of tubulin)

Aliquot, quickly freeze and store in liquid nitrogen for up to 1 year

19. Polymerization mix

BRB80 supplemented with:

50 mM KCl

1% BSA

4 mM DTT

1 mM GTP

1 mM glucose

0.05% Methyl cellulose 1,500 cP

1/50 deoxy mix (70 µg/ml catalase, 500 µg/ml glucose oxidase, 1 mg/ml glucose)

12 μ M red tubulin mix
200 nM MAP6-N-GFP

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This protocol is derived from Cuveillier *et al.* (2020).

Competing interests

The authors declare no competing interests.

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