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Chapter 4

Tau Interaction with Tubulin and Microtubules: From Purified Proteins to Cells

Tiphany De Bessa, Gilles Breuzard, Diane Allegro, François Devred,
Vincent Peyrot, and Pascale Barbier

Abstract

Microtubules (MTs) play an important role in many cellular processes and are dynamic structures regulated by an important network of microtubules-associated proteins, MAPs, such as Tau. Tau has been discovered as an essential factor for MTs formation *in vitro*, and its region implicated in binding to MTs has been identified. By contrast, the affinity, the stoichiometry, and the topology of Tau–MTs interaction remain controversial. Indeed, depending on the experiment conditions a wide range of values have been obtained. In this chapter, we focus on three biophysical methods, turbidimetry, cosedimentation assay, and Förster Resonance Energy Transfer to study Tau–tubulin interaction both *in vitro* and *in cell*. We highlight precautions that must be taken in order to avoid pitfalls and we detail the nature of the conclusions that can be drawn from these methods about Tau–tubulin interaction.

Key words Tau protein, Microtubules, Tubulin, Turbidimetry, FRET, Cosedimentation essay, Affinity constant

1 Introduction

Microtubules cytoskeleton plays an important role in many cellular processes such as mitosis, cellular integrity, cellular shaping, intracellular trafficking, and cell migration. Microtubules (MTs) are constantly alternating between polymerization and depolymerization phases. This process, called dynamic instability of MT [1], is highly regulated by an important network of microtubules-associated proteins, MAPs, [2] such as Tau. Since Weingarten's discovery of Tau as an essential factor for microtubule formation *in vitro* [3], the interaction of Tau with tubulin has been intensively studied. However, differential experimental conditions (different buffer, Tau and tubulin peptides, Tau constructs) lead to surprising range of Tau–MT affinity values. Studying this interaction is difficult for at least two reasons: the intrinsically disordered structure of Tau and the ability of $\alpha\beta$ -tubulin dimers to self-assemble

into different oligomers depending on the temperature. MTs are hollow cylinders formed at 37 °C by a combination of linear protofilaments, which themselves are formed by $\alpha\beta$ -tubulin dimers associated heads to tails, forming a pseudohelical lattice [4–6]. Tubulin can also self-assemble into other oligomers than MT. For example, in solution at 20 °C, addition of magnesium to tubulin dimers induces formation of single and doubles rings [7–9]. These rings were also described as depolymerization products resulting of protofilaments peeling at MTs end [10–12].

Human brain expresses six isoforms of Tau protein produced by alternative splicing (exons 2–3 and 10) of a single mRNA [13]. The interaction domain of Tau with microtubules, called MT binding domain (MTBR), is well documented: it is constituted of three or four (depending on the isoform) partially repeated sequences and a part of N-terminal proline-rich region flanking the MTBR [14–17]. By contrast, despite the great number of methods used to characterize this interaction, its affinity, the stoichiometry, and the topology of binding site on tubulin/MT are still under debate.

In this chapter, we focus on three biophysical methods namely turbidimetry, cosedimentation assay, and Förster Resonance Energy Transfer (FRET) in living cells used to study Tau–tubulin interaction; the use of other useful methods such as analytical ultracentrifugation or microcalorimetry has been recently described [18–20]. We highlight precautions that must be taken in order to avoid pitfalls and we detail the nature of the conclusions that can be drawn from these methods about Tau–tubulin interaction.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents.

2.1 Tubulin Purification

2.1.1 Reagents and Solutions

1. 1 M magnesium chloride (MgCl_2) commercial aqueous solution.
2. 1 M ethylene glycol-bis(2-aminoethyl-ether)-N,N,NP,NP-tetraacetic acid (EGTA): weigh 7.6 g EGTA in a 20 mL glass beaker and add ultrapure water to have just less than 20 mL. Transfer in a 20 mL graduated flask and adjust volume to 20 mL. Put in a plastic tube or glass bottle and store at room temperature.
3. 0.1 M guanosine triphosphate sodium salt hydrate (GTP): weigh 0.0567 g GTP in a microtube and add 970 μL of ultrapure water. Check the concentration by measuring the absorbance at 256 nm with a molecular extinction coefficient $\epsilon_{256 \text{ nm}} = 13,700 \text{ M}^{-1} \text{ cm}^{-1}$ and store at $-20 \text{ }^\circ\text{C}$ (*see Note 1*).

4. 400 mM phosphate buffer stock (1 L): mix 27.6 g of NaH_2PO_4 H_2O with 28.39 g anhydrous Na_2HPO_4 and add ultrapure water to 1 L in graduated flask.
5. 20 mM phosphate buffer (1 L): put 50 mL of 400 mM phosphate buffer in graduated flask and adjust volume to 1 L. pH must be at 6.95 at 25 °C.
6. PM buffer: 20 mM phosphate buffer, 0.5 mM MgCl_2 . In a 500 mL glass beaker, add 25 mL 400 mM phosphate buffer and 250 μL of 1 M MgCl_2 to 450 mL of ultrapure water. Adjust pH to 7.0 with 10 M NaOH. Transfer the buffer in a 500 mL graduated flask and complete volume to 500 mL. Then, transfer to a 500 mL cylinder and store at 4 °C.
7. PMS buffer: 20 mM phosphate buffer, 0.5 mM MgCl_2 , 0.24 M sucrose. In a 4 L glass beaker, add 200 mL 400 mM phosphate buffer, 2 mL 1 M MgCl_2 and 328.6 g sucrose to 3.5 L of ultrapure water. The pH must be at 6.90 without adjustment. Transfer in 4 L graduated flask and complete volume to 4 L. Put in a glass beaker to store at 4 °C.
8. PMG buffer: 20 mM phosphate buffer, 0.5 mM MgCl_2 , 0.1 mM GTP. In a 2 L glass beaker, add 100 mL 400 mM phosphate buffer, 1 mL of 1 M MgCl_2 to 1850 mL of ultrapure water. Adjust pH to 7 with 10 M NaOH. Transfer and fill it up to 2 L in a graduated flask and then transfer 250 mL in a 500 mL glass beaker to be used to prepare PMG 0.4 M KCl and keep the 1750 mL left in the 2 L glass beaker and store at 4 °C. Weigh 0.1056 g of GTP in a microtube and store it at -20 °C. GTP will be added to the 1.750 L of PMG Buffer on purification day.
9. PMG 0.8 M KCl: 20 mM phosphate buffer, 0.5 mM MgCl_2 , 0.8 M KCl, 0.1 mM GTP. In a 500 mL glass beaker, add 25 mL of 400 mM phosphate buffer, 250 μL of 1 M MgCl_2 , and 29.84 g KCl to 450 mL of ultrapure water. Adjust pH to 7.0 with 10 M NaOH and transfer in a 500 mL graduated flask to fill it up to 500 mL and transfer 250 mL in another beaker to prepare PMG 0.4 M KCl. Keep the 250 mL left in a glass beaker to store at 4 °C. Weigh 0.01507 g of GTP in a microtube and store it at -20 °C. GTP will be added in 250 mL PMG 0.8 M KCl on purification day.
10. PMG 0.4 M KCl: 20 mM phosphate buffer, 0.5 mM MgCl_2 , 0.4 M KCl, 0.1 mM GTP. In a 500 mL glass beaker, mix 250 mL PMG buffer and 250 mL of PMG 0.8 M KCl. Verify pH and adjust it to 7 with 10 M NaOH if it is necessary and store at 4 °C. Weigh 0.03016 g of GTP in a microtube and store at -20 °C. GTP will be added in the 250 mL of PMG 0.4 M KCl on purification day.

