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**TITLE:**

**Purification of Tubulin with Controlled Posttranslational Modifications and Isoforms from Limited Sources by Polymerization-Depolymerization Cycles**

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**KEYWORDS:**

Microtubules, in vitro reconstitution, tubulin, tubulin purification, polymerization, microtubule dynamics, tubulin code, tubulin isoforms, suspension culture, tubulin posttranslational modifications

**SUMMARY:**

This protocol describes tubulin purification from small/medium-scale sources such as cultured cells or single mouse brains, using polymerization and depolymerization cycles. The purified tubulin is enriched in specific isoforms or has specific posttranslational modifications and can be used in in vitro reconstitution assays to study microtubule dynamics and interactions.

**ABSTRACT:**

One important aspect of studies of the microtubule cytoskeleton is the investigation of microtubule behavior in in vitro reconstitution experiments. They allow the analysis of the intrinsic properties of microtubules, such as dynamics, and their interactions with microtubule-associated proteins (MAPs). The “tubulin code” is an emerging concept that points to different tubulin isoforms and various posttranslational modifications (PTMs) as regulators of microtubule properties and functions. To explore the molecular mechanisms of the tubulin code, it is crucial to perform in vitro reconstitution experiments using purified tubulin with specific isoforms and PTMs.

To date, this was technically challenging as brain tubulin, which is widely used in in vitro experiments, harbors many PTMs and has a defined isoform composition. Hence, we developed this protocol to purify tubulin from different sources and with different isoform compositions and controlled PTMs, using the classical approach of polymerization and depolymerization cycles. Compared to existing methods based on affinity purification, this

approach yields pure, polymerization-competent tubulin, as tubulin resistant to polymerization or depolymerization is discarded during the successive purification steps.

We describe the purification of tubulin from cell lines, grown either in suspension or as adherent cultures, and from single mouse brains. The method first describes the generation of cell mass in both suspension and adherent settings, the lysis step, followed by the successive stages of tubulin purification by polymerization-depolymerization cycles. Our method yields tubulin that can be used in experiments addressing the impact of the tubulin code on the intrinsic properties of microtubules and microtubule interactions with associated proteins.

## INTRODUCTION:

Microtubules play critical roles in many cellular processes. They give cells their shape, build meiotic and mitotic spindles for chromosome segregation, and serve as tracks for intracellular transport. To perform these diverse functions, microtubules organize themselves in different ways. One of the intriguing questions in the field is to understand the molecular mechanisms that allow the structurally and evolutionarily conserved microtubules to adapt to this plethora of organizations and functions. One potential mechanism is the diversification of microtubules, which is defined by the concept known as the 'tubulin code'<sup>1-3</sup>. The tubulin code includes two principal components: differential incorporation of  $\alpha$ - and  $\beta$ -tubulin gene products (tubulin isotypes) into the microtubules and tubulin posttranslational modifications (PTMs).

Since the 1970s, in vitro reconstitution experiments, combined with evolving light microscopy techniques, have paved the way for important discoveries about the properties of microtubules: dynamic instability<sup>4</sup> and treadmilling<sup>5</sup>, and their other mechanisms and functions<sup>6-15</sup>. Almost all the in vitro experiments performed so far have been based on tubulin purified from brain tissue using repeated cycles of polymerization and depolymerization<sup>16,17</sup>. Although purification from the brain tissue confers the advantage of obtaining high-quality tubulin in large quantities (usually gram amounts), one important drawback is the heterogeneity as tubulin purified from brain tissue is a mixture of different tubulin isotypes and is enriched with many tubulin PTMs. This heterogeneity makes it impossible to delineate the role of a particular tubulin PTM or isotype in the control of microtubule properties and functions. Thus, producing assembly-competent tubulin with controlled tubulin PTMs and homogenous isotype composition is essential to address the molecular mechanisms of the tubulin code.

Recently, an approach to purify tubulin by affinity chromatography using the microtubule-binding TOG (tumor-overexpressed gene) domain of yeast Stu2p has been developed<sup>18</sup>. In this method, tubulin in crude lysates of cells or tissue is passed through a column where it binds to the matrix-immobilized TOG domain, which allows the analysis of the whole tubulin pool of a given, even very small, sample. A long-awaited approach to purify recombinant tubulin has also been described in recent years. It is based on the baculovirus system, in which a bi-cistronic vector containing  $\alpha$ - and  $\beta$ -tubulin genes is expressed in insect cells<sup>19</sup>. However, this method is very cumbersome and time-consuming and is therefore mostly used for studying the impact of tubulin mutations<sup>20</sup> and tubulin isotypes<sup>21-23</sup> in vitro.

In the current protocol, we describe a method that uses the well-established and widely used polymerization-depolymerization approach as a blueprint to generate tubulin with different levels of modification either from cell lines or from mouse brain tissue<sup>24</sup>. In this procedure, tubulin is cycled between the soluble (tubulin dimer at 4 °C) and polymerized form (microtubule at 30 °C in the presence of guanosine 5'-triphosphate [GTP]). Each form is separated through successive steps of centrifugation: tubulin dimers will remain in the supernatant after a cold (4 °C) spin, whereas microtubules will be pelleted at 30 °C. Furthermore, one polymerization step is carried out at high piperazine-*N,N'*-bis(2-ethanesulfonic acid (PIPES) concentration, which allows the removal of microtubule-associated proteins from the microtubules and thus, from the finally purified tubulin. Tubulin purified from HeLa S3 cells grown as suspension or adherent cultures is virtually free of any tubulin PTM and has been used in recent in vitro reconstitution experiments<sup>25-28</sup>. We have further adapted the method to purify tubulin from single mouse brains, which can be used for a large number of mouse models with changes in tubulin isotypes and PTMs.

In the protocol, we first describe the generation of the source material (cell mass or brain tissue), its lysis (**Figure 1A**), followed by the successive steps of tubulin polymerization and depolymerization to purify the tubulin (**Figure 1B**). We further describe the process to assess the purity (**Figure 2A, B**) and quantity (**Figure 3A, B**) of the purified tubulin. The method can be adapted to produce tubulin enriched with a selected PTM by overexpressing a modifying enzyme in cells prior to tubulin purification (**Figure 4B**). Alternatively, tubulin-modifying enzymes can be added to tubulin during the purification process. Finally, we can purify tubulin lacking specific isotypes or PTMs from the brains of mice deficient in the corresponding tubulin-modifying enzymes (**Figure 4B**)<sup>29</sup>.

The method we describe here has two main advantages: (i) it allows the production of sufficiently large amounts of tubulin in a relatively short time, and (ii) it generates high-quality, pure tubulin, with either specific tubulin isotype composition or PTMs. In the associated video of this manuscript, we highlight some of the critical steps involved in this procedure.

## PROTOCOL:

Animal care and use for this study were performed in accordance with the recommendations of the European Community (2010/63/UE). Experimental procedures were specifically approved by the ethics committee of the Institut Curie CEEA-IC #118 (authorization no. 04395.03 given by National Authority) in compliance with the international guidelines.

### 1. Preparation of Reagents for Tubulin Purification

NOTE: All the buffers used for tubulin purification should contain potassium salts and NOT sodium salts<sup>30</sup>.

1.1. Prepare 1 L of complete medium: Dulbecco's modified Eagle medium (DMEM), with 10% fetal bovine serum (FBS, 100 mL), 200 mM L-glutamine (10 mL of 2 M stock), and 1x penicillin-streptomycin (10 mL of 100x stock). Store at 4 °C.

1.2. Prepare 10 M potassium hydroxide (KOH) by dissolving 140 g of KOH in water, adjust the final volume to 250 mL, and store at room temperature.

1.3. Prepare 0.5 M ethylenediamine tetraacetic acid (EDTA), pH 8, by dissolving 36.5 g of EDTA in water, adjust the pH to 8.0 using KOH (otherwise EDTA will not dissolve) and the final volume to 250 mL, filter-sterilize, and store at room temperature.

1.4. Prepare 5 mM phosphate-buffered saline (PBS)-EDTA by adding 5 mL of 0.5 M EDTA to 500 mL of PBS, filter-sterilize, and store at room temperature.

1.5. Prepare 0.5 M K-PIPES, pH 6.8, by dissolving 75.5 g of PIPES in water, adjust to pH 6.8 with KOH (otherwise PIPES will not dissolve) and the final volume to 500 mL, filter-sterilize, and store at 4 °C.

1.6. Prepare 1 M K-PIPES, pH 6.8, by dissolving 15.1 g of PIPES in water, adjust to pH 6.8 with KOH and the final volume to 50 mL, filter-sterilize, and store at 4 °C.

1.7. Prepare 0.5 M potassium-ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (K-EGTA, pH 7.7) by dissolving 47.5 g of EGTA in water, adjust to pH 7.7 with KOH and the final volume to 250 mL, filter-sterilize, and store at room temperature.

1.8. Prepare BRB80 (80 mM K-PIPES, pH 6.8; 1 mM K-EGTA; 1 mM magnesium chloride [MgCl<sub>2</sub>]) solution by mixing 3.2 mL of 0.5 M PIPES, 40  $\mu$ L of 0.5 M K-EGTA, and 20  $\mu$ L of 1 M MgCl<sub>2</sub> and adjust the final volume to 20 mL. Store at 4 °C.

1.9. Prepare 0.1 M phenylmethanesulfonyl fluoride (PMSF) by dissolving 435 mg of PMSF in isopropanol to obtain a final volume of 25 mL and store at -20 °C.

1.10. Prepare protease inhibitors mix (200x) by dissolving 10 mg of aprotinin, 10 mg of leupeptin, and 10 mg of 4-(2-aminoethyl)-benzenesulfonyl fluoride in water to obtain a final volume of 2.5 mL, make aliquots of 100  $\mu$ L, and store at -20 °C.