11. PMG 1 M sucrose: 20 mM phosphate buffer, 0.5 mM MgCl₂, 1 M sucrose, 0.1 mM GTP. In a 500 mL glass beaker, add 171.2 g sucrose, 25 mL of 400 mM phosphate buffer, 250 μL of 1 M MgCl₂ and ultrapure water to a volume of 450 mL. Adjust pH to 7.0 with 10 M NaOH and transfer in a graduated flask to fill it up to 500 mL. Store at 4 °C. Prepare 500 μL of 1 M GTP as described in **step 3**. It will be added in this buffer on the purification day.
12. Microtubule formation buffer: 20 mM phosphate buffer, 10 mM MgCl₂, 3.4 M glycerol, 1 mM EGTA, pH 6.5, 0.1 mM GTP. In a 500 mL glass beaker, add 156.4 g glycerol to 300 mL ultrapure water and mix few minutes to dilute glycerol (*see Note 2*). Then add 25 mL 400 mM phosphate buffer, 5 mL of 1 M MgCl₂, 500 μL of 1 M EGTA and ultrapure water to 450 mL. Adjust pH to 6.5 and complete volume to 500 mL in a graduated flask. Store at 4 °C. 0.1 mM GTP will be added freshly.
13. Sephadex G25 ultra fine: 50 g of Sephadex G25 beads are preincubated overnight in 1 L water ultrapure before use (*see Note 3*).
14. DEAE A50: weigh 10 g of DEAE A50 in 1 L of 20 mM phosphate buffer, pH7.
15. Guanidine-HCl 6 M: weigh 11.46 g of guanidine-HCl and add 20 mL of water (*see Note 4*).

2.1.2 Special Equipment

1. For slaughterhouse: ice box, plastic bag to put brains, gloves, lab coat, and disposable paper.
2. For purification: big container to boil water, cold room, blender, centrifuge and ultracentrifuge and matching tubes (18×250 mL and 8×50 mL), sintered-glass filter and vacuum flask, column 3 cm×50 cm and collector, dialysis tubing : cellulose molecular weight cutoff 3500 Spectra/Por® molecular porous n°3 18 mm×50 ft, nitrogen tank.

2.2 Tau Purification

2.2.1 Reagents and Buffers

1. Transformed bacteria *Escherichia coli* BL21DE3 with pET containing Tau DNA.
2. Medium Luria Broth Miller (200 mL) sterilized. Weigh 5 g medium in a 200 mL glass beaker and add ultrapure water to 200 mL. Adjust volume in a graduated flask and transfer in a bottle to sterilize and store at 4 °C.
3. Medium Luria Broth Buffered (1 L) sterilized and supplemented with glucose and ampicillin. Weigh 54.8 g medium in a 1 L glass beaker and add ultrapure water. Adjust volume in a graduated flask and transfer in two bottles to sterilize it. Store it at 4 °C.

4. Ampicillin 100 mg/mL. Weigh 1 g ampicillin in 15 mL tube and add 10 mL ultrapure water. Make 1 mL aliquots and store them at -20°C .
5. 1 M Isopropyl-beta-D-1-thiogalactopyranoside (IPTG): weigh 2.4 g in a 15 mL tube and add 10 mL ultrapure water. Make 1 mL aliquots and store them at -20°C .
6. 1 M DL-Dithiothreitol (DTT): weigh 1.54 g in a 15 mL tube and add 10 mL ultrapure water. Make 1 mL aliquots and store them at -20°C .
7. Lysis Buffer (100 mL): 8 mM Triton X100, 45 mM 2-(N-morpholino) ethanesulfonic acid (MES), 1 mM DTT. Weigh 0.5 g Triton and 0.9 g MES. Mix in a 100 mL glass beaker with 95 mL of ultrapure water and add 100 μL DTT 1 M. pH should be around 3.9. Adjust volume with a graduated flask. Make 10 mL aliquots and store them at -20°C .
8. Activation Buffer (500 mL): 45 mM MES, 1 M NaCl. Dissolve 4.4 g MES and 29 g NaCl in 450 mL ultrapure water in a 500 mL glass beaker. Increase pH to 6.5 with NaOH and complete volume to 500 mL in a graduated flask (*see Note 5*).
9. Equilibration Buffer (500 mL): 45 mM MES. Weigh 4.4 g MES and dissolve in 450 mL ultrapure water in a 500 mL glass beaker. Adjust pH to 6.5 with KOH and complete volume to 500 mL in a graduated flask (*see Note 5*).
10. Elution Buffer (500 mL): 45 mM MES, 0.5 M NaCl. Weigh 4.4 g MES and 14.5 g NaCl and dissolve them in 450 mL ultrapure water in a 500 mL glass beaker. Adjust pH to 6.5 with NaOH and complete volume to 500 mL in a graduated flask (*see Note 5*).

2.2.2 Special Equipment

Incubator, French press, centrifuge, FPLC apparatus, dialysis tubing: cellulose molecular weight cutoff 3500 Spectra/Por® molecular porous n°3 18 mm \times 50 ft, freeze-dryer.

2.3 Tau-Tubulin Interaction Experiments in Solution

2.3.1 Microtubule Formation in Solution

1. 400 mM phosphate buffer, 1 M EGTA, 1 M GTP is prepared as described in Subheading 1.
2. 1 M MgCl_2 commercial aqueous solution.
3. 1 M Tris(2-carboxyethyl)phosphine (TCEP): weigh 1.4 g in a 5 mL plastic tube, add 4.5 mL ultrapure water. Use a Vortex to strongly mix the solution for few minutes (*see Note 6*). Make 500 μL aliquots and store them at -20°C .
4. Assembly Condition Buffer : 3.4 M glycerol, 20 mM phosphate buffer, 1 mM EGTA, 10 mM MgCl_2 , pH 6.5, 0.1 mM GTP, 1 mM TCEP. Microtubule formation buffer is prepared as described in Subheading 2.1.1 and 0.1 mM GTP and 1 mM TCEP are added freshly before used.

5. Non Assembly Condition Buffer: 20 mM phosphate buffer pH 6.5, 0.1 mM GTP, 1 mM TCEP. In a 500 mL glass beaker, add 25 mL of 400 mM phosphate buffer to 450 mL of ultra-pure water. Adjust pH to 6.5 with 10 M NaOH. Transfer the buffer in a 500 mL graduated flask and complete volume to 500 mL. Add 0.1 mM GTP and 1 mM TCEP freshly just before used.
6. 10^{-3} M Paclitaxel: weigh 0.8 mg of paclitaxel in 1 mL in dimethyl sulfoxide (DMSO). Its concentration is measured spectrometrically with $\epsilon_{273\text{nm}} = 1,700 \text{ M}^{-1} \text{ cm}^{-1}$ in ethanol.
7. Gravity column (1 cm \times 25 cm).
8. Centrifuge to run at $1,200 \times g$ at 4 °C.
9. Spectrophotometer and quartz cuvettes.

2.3.2 Cosedimentation Assay

1. Glycerol purity superior to 99.5 %.
2. All buffers and reagents necessary to make SDS-PAGE (polyacrylamide gel electrophoresis in denaturing conditions) with 12 % acrylamide.
3. SDS PAGE materials.
4. Ultracentrifuge to run at $88,000 \times g$ at 37 °C.

2.3.3 Electronic Microscopy

1. Uranyl acetate 2%: weigh 0.2 g uranyl acetate and add 10 mL ultrapure water. Mix with vortex and check carefully for the dissolution of crystals (*see Note 7*).
2. 200 mesh carbon-coated formvar films on copper grids.
3. Thermostated room at 37 °C.
4. Transmission electron microscope.