1.11. Prepare 10% Triton X-100 by mixing 5 mL of Triton X-100 in 45 mL of water, filter-sterilize, and store at room temperature.

1.12. Prepare Lysis buffer (BRB80 supplemented with 1 mM 2-mercaptoethanol, 1 mM PMSF, 1x protease inhibitors mix and, optionally for HEK-293 cells, 0.2% Triton X-100) on the day of tubulin purification by mixing 20 mL of BRB80 with 1.5  $\mu$ L of 2-mercaptoethanol, 200  $\mu$ L of 0.1 M PMSF, 100  $\mu$ L of the protease inhibitors mix and, optionally for HEK-293 cells, 400  $\mu$ L of 10% Triton X-100.

NOTE: 2-mercaptoethanol is toxic and should be used in the fume-hood.

1.13. Prepare 0.2 M GTP by dissolving 1 g of GTP in 9.5 mL of water, adjust the pH to 7.5 using KOH, make aliquots of 20  $\mu$ L, and store at -20 °C. Avoid repeated freeze-thaw cycles.

1.14. Prepare 1 M tris(hydroxymethyl) aminomethane-hydrochloride (Tris-HCl) by dissolving 60.56 g of Tris in water, adjust to pH 6.8 with HCl, complete to a final volume of 500 mL, filter-sterilize, and store at room temperature.

1.15. Prepare 5x Laemmli sample buffer (450 mM dithiothreitol (DTT); 10% sodium dodecylsulfate (SDS); 400  $\mu$ M Tris-HCl, pH 6.8; 50% glycerol; ~0.006% bromophenol blue) by adding 4 g of SDS to 16 mL of preheated 1 M Tris-HCl, pH 6.8, and mix the solution gently. Add 2.6 g of DTT and 20 mL of 100% glycerol to the mix and stir until the solution becomes homogenous. Add the desired amount of bromophenol blue (2.5 mg) to reach the required color intensity. Make 5 mL aliquots and store at -20 °C. Prepare the 2x working solution of Laemmli sample buffer by diluting the 5x stock in distilled water.

## **2. Amplification and harvesting sources of tubulin**

NOTE: In this protocol, three sources of tubulin were used: (i) cells (HeLa S3 and HEK-293) grown as suspension cultures; (ii) cells grown as adherent cultures (HEK-293, HeLa, and U-2 OS); and (iii) mouse brain tissue. This protocol considers the day of tubulin purification as 'day 0' and accordingly, other steps have been described relative to day 0.

### **2.1. Amplification of Cells**

#### **2.1.1. Cells Grown as Suspension Cultures**

NOTE: To successfully purify tubulin from suspension cultures, use at least 2 L of suspension culture.

2.1.1.1. For 2 L of suspension culture, revive and grow the preferred cell type to obtain  $6 \times 10^7$  cells 10 days before the day of preparation. On day -10, plate cells on six 15 cm diameter dishes at  $10^7$  cells per plate.

2.1.1.2. On day -8, preheat the required amount of complete medium to 37 °C. Add 1 L of pre-heated medium to each spinner bottle under sterile conditions. Place the spinners on a stirrer table set at 20–25 rpm inside the cell culture incubator, slightly open the lateral spinner caps to allow the medium to equilibrate to the incubator's atmosphere.

NOTE: To avoid any contamination, thoroughly clean the outer surface of the media and spinner bottles using 70% ethanol.

2.1.1.3. On day -7, trypsinize and collect the cells grown to 80–90% confluence (approximately  $1.8 \times 10^8$  cells). Collect cells from 3 dishes at a time, spin down ( $200 \times g$ , 5 min, room temperature), and re-suspend all cells in 10 mL of DMEM.

NOTE: Thorough dissociation of the cells at this point is very important to avoid the formation of larger aggregates in the spinner bottles, which affects cell survival and results in low tubulin yield.

2.1.1.4. Add 5 mL of the cell suspension to each spinner bottle containing 1 L of DMEM, return the spinners to the stirrer table in the cell culture incubator, and allow cells to grow for one week.

## 2.1.2. Cells Grown as Adherent Cultures

NOTE: To successfully purify tubulin from adherent cells, use a minimum of 10 dishes of 80–90% confluence.

2.1.2.1. Revive and amplify the desired cell type to obtain  $1 \times 10^8$  cells three days before the day of the tubulin preparation.

2.1.2.2. On day -3, plate these cells on ten 15 cm dishes at  $1 \times 10^7$  cells per dish and allow them to grow 80–90% confluence.

2.1.2.3. On day -1, if required, transfect cells with a plasmid to express a tubulin-modifying enzyme or a particular tubulin isotype.