2.4 Tau–Tubulin Interactions by FRET in Living Cells

2.4.1 Cell Culture, Plasmids, and Transfection Reagent

1. 1.5×10^5 cells (purchased from ATCC, USA).
2. Growth media: media supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (*see Note 8*).
3. 8-well Lab-Tek chamber cover glass (Nunc).
4. Media without FBS (*see Note 8*).
5. $10 \times$ phosphate buffer saline stock solution.
6. 10 mM HEPES buffer.
7. Two plasmid DNA species (1 mg/mL): pEGFP-Tau DNA coding for hTau40 protein, the longest human isoform of Tau [21], coupled to the fluorescent protein EGFP as an energy donor; and pmCherry- α -tubulin DNA coding for α -tubulin protein (wild-type α 1B isotype) coupled to the fluorescent mCherry protein [22] as the energy acceptor.
8. Commercial transfection reagent such as LipofectAmine 2000 (Life Technologies, Invitrogen).

2.4.2 Confocal Laser Scanning Microscopy

1. An inverted microscope equipped with a Plan-Apochromat 63× oil immersion objective (Numerical Aperture = 1.4).
2. A Confocal Laser Scanning Microscope (CLSM) equipped with Argon (488 nm) and Helium–Neon (543 nm) laser lines, specific dichroic filter and a spectral mode to select specific domains of the emission spectrum (*see Note 9*).
3. A photobleaching module coupled to CLSM including an observation beam, a photo-destruction beam and a time-resolved recording system.
4. The public-domain ImageJ software [23] and the plug-in PixFRET [24] for image analysis.

3 Methods

3.1 Tubulin Purification

Tubulin purification necessitates three consecutive days, one for materials and buffer preparations and two others for the purification.

3.1.1 Tubulin Purification (Day 1)

1. Equilibrate the ionic exchange resin in 20 mM phosphate buffer. From stock solution 400 mM phosphate buffer, prepare 1 L of phosphate buffer 20 mM by adding 50 mL of 400 mM phosphate buffer in graduated flask and adjust volume to 1 L with ultrapure water. Then, weigh 10 g of DEAE-Sephadex A50 in a 2 L flask and add 1 L 20 mM phosphate buffer. Put this flask in boiling water and let DEAE-Sephadex A50 swell for 5 h (*see Note 10*).
2. G25 Preparation: pack the Sephadex G25 beads (preincubated in ultrapure water overnight) in a column of 50 cm high and 3 cm diameter (3 cm × 50 cm). Leave 2 cm at the top of the column to add the sample. Equilibrate it with 400 mL PM buffer at a 1 mL/min flow rate (*see Note 11*).
3. Chemical preparation: crush ammonium sulfate crystals in powder to dissolve it easier.
4. Weigh GTP samples as described in Subheading 2.1.1 and store it at −20 °C.
5. Material and room preparation: clean and organize the cold storage room. Dispose of materials.

3.1.2 Tubulin Purification (Day 2)

The brains (fifteen lamb or pig brains) should be collected just after the animal death in the slaughterhouse and put them in plastic bag and rapidly in ice box for the transport. From there, conduct all the experiments in a cold room to prevent tubulin denaturation. Nitrile gloves are used for all purification steps and workers must wear mask during brain homogenization.

1. Brain homogenization: remove superficial blood vessels and meninges from brains with dissection pliers. Cut and rinse them with an equal volume of PMS (do not exceed 2.5 L) that will be eliminated through gauze. Repeat this step three times. Add PMS (total volume must not exceed 3 L to limit centrifuge run numbers) to brains and homogenize them in a blender for 30 s.
2. To eliminate membranes and cellular fragments, homogenate is centrifuged in 12 tubes (250 mL) at $13,000 \times g$ for 30 min at 4 °C (*see Note 12*). During this centrifugation, add GTP to PMG buffer, PMG 0.4 M KCl and PMG 0.8 M KCl. Equilibrate G25 with 400 mL PMG at a flow rate of 1 mL/min. Begin DEAE-Sephadex A50 equilibration: get rid of the buffer by filtration with sintered-glass filter and vacuum flask. Add 250 mL PMG buffer to Sephadex beads and increase pH to 6.7 with few drops of 10 M NaOH. Get rid of the buffer as described above and add 250 mL PMG buffer.
3. Ammonium sulfate fractionation 32%: discard pellets (*see Note 13*) and measure supernatant volume with cylinder. Put it in a 2 L glass beaker and add slowly 177 g of ammonium sulfate per liter of supernatant. When ammonium sulfate is completely dissolved, wait for 10 min.
4. Centrifuge the precipitate at $13,000 \times g$ for 30 min at 4 °C in six tubes of 250 mL (*see Note 12*). During this second centrifugation, continue to equilibrate DEAE-Sephadex A50: increase pH to 6.8 with 10 M NaOH, throw out buffer and add 250 mL PMG buffer. Increase pH to 6.9 and throw out buffer. Add 250 mL PMG buffer. DEAE-Sephadex A50 is ready to use.
5. Ammonium sulfate fractionation 43%: discard pellets (*see Note 13*) and measure the supernatant volume with cylinder. Put it in a 2 L glass beaker and add slowly 71 g of ammonium sulfate per liter of supernatant. When ammonium sulfate is completely dissolved, wait for 10 min.
6. Centrifuge the precipitate at $13,000 \times g$ for 30 min at 4 °C (*see Note 12*). Discard the supernatant. Collect pellets with spatula and suspend them by gentle mixing in a 100 mL glass beaker. Wash thoroughly centrifugation tubes with PMG buffer. Do it three times but do not exceed a final volume of 40 mL PMG buffer (*see Note 14*).
7. DEAE A50: the protein solution is then mixed with 250 mL of the DEAE-Sephadex A50 which has been previously equilibrated in PMG buffer. Incubate for 10 min with gentle agitation. Get rid of buffer by filtration on sintered-glass filter and vacuum flask. Eliminate filtrate and add 200 mL of PMG 0.4 M KCl on DEAE-Sephadex A50 containing sample.

Incubate with gentle agitation for 5 min. Get rid of buffer by filtration as described above twice. Change vacuum flask to keep filtrate. Add 125 mL PMG 0.8 M KCl, wait 5 min and eluate tubulin by filtration. This step has to be repeated twice.

8. Add slowly 62 g ammonium sulfate to the 250 mL of filtrate in a 500 mL glass beaker and wait for 5 min with gentle agitation after total ammonium sulfate dissolution.
9. Centrifuge at $66,000\times g$ for 25 min at 4 °C (*see Note 12*). Discard the supernatant and collect pellets with spatula and suspend them by gentle mixing in a 100 mL glass beaker. Wash thoroughly centrifugation tubes with PMG buffer. Do it three times but do not exceed a final volume of 20 mL PMG buffer (*see Note 14*). The totally dissolved pellets will be passed through Sephadex G25 chromatography.
10. Sephadex G25 chromatography: when the PMG buffer completely entered in the resin, load carefully the protein solution. When half of the mix is in the column add buffer on the top without mixing solutions (*see Note 15*). Eluate with a 1 mL/min flow rate and collect fractions of 3–3.5 mL. In tubes containing protein where a meniscus is observed (use plastic tubes), add 1 M MgCl₂ to have a final concentration of 30 mM (110 μL for 3.5 mL). Mix by inverting and collect all samples in which white precipitate is instantaneously observed. Gather them in centrifugation tubes.
11. Centrifuge at $9,000\times g$ for 10 min at 4 °C (*see Note 12*). During this centrifugation, add 500 μL 0.1 M GTP to the 500 mL of PMG sucrose buffer.
12. Collect pellets and dissolve them in a minimum volume of PMG sucrose buffer (do not exceed 5 mL) with gentle agitation (*see Note 14*).
13. The solution is placed in a dialysis tubing (cellulose molecular weight cutoff 3500, Spectra/Por® molecular porous n°3 18 mm × 50 ft) and dialyzed for 16 h against PMG sucrose buffer supplemented with 0.1 mM GTP.