## 2.2. Harvesting the Cells/Brain Tissue

### 2.2.1. Cells Grown as Suspension Cultures

2.2.1.1. Transfer the cell suspension from spinners into 1 L centrifuge bottles (**Table of Materials**) and pellet cells at  $250 \times g$ , 15 min, room temperature. For immediately starting another culture of HeLa S3 cells in the spinner bottles, leave 100 mL of cell suspension in the spinners, and add 1 L of complete, pre-heated DMEM to the spinner bottle.

NOTE: Carefully check for bacterial contamination before proceeding for tubulin purification.

2.2.1.2. Resuspend pelleted cells from each centrifuge bottle in 10 mL of ice-cold PBS, and transfer all the cells into 50 mL screw-cap tubes. During re-suspension, keep the cells on ice. Pellet the cells at  $250 \times g$ , 15 min, 4 °C.

NOTE: Follow recommendations for spinner bottle cleaning and storage (see **Table of materials**).

2.2.1.3. Discard the supernatant and determine the volume of the cell pellet. From 2 L of suspension culture (two spinner bottles), expect a cell pellet of 5–6 mL.

NOTE: In the protocol described below, the cell pellet volume is assumed to be 10 mL. Adjust the experiment according to the pellet volumes.

2.2.1.4. Add 1 volume (10 mL) of lysis buffer and re-suspend the cell pellet.

NOTE: The ratio of cell pellet volume to lysis buffer volume is very important for successful tubulin purification. Adding more lysis buffer decreases tubulin concentration, which then fails

to reach the critical concentration needed for polymerization, thus greatly reducing the tubulin yield.

NOTE: Cells resuspended in lysis buffer can be snap-frozen in liquid nitrogen and stored at -80 °C for two months.

#### 2.2.2. Cells Grown as Adherent Cultures

NOTE: Cells from adherent cultures must be harvested very quickly for successful tubulin purification (approximately 15 mins for harvesting ten 15 cm dishes). Three people participated in this step of the protocol.

2.2.2.1. Remove the medium from 15 cm dishes by inclining the dishes, and then gently wash the cells with 7 mL of PBS-EDTA at room temperature (person 1). Work only with three 15 cm dishes at a time to avoid leaving the cells without medium or buffer.

2.2.2.2. Add 5 mL of PBS-EDTA to the cells and incubate them for 5 min at room temperature.

2.2.2.3. Use a cell lifter to gently detach the cells by shoveling them to one edge of the dish (person 2), and collect all the cells in a 50 mL screw-cap tube (person 3). Rinse each plate with an additional 2 mL of PBS-EDTA to collect any remaining cells from the dishes. During this step, keep the 50 mL screw-cap tube containing the cell suspension on ice.

2.2.2.4. Pellet the cells at  $250 \times g$ , 10 min, 4 °C. Discard the supernatant and determine the volume of the cell pellet. Expect a volume of ~1 mL from ten 15 cm dishes.

NOTE: In the protocol described below, the cell pellet volume is assumed to be 10 mL. Adjust the experiments according to the pellet volumes.

2.2.2.5. Resuspend the cells in 1 volume (10 mL) of lysis buffer.

NOTE: Cells resuspended in lysis buffer can be stored at -80 °C for up to two months.

#### 2.2.3. Brain Tissue

NOTE: Mice of any age, sex, or genetic background can be used. The choice of the transgenic mouse strain will depend on the scientific question to be addressed. In this manuscript, we show the example of tubulin purified from the *ttl1*<sup>-/-</sup> mouse, lacking a major brain glutamylating enzyme, the tubulin tyrosine ligase-like 1 (TTL1) protein<sup>31</sup>.

2.2.3.1. Sacrifice the mouse by cervical dislocation, quickly decapitate, and collect the brain into a round-bottom tube. If there is excess blood on the brain, quickly wash with lysis buffer. Collect the brain as soon as the mouse is sacrificed as a post-mortem delay can affect the success of tubulin purification. Use round-bottom tubes to accommodate the width of the probe used for homogenization.



NOTE: Collected mouse brains can be snap-frozen in liquid nitrogen and stored at -80 °C for up to 3 years.

2.2.3.2. Add 500 µL of lysis buffer to a single brain extracted from an adult mouse. For the rest of the protocol, the volume of lysis buffer added is assumed to be 10 mL. Adjust for your experiment according to the number of brains to be used.

### 3. Lysis of Cells or Brain Tissue

#### 3.1. Cells Grown as Suspension Cultures

3.1.1. For HEK-293, lyse the cells on ice by repetitively pipetting up and down using pipette tips of different widths. First, attach a p1000 tip to a 10 mL pipette, and pipette the cell suspension up and down every 5 min, for 10 min (three cycles of pipetting). Second, attach a p200 tip to a p1000 tip and further pipette every 5 min, for 20 min (five cycles of pipetting).

3.1.2. For HeLa S3, lyse the cells using a French press (see **Table of Materials** for settings).

3.1.3. Take 1/100<sup>th</sup> volume of the lysis mix (L) (200 µL for 20 mL of L), and add the same volume of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analysis.

#### 3.2. Cells Grown as Adherent Cultures

3.2.1. Transfer the cells into a 14 mL round-bottom tube whose height has been reduced to accommodate the sonicator probe (see **Table of Materials** for settings). Sonicate the cells for ~45 pulses, and confirm cell lysis by sampling a drop of the lysis mix under a microscope.

NOTE: The number of pulses could vary according to the cell type used for tubulin purification. Sonicating cells too much could cause tubulin to precipitate and will negatively affect the purification yield.

3.2.2. Pipette the cells up and down on ice every 5 min for 20 min (five cycles of pipetting), using a p200 tip.

3.2.3. Take 1/100<sup>th</sup> volume of lysis mix (L) (200 µL for 20 mL of L) and add the same volume of 2x Laemmli buffer, boil for 5 min and store at -20 °C for further analysis.

#### 3.3. Brain Tissue

3.3.1. Lyse the brain tissue using a tissue blender (see **Table of Materials** for settings). Alternatively, lyse the tissue using a microtube pestle or an equivalent equipment and pipette up and down on ice with a 1 mL syringe with an 18 G needle.

3.3.2. Take 1/100<sup>th</sup> volume of lysis mix (L) (200 µL for 20 mL of L), and add the same volume of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analysis.

### 4. Purification of Tubulin

#### 4.1. Lysate Clarification

4.1.1. Clear the lysate (separating pellet and soluble fraction of the lysis mix) by centrifugation at  $150,000 \times g$ , 4 °C, 30 min. See **Table of Materials** for details about ultracentrifuge rotors and tubes. For cell extracts, a white floating layer is often formed after centrifugation. Do not transfer this floating layer along with the supernatant, as it interferes with tubulin polymerization. Use a syringe of appropriate volume attached to a long 20 G or 21 G needle to gently remove the supernatant without disturbing the floating layer. If the supernatant is still cloudy, centrifuge at  $5,000 \times g$ , 4 °C for 10 min.

4.1.2. Transfer the supernatant (SN1) to an ultracentrifuge tube and note its volume. For a 10 mL cell pellet, expect a volume of ~12 mL for SN1.

4.1.3. Take 1/100<sup>th</sup> volume of SN1 (120 µL for 12 mL of SN1), and add the same volume of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analysis.

4.1.4. Resuspend the pellet (P1) in BRB80 (**Table of Materials**) using the same volume as SN1. Take 1/100<sup>th</sup> volume of P1 (200 µL for 20 mL of P1), and add the same volume of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analysis.

#### 4.2. First Polymerization in Low-Molarity Buffer

4.2.1. Prepare the polymerization mix by combining 1 volume of SN1 (12 mL), 1/200<sup>th</sup> volume of 0.2 M GTP (60 µL; final concentration 1 mM), and 0.5 volume of pre-heated glycerol (6 mL) in a screw-cap tube of the appropriate volume.

NOTE: Glycerol is used as a crowding agent in the polymerization steps throughout the protocol and thus is not considered in the calculations of other components' concentrations.

4.2.2. Pipette the mix up and down, gently avoiding the formation of air bubbles and transfer it to the appropriate ultracentrifuge tubes.

NOTE: While transferring the mix to the tubes, adjust the weight of the tubes (in pairs). This allows the experimenter to directly proceed to the sedimentation of microtubules after the polymerization step. Do this for all polymerization steps throughout the protocol.

4.2.3. Cover the tubes with parafilm, transfer to a water bath set at 30 °C, and incubate for 20 min.

4.2.4. Centrifuge the tubes at  $150,000 \times g$ , 30 °C for 30 min. Remove the supernatant (SN2), and keep the pellet of polymerized microtubules (P2).

NOTE: Microtubule pellet can be snap-frozen and stored at -80 °C for up to 1 year.

4.2.5. Take 1/200<sup>th</sup> volume of SN2 (90 µL for 18 mL SN2) and add the same volume of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analysis.

### 4.3. First Depolymerization

4.3.1. Depolymerize microtubules by adding ice-cold BRB80 to the pellet P2, and leave on ice for 5 min: for tubulin from cells, add 1/60<sup>th</sup> (200  $\mu$ L), and for tubulin from brains, add 1/20<sup>th</sup> (600  $\mu$ L) of the volume of the SN1.

NOTE: The volume of ice-cold BRB80 added to the pellet during depolymerization steps is always relative to the volume of SN1.

4.3.2. Resuspend the microtubule pellet gently, avoiding air bubbles, until the solution is completely homogeneous. Use a p1000 tip for a couple of cycles of pipetting followed by a p200 tip every 5 min, for 20 min (five cycles of pipetting). This is a crucial step for the success of the tubulin purification.