3.1.3 Tubulin Purification and Storage (Day 3)

1. Collect tubulin from the dialysis tubing and centrifuge it at $12,000\times g$ for 30 min at 4 °C (*see Note 12*). Discard pellets and keep the supernatant. Make 300 μL aliquot in cryotubes and stock them in liquid nitrogen. Store 2 mL of dialysis buffer.
2. Tubulin concentration is determined by spectrophotometry. Put 5 μL of dialysis buffer in 500 μL of 6 M Guanidine-HCl and make the blank. Put in triplicate 5 μL of tubulin sample in 500 μL and measure the absorbance at 275 nm. Determine the concentration using the Lambert–Beer law with a molecular extinction coefficient of $\epsilon_{275\text{ nm}} = 1.09\text{ mL mg}^{-1}\text{ cm}^{-1}$ taking into account the dilution factor (here 101).

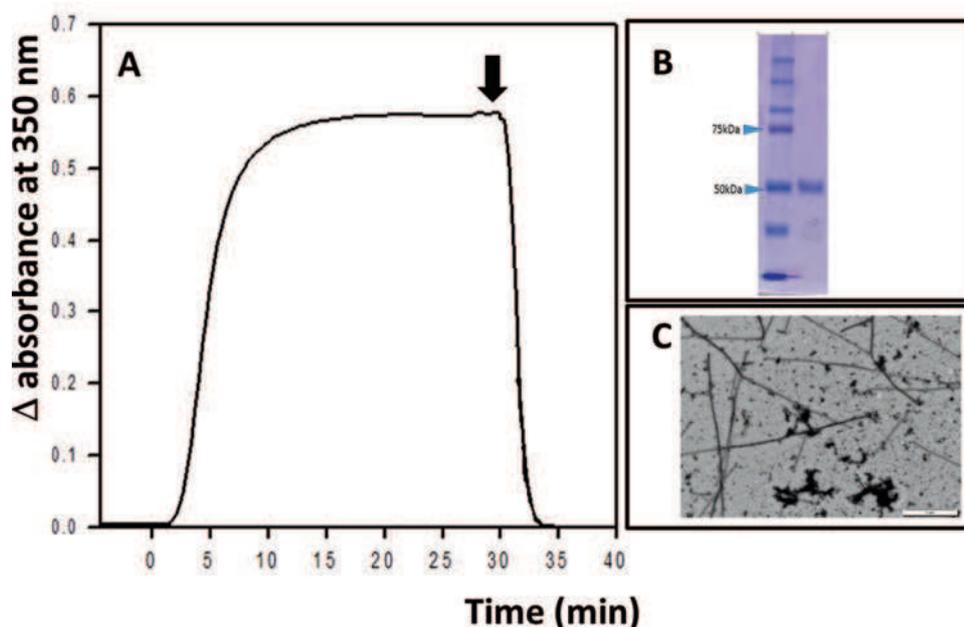


Fig. 1 Tubulin purification: **(A)** MT formation time course at 350 nm of 30 μM of purified tubulin in assembly conditions at 37 $^{\circ}\text{C}$. At time indicated by *arrow*, the temperature is decreased to 10 $^{\circ}\text{C}$. **(B)** 12% SDS-PAGE of Tubulin that migrates in one band at 50 kDa. **(C)** Electronic microscopy of the MTs sample. The *white scale bar* represents 1 μm

3. Test the tubulin capacity to form microtubules using a turbidimetry assay (*see* Subheading 3.3.2).

At the end of the purification, you should have 200–300 mg of pure tubulin at 40–80 mg/mL and an absorbance variation of about 0.5 for 30 μM (3.0 mg/mL) of tubulin corresponding to microtubule formation (Fig. 1).

3.2 Tau Purification

3.2.1 Bacterial Pregrowth

1. Switch on the “Hoffman heater” 30 min before the beginning of the bacterial growth (*see* Note 16).
2. Near the “Hoffman heater” in a 500 mL Erlenmeyer flask, mix 100 mL sterilized medium Luria Broth Miller with 100 μL Ampicillin and 100 μL stock bacteria. Place it in an incubator at 37 $^{\circ}\text{C}$ overnight with agitation.

3.2.2 Bacterial Culture

1. Switch on the “Hoffman heater” 30 min before the beginning of bacterial growth (*see* Note 16).
2. Near the “Hoffman heater” first, remove 5 mL medium without bacteria to make blank to read absorbance and second, in each two 2 L Erlenmeyer flask, 500 mL sterilized medium Luria Broth Buffered are mixed with 1.85 g glucose, 100 μL ampicillin, and 10 mL pregrowth bacteria. Place them in an incubator at 37 $^{\circ}\text{C}$ with agitation for few hours. Read absorbance at

600 nm every hour until it reaches a value between 0.6 and 1. At this moment add 750 μL of 1 M IPTG to induce protein expression and incubate for 2 h 30 min.

3. Centrifuge at $5,000\times g$ for 10 min at 4 °C and remove supernatant. Pellets are recovered and dissolved in lysis buffer (*see Note 17*).

3.2.3 Proteins Extraction

1. Apply twice on the dissolved pellet a 4 t pressure in French press.
2. Boil the bacterial lysate at 90 °C for 10–15 min, to select thermal-resistant proteins like Tau.
3. Centrifuge for 30 min at $30,000\times g$ at 4 °C (*see Note 12*). Keep the supernatant (*see Note 17*).

3.2.4 Tau Purification

1. Chromatography preparation (*see Note 18*): A SP Sephadex® High Performance 5 mL column is activated by passing 5 mL (one column volume) of activation buffer (45 mM MES, 1 M NaCl) with a NaCl gradient of 0–100% (mix with equilibration buffer constituted of 45 mM MES) at a flow rate of 1 mL/min. Beads are rinsed with 3 column volumes (15 mL) of activation buffer. Reverse the NaCl gradient from 100 to 0% in 5 mL. Before sample injection, column is washed with 15 mL of equilibration buffer.
2. Start to collect 5 mL fractions and inject sample with equilibration buffer at a flow rate of 0.5 mL/min. Absorbance increases during sample deposit (resin–protein interaction). Rinse column with equilibration buffer as far as absorbance comes back to baseline level and then begin elution.
3. Apply a 30% NaCl gradient: increase the salt concentration to 30% in two column volumes (10 mL) with a flow rate of 1 mL/min. Apply it as long as absorbance is constant.
4. Increase the salt gradient to 70% in one column volume (5 mL) with a flow rate of 1 mL/min. Tau is released at this step. When Tau protein has been released from the column, a 100% gradient is applied to wash column for few minutes at a flow rate of 1 mL/min.
5. To verify that Tau is present in fractions or to know if there is no Tau in flow through, you may take 20 μL of each fraction, add 5 μL of loading buffer 5 \times and depose them on SDS PAGE 12% acrylamide (*see Note 17*).
6. Fractions containing Tau are pooled in a dialysis tubing (cellulose molecular weight cutoff 3500, Spectra/Por® molecular porous n°3 18 mm \times 50 ft) and dialyzed for 2 h, three times against ultrapure water. Then, Tau is collected and frozen at -80 °C to be lyophilized. Once lyophilized, Tau can be stored at -20 °C for several months.

- Before use, Tau is dissolved in the desired buffer, centrifuged at $3,000 \times g$ for 10 min and its concentration is determined by spectrophotometry using the extinction coefficient $\epsilon_{280\text{nm}} = 7,700 \text{ M}^{-1} \text{ cm}^{-1}$ after light scattering correction (Fig. 2).