4.3.3. Transfer the solution to appropriate ultracentrifuge tubes, and spin down at 150,000  $\times g$ , 4  $^{\circ}$ C for 20 min. Transfer the SN3 to a new 1.5 mL ultracentrifuge tube. The pellet formed after this centrifugation step (P3) contains precipitated proteins (microtubule-associated proteins or MAPs) and non-depolymerized microtubules. The supernatant (SN3) contains soluble components: depolymerized tubulin dimers and MAPs, which have detached from the depolymerized microtubules.

4.3.4. Take 1–4  $\mu$ L of SN3, and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20  $^{\circ}$ C for further analyses.

4.3.5. Resuspend the pellet P3 in BRB80 (in the same volume of SN3), take 1–4  $\mu$ L, and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20  $^{\circ}$ C.

### 4.4. Second Polymerization (in High-Molarity Buffer)

4.4.1. Prepare the polymerization mix by combining 1 volume of SN3 (200  $\mu$ L), 1 volume of pre-heated 1 M PIPES (200  $\mu$ L, final concentration 0.5 M), 1/100<sup>th</sup> volume of 0.2-M GTP (2  $\mu$ L, final concentration 1 mM), and 1 volume of pre-heated glycerol (200  $\mu$ L) in a tube of the appropriate volume.

4.4.2. Pipette the mix up and down, avoiding the formation of air bubbles, and transfer it to ultracentrifuge tubes.

4.4.3. Cover the tubes with parafilm, transfer them to a water bath set at 30  $^{\circ}$ C, and incubate for 20 min.

4.4.4. Centrifuge the tubes at 150,000  $\times g$ , 30  $^{\circ}$ C for 30 min. Remove the supernatant (SN4), and keep the pellet of polymerized microtubules (P4). The pellet P4 contains the polymerized microtubules, and the supernatant SN4 contains unpolymerized tubulin, MAPs, and other soluble proteins.

NOTE: The microtubule pellet after the second polymerization step can be snap-frozen and stored at -80 °C for up to 1 year.

4.4.5. Take 1–4 µL and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analyses.

#### 4.5. Second Depolymerization

4.5.1. Depolymerize microtubules by adding ice-cold BRB80 to the pellet P4, and leave on ice for 5 min: for tubulin from cells, add 1/100<sup>th</sup> (120 µL), and for tubulin from brains, add 1/40<sup>th</sup> (300 µL) of the volume of the SN1.

4.5.2. Pipette up and down with a p200 tip every 5 min, for 20 min (five cycles of pipetting).

4.5.3. Transfer the solution to a 1.5 mL ultracentrifuge tube, and spin down at 150,000 × *g*, 4 °C for 20 min. Transfer the SN5 to a new 1.5 mL ultracentrifuge tube. The pellet formed after this centrifugation step (P5) contains non-depolymerized microtubules. The supernatant (SN5) contains the soluble tubulin.

4.5.4. Take 1–4 µL and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analyses.

4.5.5. Resuspend the pellet P5 in BRB80 (same volume of SN5), take 1–4 µL, and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analyses.

#### 4.6. Third Polymerization (in Low-Molarity Buffer)

4.6.1. Prepare the polymerization mix: 1 volume of SN5 (120 µL), 1/200<sup>th</sup> volume of 0.2-M GTP (0.6 µL, final concentration is 1 mM), and 0.5 volume of pre-heated glycerol (60 µL) in a tube of the appropriate volume.

4.6.2. Pipette the mix up and down, gently avoiding formation of air bubbles, and transfer it to the appropriate ultracentrifuge tubes.

4.6.3. Cover the tubes with parafilm, transfer them to a water bath set at 30 °C, and incubate for 20 min.

4.6.4. Centrifuge the tubes at 150,000 × *g*, 30 °C for 30 min. The pellet (P6) contains polymerized microtubules and the supernatant SN6 contains small amounts of non-polymerized tubulin.

NOTE: Microtubule pellets can be snap-frozen and stored at -80 °C for up to 1 year.

4.6.5. Take 1–4 µL and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analyses.

#### 4.7. Third Depolymerization

4.7.1. Depolymerize microtubules by adding ice-cold BRB80 to the pellet P6, and leave on ice for 5 min: for tubulin from cells, add 1/100<sup>th</sup> (120  $\mu$ L), and for tubulin from brains, add 1/40<sup>th</sup> (300  $\mu$ L) of the volume of the SN1.

4.7.2. Pipette up and down with a p200 tip every 5 min, for 20 min (five cycles of pipetting).

4.7.3. Transfer the solution to the appropriate ultracentrifuge tubes, and spin down at 150,000  $\times g$ , 4  $^{\circ}$ C for 20 min. Transfer SN7 to a new 1.5 mL ultracentrifuge tube. The pellet (P7) contains small amounts of non-depolymerized microtubules. The supernatant (SN7) contains exclusively depolymerized microtubules (soluble tubulin).

4.7.4. Take 1–4  $\mu$ L and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20  $^{\circ}$ C for further analyses.

4.7.5. Resuspend the pellet P7 in BRB80 (same volume of SN7), take 1–4  $\mu$ L, and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20  $^{\circ}$ C for further analyses.