3.3 Tau–Tubulin/ Microtubule Interaction in Solution

Microtubule formation is a cooperative system that needs in vitro a tubulin minimum concentration called “tubulin critical concentration” (C_r). C_r is defined as the inverse of the apparent MT-elongation constant K_{app} [25]. To determine C_r , we measured microtubule amount by turbidimetry or sedimentation assay (tubulin concentration in the pellet) as a function of total tubulin concentration and fit the results to a linear regression. The C_r corresponds in this case to the value of x (tubulin concentration) found for $y=0$ (no microtubule formation). More accurately, measuring the concentration found in the supernatant of sedimentation assay of microtubule solution leads directly the C_r value. This value is dependent of the experimental conditions. It is well known that magnesium ions, glycerol, dimethyl sulfoxide, sucrose are positive factors that reduce the C_r . For example, C_r of tubulin is $7 \mu\text{M}$

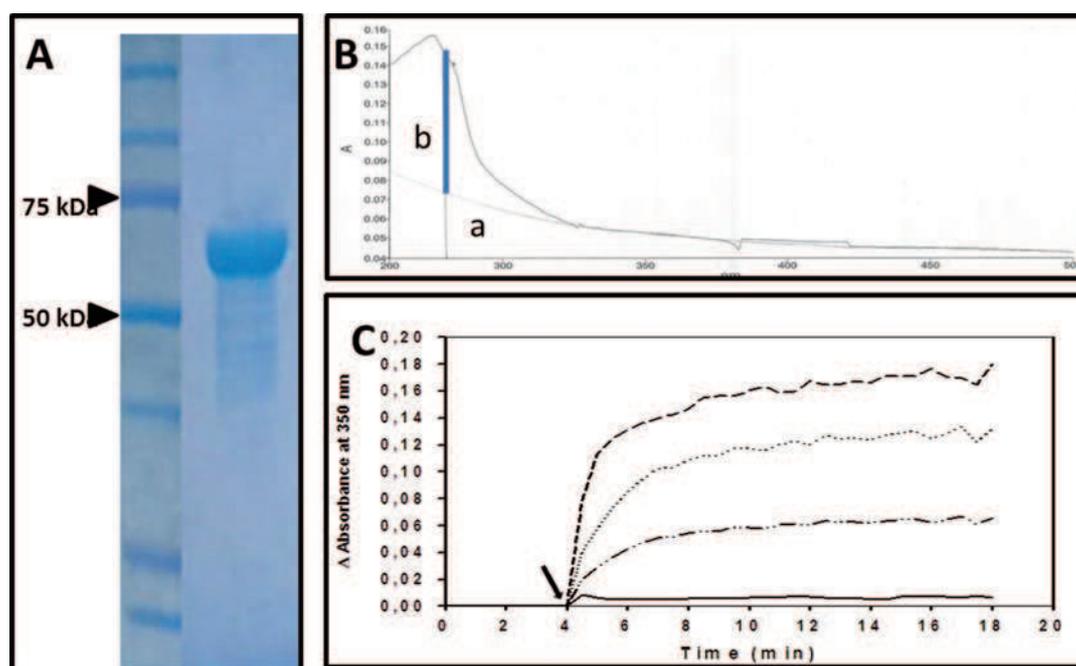


Fig. 2 Tau purification: (A) 12 % SDS-page of purified Tau protein. As largely described in literature, the largest 441 amino acid long isoform migrate at an apparent molecular mass of approximately 65 kDa. (B) Concentration determination by UV–visible wavelength scan of Tau protein. The *line A* corresponds to the light diffusion that should be subtracted to the scan to not sur-estimate the concentration. The corrected absorbance at 280 nm (*line B*) is then used to determine the Tau concentration. (C) Typical time course of MTs formation at 37 °C induced by Tau. Tubulin concentration is 5 μM is mixed with no (*dark line*) or 3.5 (*dash-dot line*), 4.2 (*dot line*) and 5 μM of Tau (*dash line*). The *arrow* indicates the Tau addition

in 20 mM phosphate buffer, 3.4 M glycerol, 1 mM EGTA, 10 mM MgCl₂, 0.1 mM GTP, pH 6.5 [26] and 20 μM in 80 mM K-Pipes, 1 mM MgCl₂, 1 mM EGTA, GTP 1 mM pH 6.8 [27]. In these conditions, called in this chapter as “assembly conditions buffer,” presence of Tau leads to *a microtubule formation facilitated by ligand binding*. In 2004, we found experimental conditions in which the presence of Tau induces the microtubule formation in the absence of any other known polymerization factors (20 mM phosphate buffer pH 6.5, 0.1 mM GTP, 1 mM TCEP) (*see Note 6*). In this condition, called in this chapter “non-assembly condition,” presence of Tau leads to *a microtubule formation induced by ligand binding* [28].

3.3.1 Tubulin Preparation

Before using tubulin it is necessary to eliminate sucrose. Tubulin is equilibrated (*see Note 19*) in the appropriate buffer (assembly or non-assembly condition) on gel filtration Sephadex G25 resin on a gravity column (1 cm*25 cm).

1. Equilibrate the column with cold 40 mL of appropriate buffer at a flow rate of 1 mL/min and defreeze rapidly tubulin sample.
2. When the buffer is removed (without completely drying the resin), put tubulin solution (*see Note 20*) on the top. When all the protein entered in the resin, put the buffer and start elution at a flow rate of 1 mL/min. Collect 500 μL fractions and put them on ice. The tubulin concentrations into the tubes with meniscus (*see step 10* in Subheading 3.1.2) are determined by spectrophotometry as described in Subheading 3.1.3 with elution as control instead of dialysis buffer.

3.3.2 Turbidimetry Assay

Tubulin prepared as described above is diluted to 7.5 μM in the appropriate buffer to be at a tubulin final concentration of 5 μM (this concentration is under the 7 μM Cr for the assembly condition). Sample volume and number of samples depend on the spectrophotometer (for us, it is 300 μL and six samples).

1. Before starting the time-driven microtubule formation, make blank on the spectrophotometer with buffer alone.
2. Put 200 μL of tubulin at 7.5 μM in the six different cuvettes and put them on ice.
3. Put rapidly the cuvettes in the spectrophotometer thermostated at 37 °C and measure the absorbance time course at 350 nm for 5 min. You may have to wipe condensation off.
4. Add rapidly in each cuvettes 100 μL of buffer for control or different Tau concentrations (from 0.5 to 5 μM). Do not use Tau concentration above the tubulin concentration because it induces microtubule bundling [28]. The signal should increase rapidly indicating microtubule formation. The turbidity is

observed for 30 min (Fig. 2). The samples then can be used for cosedimentation assay and/or electron microscopy.

5. Mix 15 μM of tubulin prepared as described in Subheading 3.3.1 in 20 mM phosphate buffer, 8 mM MgCl_2 , pH 6.5, 1 mM TCEP with 15 μM of paclitaxel, a microtubule inducer and stabilizing agent.
6. Incubate for 25 min at 37 °C. At this stage the solution is opaque due to the microtubule formation.
7. Dilute the paclitaxel-stabilized microtubules (200 μL) to 7.5 μM .
8. Mix 200 μL of paclitaxel-stabilized MT with 100 μL of buffer or different concentrations of Tau from 0.5 to 20 μM . After 5 min, take 10 μL for transmission electron microscopy and 200 μL for cosedimentation assays.

3.3.3 Cosedimentation Assays: Microtubules Bound Tau, Free Tau, and Free Tubulin Separation

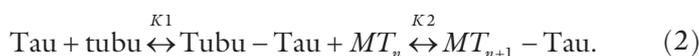
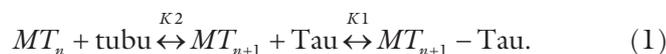
Microtubule and Tau bound to microtubule are separated from free Tau and tubulin by centrifugation. To prevent nonspecific association, centrifugation is conducted on 60% glycerol cushion.