4.7.6. Quantify the amount of tubulin (see **Representative Results**) and aliquot SN7 into small volumes, snap-freeze, and store at -80  $^{\circ}$ C.

#### **REPRESENTATIVE RESULTS:**

The main goal of this method is to produce high-quality, assembly-competent tubulin in quantities sufficient to perform repeated in vitro experiments with the purified components. Microtubules assembled from this tubulin can be used in reconstitution assays based on the total internal reflection fluorescence (TIRF) microscopy technique with either dynamic or stable microtubules, in experiments testing microtubule dynamics, interactions with MAPs or molecular motors, and force generation by the motors<sup>25</sup>. They can also be used in microtubule-MAP co-pelleting assays and solid-state NMR spectroscopy<sup>28</sup>.

The enrichment and purity of tubulin throughout the purification process can be monitored by using a Coomassie-stained SDS-polyacrylamide gel electrophoresis (PAGE) gel, preferably the 'TUB' SDS-PAGE gels, that allow for the separation of  $\alpha$ - and  $\beta$ -tubulins, which co-migrate as a single band in classical gels<sup>32</sup>. Lysates collected at different steps (except for the very last depolymerization, see protocol) are loaded onto the gel in comparable amounts for assessing the success of tubulin purification (**Figure 2A**)<sup>24</sup>. The final tubulin sample, which is very precious, is only loaded on the gel run for the determination of tubulin concentration. It is normal to lose some tubulin in the process of repeated cycles of polymerization and depolymerization. A lower-than-expected yield of the final purified tubulin can be due to either (i) incomplete depolymerization of microtubules, visualized by the presence of an important amount of tubulin in fractions P3, P5, and P7, or (ii) an inefficient tubulin polymerization into microtubules, in which case a lower amount of tubulin is present in fractions P2, P4, and P6 and higher in fractions SN2, SN4, and SN6 (**Figure 2B**). If the tubulin is lost during polymerization steps (lower amounts of P2 and P4) (i) ensure sufficient tubulin concentration during polymerization (ii) use a fresh aliquot of GTP, and/or (iii) reconfirm the temperature of the polymerization reaction. If the tubulin is lost during depolymerization

steps (lower amounts of SN3 and SN5), increase the time as well as pipetting of the mix on ice.

For the quantification of purified tubulin, run the samples along with the known quantities of bovine serum albumin (BSA, 0.5  $\mu$ g – 1  $\mu$ g – 2  $\mu$ g – 4  $\mu$ g) (**Figure 3A**) on SDS-PAGE. Gels are stained with Coomassie brilliant blue, scanned, and the intensities of BSA and tubulin bands are measured by quantitative densitometry (**Figure 3B**) as described at [https://openwetware.org/wiki/Protein\\_Quantification\\_Using\\_ImageJ](https://openwetware.org/wiki/Protein_Quantification_Using_ImageJ). Please note that the same analysis can be done in Fiji, an upgraded version of ImageJ<sup>33</sup>. Values from the BSA bands were used to determine the linear regression equation, which was used to calculate the amount of protein in the tubulin bands. Only tubulin band intensities within the range of the BSA curve are used to determine tubulin concentration. Based on the calculated tubulin concentration, aliquots of desired volumes of tubulin are prepared, snap-frozen in liquid nitrogen, and stored at -80 °C. We usually obtain about ~2 mg of tubulin from four spinner bottles of HeLa S3 suspension cultures (~15 g of cells), ~250  $\mu$ g of tubulin from ten 15-cm diameter dishes (~1.2 g of cells), and ~1 mg of tubulin from 1 g of mouse brain tissue.

To confirm the enrichment of a particular tubulin isotype or modification, ~0.1  $\mu$ g of the purified tubulin can be immunoblotted using respective antibodies<sup>34,35</sup>. The control tubulin will vary depending on the tubulin of interest. For tubulin modified in vitro with a modifying enzyme, use non-treated tubulin as control. For tubulin modified in cellulo by the overexpression of a modifying enzyme, use tubulin purified from cells that do not express the enzyme as control (**Figure 4A**). Control tubulin for tubulin purified from knockout-mouse brains will be tubulin from wild type mice (**Figure 4B**). In all immunoblot analyses, an equal load of tubulin is verified by using a PTM-independent anti- $\alpha$ -tubulin antibody (12G10).

#### FIGURE LEGENDS:

**Figure 1: Tubulin purification from different sources using polymerization-depolymerization cycles. (A)** Different sources of tubulin are lysed using specific strategies. HeLa S3 cells cultured in suspension are lysed using a French press; HEK-293 cells are lysed by repetitive pipetting. Adherent cells were lysed using short pulses of sonication and mouse brain tissue using a tissue homogenizer. **(B)** Schematic representation of the successive steps of the tubulin purification protocol using cycles of cold-depolymerization and warm-polymerization. After lysis and lysate clarification, microtubules are polymerized and pelleted. Microtubules are then depolymerized and subsequently allowed to polymerize in a high-molarity buffer, preventing microtubule-associated protein (MAP) co-sedimentation with the microtubules. MAP-free microtubules are then depolymerized and can be further subjected to a third cycle of polymerization-depolymerization to remove trace amounts of the high-molarity buffer.