1. Prepare the glycerol cushion by adding 60 mL of glycerol to 40 mL of appropriate buffer and form microtubules as described in the Subheading 3.3.2.
2. Place 200 μL samples on 200 μL glycerol 60% in ultracentrifuge tubes pre-warmed at 37 °C and centrifuge at $88,000\times g$ for 20 min at 37 °C in a pre-warmed rotor.
3. Collect supernatant (200 μL) containing free Tau and tubulin and mix it with 40 μL of 5 \times SDS-PAGE loading buffer. Turn upside down the tubes containing the pellets to remove the rest of supernatant.
4. Wash the pellets containing MT and bound Tau tactfully with 200 μL of warm (37 °C) buffer to eliminate free Tau and free tubulin. Repeat this step three times.
5. Dissolve the pellet in 200 μL cold buffer to depolymerize microtubules. A manual action may be necessary to suspend correctly pellets. Add 40 μL of 5 \times SDS-PAGE loading buffer to the 200 μL dissolved pellets.
6. Heat samples at 95 °C before loading it in a 12% SDS-PAGE.
7. 10 μL of pellets and supernatants are loaded on a same 12% SDS-PAGE to eliminate error due to coloration variation. Protein content is revealed by Coomassie Brilliant Blue staining. To quantify free and bound tubulin and free and bound Tau, loading of each sample must be readjusted to obtain comparable and non-saturated spots. Protein quantification is determined with band intensity quantification by ImageJ.

The experiments must be done three times to have a representative average. Because Tau concentration in the pellet represents MT-bound Tau concentration and that in the supernatant the free one, Scatchard plot can be used to determine affinity constant and the stoichiometry of the reaction. For analysis used the following equation:

$$\frac{B}{[\text{Tau}]_{\text{free}}} = nK_a - BK_a$$

where B is the ratio of the concentration of bound ligand (here Tau in the pellet) to total available binding sites (total tubulin concentration), $[\text{Tau}]_{\text{free}}$ is the concentration of free Tau, n is the number of binding sites, and K_a the affinity constant of Tau for the microtubule. Plotting this data, $B/[\text{Tau}]_{\text{free}}$ versus B , yields the Scatchard plot with a slope $-K_a$ and an x -intercept leads to n . This approach can be used only in the case of studying Tau interaction with paclitaxel-stabilized MT. For the two other conditions, Scatchard plots are curved downward reflecting the fact that ligand-induced tubulin assembly may proceed by a ligand facilitated elongation (in assembly conditions) in which the elongation precedes the binding of Tau (Eq. 1) or a ligand-mediated elongation pathway (in non-assembly conditions), in which the binding of Tau, to either tubulin or microtubule, precedes the elongation pathways (Eq. 2) [28].



Equation 1 is characterized by an apparent elongation constant $K_{\text{app}} = K_2(1 + K_1[\text{Tau}]_{\text{free}})$ (for more details, *see* the appendix of ref. [29]) and Eq. 2 is characterized by an apparent elongation constant $K_{\text{app}} = K_1K_2[\text{Tau}]_{\text{free}}/(1 + K_1[\text{Tau}]_{\text{free}})$. As already mentioned in Subheading 3.3, the K_{app} is related to $1/\text{Cr}$ and the Cr corresponds to the free tubulin concentration in the supernatant of the cosedimentation assay. So plot K_{app} in function of free Tau concentration and fit the data with the equation of K_{app} described above. Use Eq. 1 in assembly condition and Eq. 2 in non-assembly condition.

3.3.4 Transmission Electronic Microscopy

1. Removed 10 μL at the end of microtubule formation reaction (*see* **Note 21**).
2. Put down on a Parafilm sheet a 200 mesh carbon-coated formvar films on copper grids
3. Put a little drop of samples (3 μL) on grids and incubate for 15 s.

4. Eliminate the excess of sample by taking the grid with tweezers and absorb sample with filter paper (apply the grid perpendicularly).
5. Add 3 μL uranyl acetate on the grid for 30 s for staining.
6. Eliminate uranyl acetate excess as describe in **step 4**.
7. Dry grids overnight at 37 °C and observe it using a transmission electron microscope.

3.4 Tau–Tubulin Interactions by FRET in Living Cells

3.4.1 Transfection Protocol with Plasmids DNA

The transfection protocol is performed as recommended by the supplier (LipofectAmine 2000, Life technology, U.S.). For cell transfection, 0.4 μg of DNA (0.4 μg of pEGFP-Tau DNA in well 1, 0.4 μg of pmCherry- α -tubulin DNA in well 2, 0.2 μg of pEGFP-Tau DNA and 0.2 μg of pmCherry- α -tubulin DNA in well 3) are applied on cells. After transfection, the well 1 contains cells expressing EGFP-tau proteins (corresponding to the condition “donor alone”), the well 2 contains cells expressing mCherry- α -tubulin proteins (corresponding to the condition “acceptor alone”), and the well 3 contains cells expressing both EGFP-tau and mCherry- α -tubulin proteins (corresponding to the condition “uFRET”). Cells are observed after 24–48 h post-transfection.

3.4.2 Confocal Imaging: FRET Measurement by Sensitized Emission

1. Place cells in medium supplemented with 1 % FBS and 10 mM HEPES to reduce pH variations (*see Note 22*), and maintain cells at 37 °C.
2. Configure the CLSM’s settings such as imaging conditions are optimized before data collection (*see Note 23*). The donor EGFP-tau is excited sequentially at 488 and 543 nm and the fluorescence emission is collected using the CLSM’s spectral mode simultaneously between 496 and 535 nm (channel 1, corresponding to the donor settings: excitation of the donor, detection of the donor) and between 580 nm and 650 nm (channel 2 corresponding to the uncorrected FRET (uFRET) settings: excitation of the donor, detection of the acceptor) (*see Note 23*). The acceptor mCherry- α -tubulin is then excited at 543 nm and the fluorescence emissions are collected between 580 and 650 nm (channel 3 corresponding to the acceptor setting: excitation of the acceptor, detection of the acceptor).
3. Adjust gain and offset of the donor and uFRET channels with cells expressing EGFP-tau alone. The pixel intensities must be linear with the total dynamic scale of fluorescence intensities (for example 256-grey scale for an 8-bit image depth) (*see Notes 24 and 25*). Collect three images of cells on the field.
4. Adjust gain and offset of the acceptor channel exclusively with cells expressing mCherry- α -tubulin alone (*see Note 26*). Collect three images of cells on the field.

5. Image cells expressing the donor and acceptor pairs through the donor, the uFRET and the acceptor settings previously adjusted in **steps 3** and **4**.

**3.4.3 Confocal Imaging:
FRET Measurement
by the Acceptor
Photobleaching**

An alternative method to quantify the absolute efficiency of FRET between the donor EGFP-tau and the acceptor mCherry- α -tubulin is to measure the EGFP emission before and after selective photobleaching of the mCherry [30].

1. Place cells expressing both the donor and the acceptor in medium supplemented with 1% FBS and 10 mM HEPES to reduce pH variations (*see Note 22*), and maintain cells at 37 °C.
2. Configure the CLSM settings such as: EGFP-tau is excited at 488 nm and the fluorescence emission is collected using the CLSM's spectral mode between 496 and 535 nm; and mCherry- α -tubulin is excited at 543 nm and the fluorescence emission is collected between 580 and 650 nm. The donor and acceptor fluorophores are sequentially excited.
3. Adjust gain and offset of the donor channel with cells expressing EGFP-tau alone so that as the fluorescence intensity of EGFP-tau does not saturate PMT (*see Note 27*).
4. Adjust gain and offset of mCherry- α -tubulin channel with cells expressing mCherry- α -tubulin alone. The pixel intensities must be linear with the total dynamic scale of fluorescence intensities (for example 256-grey scale for an 8-bit image depth).
5. Collect two images of cells with the 488 and 543 nm excitation lines with donor and acceptor settings as previously described in **steps 3** and **4** (*see Note 28*).
6. Irradiate the acceptor on a region of interest (ROI) in the cell using 100% laser power with a 543 nm exciting beam. As reported in ref. [30], mCherry- α -tubulin was photobleached for 31.56 s (25 frames) on a 6 \times 6 μ m² ROI (*see Note 29*).
7. Recollect two images of cells with the 488 and 543 nm excitation lines after the mCherry- α -tubulin photobleaching in the ROI.

**3.4.4 FRET Calculation
for Sensitized-Emission
FRET**

The ImageJ plug-in PixFRET (Center for Integrative Genomics, Faculty of biology and medicine, University of Lausanne, Switzerland) [24] can generate images of sensitized-emission FRET, and hence show where FRET occurs within a cell or a group of cells, by computing pixel by pixel the images of a sample acquired in a three channel setting.

1. For donor Spectral Bleed-Through (SBT) determination, create a stack of donor and uFRET images of cells expressing EGFP-tau alone.

2. Select a ROI manually by outlining cells on the donor image and determine the mean fluorescence intensity of the ROI in the Analyze menu of ImageJ software; do the same measure of the mean fluorescence intensity on the uFRET image (*see Note 30*).
3. Calculate the intensity ratio of the uFRET on donor images; the mean value of all intensity ratios corresponds to the donor SBT parameter *A* (*see Note 31*).
4. Repeat **steps 1–3** to calculate the acceptor SBT parameter *B* with images of cells expressing mCherry- α -tubulin alone.
5. Before starting the PixFRET plug-in, create a stack of three images of the cell or the group of cells expressing both the donor EGFP-tau and the acceptor mCherry- α -tubulin: the first one in the uFRET setting, the second one in the donor setting and the third one in the acceptor setting.
6. Launch PixFRET from the plug-ins menu in ImageJ.
7. Enter the value for the donor and acceptor SBTs in the Donor and Acceptor Models bookmarks, respectively (*see Note 32*), and click on the FRET bookmark to select a ROI on the stack in order to determine the background outside cells; press Get button.
8. In the parameter box below, select “no Gaussian Blur” and a value of 1.0 for threshold correction factor (*see Note 33*), as well as the type of normalization of the FRET values among 4 possibilities: by dividing by the donor or the acceptor intensity [31], by the product of donor and acceptor intensities [32] or by the square root of the product of donor and acceptor intensities [33]; users can also generate an image displaying the FRET efficiencies in the sample. For example, the methodology reported in ref. [30] was to normalize corrected FRET (*F_c*) to the donor intensity.
9. Click on “Compute FRET” button to generate the normalized FRET image, in addition to the *F_c* image calculated from the formula described by ref. [34]:

$$F_c = I_{\text{FRET}} - A \times I_{\text{donor}} - B \times I_{\text{acceptor}}$$

Where I_{FRET} , I_{donor} , and I_{acceptor} are pixel intensities (subtracted from their background intensity) in cells under the distinct uFRET, donor, and acceptor settings, respectively. The parameters *A* and *B* were calculated in **steps 3** and **4**.

10. To reduce background noise, prefer to use a nonlinear filtering of the computed FRET image with for example a 1-pixel-range median filter than a Gaussian filter [35, 36].

11. In the Image menu of ImageJ, select an adequate LookUp Table (LUT) and adjust brightness and contrast to take over all the dynamic scale of fluorescence intensities (*see Note 34*).
12. Save the computed FRET images as a TIFF file.

3.4.5 FRET Calculation for FRET by the Acceptor Photobleaching

1. With ImageJ software [23], create a stack of four images of cells expressing EGFP-tau and mCherry- α -tubulin before and after the acceptor photobleaching.
2. On the post-bleaching image of mCherry- α -tubulin, select manually a ROI overlaying the bleached zone.
3. Place on the pre-bleaching image of the donor with the horizontal scrollbar in the stack and determine the mean fluorescence intensity of the ROI using the Measure function of the Analyze menu in ImageJ.
4. Do the same measure of the fluorescence intensity on the post-bleaching image of the donor.
5. Calculate the FRET efficiency (%*E*) from fluorescence recovery after the photobleaching kinetic of the donor emission in the presence of the acceptor in ROIs [37]:
6. Return to **step 2** and select manually a ROI in another zone of the cell; determine the mean fluorescence intensity of the ROI on pre- and post-bleaching images of the donor and calculate %*E* which must be significantly lower than for the bleached zone (*see Note 35*). For example, we obtained %*E* of 8.7 ± 0.2 for EGFP-tau in the mCherry- α -tubulin bleached zone vs. %*E* of 1.1 ± 0.1 in a control zone (unpublished data) (*see Note 36*).

$$\%E = 1 - \left(I_{\text{donor}} \right)_{\text{pre-bleaching}} / \left(I_{\text{donor}} \right)_{\text{post-bleaching}}$$

where $(I_{\text{donor}})_{\text{pre-bleaching}}$ and $(I_{\text{donor}})_{\text{post-bleaching}}$ are the donor emission before and after selective photobleaching of the acceptor, respectively.

7. We recommend completing data with figure showing location of the fluorescence variation of EGFP-tau and mCherry- α -tubulin before and after the acceptor photobleaching, as the example reported in Fig. 3.

4 Notes

1. GTP is dissolved in water. Make Aliquots of 100 μ L and store them at -20 °C. GTP should be added freshly to buffer to avoid hydrolysis of GTP into GDP which is inactive for MTs formation.
2. Glycerol is viscous. Precautions should be taking to be sure that it was accurately mixed with buffer or water.

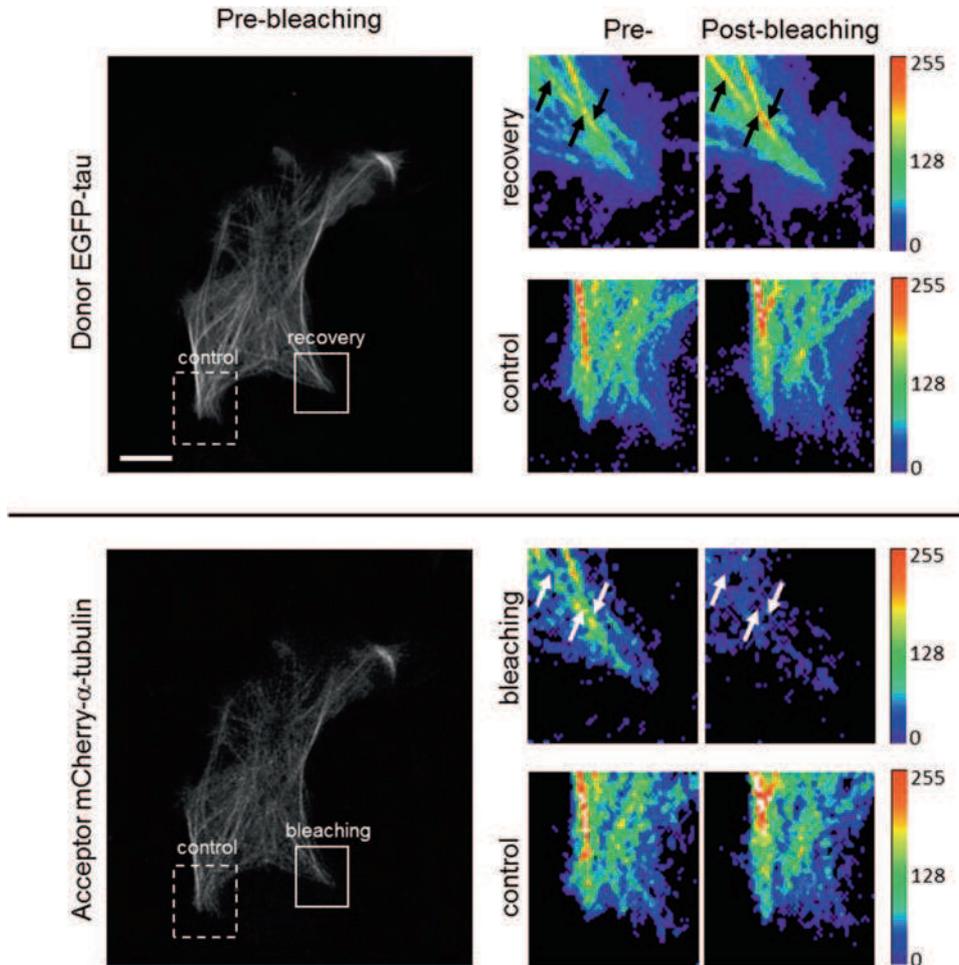


Fig. 3 Energy transfer between EGFP-tau and mCherry- α -tubulin revealed by the acceptor photobleaching in living cells. The quenched and unquenched EGFP-tau emission is compared after specific photobleaching of the mCherry- α -tubulin fluorophore (respectively, *upper* and *lower panels* in false colors under Pre- and Post-bleaching titles). Prior to the acceptor photobleaching, microtubules and uniformly labeled with EGFP-tau; after photobleaching, the fluorescence emission of EGFP-tau increased in spots along microtubules (*black arrows* in *upper panel*) confirming FRET in the initial state; no significant fluorescence intensity fluctuation is observed inside the control region. Scale bar: 10 μ m; side bars: color pixel scoring for fluorescence intensities from 0 (*dark purple*) to 255 (*red*); EGFP-tau and mCherry- α -tubulin are excited at 488 and 543 nm respectively, using the multi-tracking mode of CLSM system

3. Add a pinch of azide to prevent bacteria or fungus growth. After use, unpacked resin, wash ten times with ultrapure water. It can be used ten times.
4. In the case of guanidine-HCl, the volume increases when it is dissolved and the reaction is endothermic. Do not put a volume of water larger than the half of the total volume.

5. All FPLC buffers can be stored at room temperature for few months.
6. TCEP powder is very difficult to dissolve at concentration of 1 M. TCEP is preferred to DTT to reduce disulfide bonds because it is more stable. It is used to maintain Tau monomeric and have no effect on MTs formation.
7. This solution must be filtered just before use to remove crystals or possible pollutants which perturb coloration. To use uranyl acetate use gloves.
8. Add 2 mM L-glutamine and nonessential amino acids if absent in media both to stimulate growth and to prolong the viability of cells in culture.
9. We use a Leica DMI6000CS inverted microscope coupled to a CLSM Leica TCS SP5, with the TD488/543/633 dichroic filter equipping the system.
10. During DEAE swelling, you must check there is always enough water and that it is boiling.
11. The resin must not be dried. Keep an eye on column when already 500 mL went through.
12. Think to thermostat centrifuge and rotor and tubes at the good temperature before run.
13. Collect all wastes in a special bag to eliminate them in rules respect.
14. Wash all tubes meticulously with spatula and collect all volumes, as little as they are, to optimize the output. Start using spatula to crash pellet and then use a magnetic stirring bar which has almost the same length as glass beaker diameter and finish by gently by gently pipetting in and out the sample with a 1 mL pipetman without making bubbles. It is very important to suspend every tiny particle.
15. When you add buffer on the top of mixing solution a biphasic layer formed. It is the sign that tubulin is here and concentrated.
16. Do not wear gloves when you work near a heat source but handle carefully bacteria samples and wash hands after experiments.
17. Pellets can be kept in lysis buffer at $-20\text{ }^{\circ}\text{C}$ for few weeks or experiment can continue. If sample is frozen, defreeze and homogenize it before use.
18. Make sure that the instrument is ready to start (flow rate, good pressure, no leakages, no blocks).
19. Tubulin is sensitive to temperature. So all steps to prepare it must be made at $4\text{ }^{\circ}\text{C}$ in a cold room or ice box with cold buffer.

20. If you put 150 μL of tubulin solution at 670 μM , that corresponds to 10 mg of protein and you recuperate 6–7 mg into four tubes at an average of approximately 35 μM corresponding to the $2/3$ of the amount of protein put initially onto the column.
21. To maintain MT, grids preparation is made in a thermostated room at 37 $^{\circ}\text{C}$ at room temperature, because rings of tubulin might form.
22. Do not add 10 mM Hepes to the medium if the CLSM is equipped with a heat- and CO_2 -regulated chamber.
23. Use samples expressing the donor or the acceptor fluorophores alone to maximize uFRET signals, to minimize SBT, to avoid widespread saturation, to avoid unintended photobleaching, and to minimize background noise [34].
24. Avoid images showing pixels with saturated intensity indicating an excessive excitation laser power.
25. The same PMT settings in donor and acceptor channels and low laser power are recommended, in order to generate a robust uFRET signal at donor excitation in the acceptor channel, without at the same time saturating the donor emission signal in the donor channel.
26. Do not modify gain and offset of the uFRET channel, otherwise to calculate wrong donor and acceptor SBT (parameters *A* and *B* in the FRET calculation in the Subheading 3.4.4).
27. We recommend adjusting gain and offset of the donor channel in order to spread fluorescence intensities on two-thirds of the dynamic intensity scale.
28. Users can focus on two cells in the same image, a first cell on which users irradiate a ROI to photobleach the acceptor and another cell as control.
29. On our CLSM with a 63×1.4 NA objective, these conditions destroy mCherry with very little or no direct bleaching of the EGFP donor. The optimal exposure conditions that bleach acceptor maximally with minimal direct effect on donor should be determined for each microscope system and filter set, using for example droplets of donor and acceptor under oil, cells transfected with donor and acceptor fusion proteins alone, or cells co-transfected with non-interacting donor and acceptor fusion proteins.
30. Keep the same selected ROI in the donor image while switching on the uFRET image.
31. The main causes of bleed-through are the emission of the donor in the acceptor channel and the excitation of the acceptor when exciting the donor. To calculate donor (and acceptor) SBTs, average fluorescence intensities in cells expressing only the donor (or the acceptor) should be quantified, both in the uFRET and in donor (or acceptor) channels. The SBT ratios

are the ratios between the fluorescence intensities in each channel after background subtraction, such as:

- For the parameter A : $A = (I_{\text{FRET}}/I_{\text{Donor}})_{\text{Exc } 488\text{nm}}$
When the donor alone is expressed and excited at 488 nm;
- For the parameter B : $B = (I_{\text{FRET}})_{\text{Exc } 488\text{nm}} / (I_{\text{Acceptor}})_{\text{Exc } 543\text{nm}}$
When the acceptor alone is expressed and excited at both 488 and 543 nm.

32. Keep the value 0 for FRET and Donor/Acceptor boxes in the Background sections; press Accept button; in the Model Donor/Acceptor boxes, select “no Gaussian Blur” to compute FRET images from native uFRET, donor and acceptor images; press on Constant button just below and fill the box with the calculated parameters A (in the Donor Model bookmark) and B (in the Acceptor Model bookmark); press Accept button.
33. The Fc and normalized FRET images are calculated only if pixel values in each image are above a given threshold, set by default to average background values; the threshold correction factor is a multiplication factor applied to background values in order to modify this threshold; the pixels below the threshold are displayed in blue (or black in the new user’s LUT) in the computed Fc and normalized FRET images.
34. The computed Fc and normalized FRET images are encoded on 32 bits but visualized on 256 color levels; the minimum value is displayed in black and the maximum value in white. Hence, an artificially amplified FRET signal may be observed in a cell where no FRET occurs (negative control). Users should therefore always:
 - Check the amplitude of the FRET signal by performing a profile plot through the cell of interest and compare this amplitude to that of a negative control (such as with cells expressing the donor-acceptor couple free EGFP and mCherry- α -tubulin for example) and to that of a positive control (such as with cells expressing the donor-acceptor couple EGFP- β -tubulin and mCherry- α -tubulin).
 - Stack the image of interest with an image of a negative and a positive control for FRET in order to visualize both images with the same LUT scale.
35. This procedure uses each cell or sub-region as its own internal standard; in addition, users can calculate %E in a neighboring cell that should be similar to the one of the internal standard.
36. The significance of %E is discussed in ref. [38].

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