**Figure 2: Evaluating the success of the tubulin purification.** Samples collected at different steps of the tubulin purification protocol were run on a 'TUB' sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (see protocol for details) and stained with Coomassie brilliant blue. **(A)** In a successful tubulin purification,  $\alpha$ - and  $\beta$ -tubulins are progressively enriched throughout the process. After the second polymerization, the microtubule pellet (P4) is virtually free of contamination from other proteins or microtubule-associated proteins (MAPs). Note that it is normal to lose some tubulin during the procedure. **(B)** In an unsuccessful tubulin purification, the final tubulin yield is low, and tubulin remains

either in the pellet after depolymerization or in the supernatant after polymerization (red boxes). In the example shown here, tubulin did not polymerize efficiently in both polymerization steps.

**Figure 3: Quantification of the purified tubulin using Coomassie-stained sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and densitometry. (A)** Coomassie-stained SDS-PAGE gel with known quantities of bovine serum albumin (BSA; 0.5, 1, 2 and 4  $\mu$ g, gray gradient line) and different volumes (0.5 and 1  $\mu$ L, light and dark colors, respectively) of purified tubulin. In the example shown, tyrosinated tubulin (HeLa S3 tubulin, light and dark orange) and detyrosinated tubulin (HeLa S3 tubulin treated with carboxypeptidase A, light and dark blue) were loaded on the gel. **(B)** BSA bands from (A) were quantified using ImageJ (in arbitrary units, AU) and plotted against the amount of protein loaded (gray to black points). Those points were used to calculate the linear regression line (the gray gradient line) and equation, which were used to calculate the amounts of protein in the tubulin samples (light and dark orange and blue points) loaded on the gel. This facilitated the calculation of the concentration of the tubulin samples. Note that the points that lie beyond the BSA standard curve should not be used to determine concentration (dark orange and blue points).

**Figure 4: Immunoblot analysis of purified tubulin with different PTMs. (A)** Tubulins purified from HEK-293 cells: wild type, or cells overexpressing TTLL5 or TTLL7 were analyzed for the specific enrichment of polyglutamylation using the GT335 antibody. While TTLL5 overexpression increases polyglutamylation on  $\alpha$ - and  $\beta$ -tubulin, TTLL7 overexpression specifically enriches  $\beta$ -tubulin glutamylation. **(B)** Tubulin purified from brain tissues of wild type and *ttll1*<sup>-/-</sup> mice were analyzed for patterns of glutamylation. Note the strong reduction of polyglutamylation of tubulin from *ttll1*<sup>-/-</sup> mice, which lack the major brain glutamylase TTLL1<sup>36</sup>. 'TUB' gels were used to separate  $\alpha$ - and  $\beta$ -tubulin. An equal amount of tubulin load was confirmed by 12G10, an anti- $\alpha$ -tubulin antibody.

## DISCUSSION:

The method described here provides a platform to rapidly generate high-quality, assembly-competent tubulin in medium-large quantities from cell lines and single mouse brains. It is based on the gold-standard protocol of tubulin purification from bovine brains used in the field for many years<sup>16,17</sup>. One particular advantage of the approach is the use of suspension cultures of HeLa S3 cells, which, once established, yields large amounts of cells while requiring little hands-on time. This makes the protocol relatively easy to perform in any cell biology lab, whereas other tubulin purification methods<sup>18,19,32,37</sup> require specific equipment and expertise and are thus mostly used by laboratories with a strong background in protein purification. When producing smaller quantities of tubulin from adherent cell lines, a variety of cell lines can be used. We have successfully purified tubulin from HeLa, U-2 OS, and HEK-293 cells. If a larger-scale purification is needed, harvested cells or brains can be snap-frozen in lysis buffer and stored at -80 °C, and multiple cell pellets or brains can be pooled together to purify larger amounts of tubulin.

Tubulin purified from cell lines is virtually free of tubulin PTMs. This Tyr-tubulin can readily be converted to detyrosinated (deTyr-) tubulin in a single straightforward step<sup>25</sup>. To produce tubulin with other PTMs, specific tubulin-modifying enzymes can be overexpressed in cells

prior to tubulin purification. Furthermore, using cell lines of human origin as the source of material helps avoid potential cross-species issues when studying interactions between microtubules and human MAPs. Further, tubulin from untransformed (such as HEK293) or transformed (such as HeLa) cells can provide information about the effects of microtubule-directed drugs (e.g., taxanes) on normal- vs. tumor-cell microtubules.

Finally, our protocol facilitates the purification of tubulin from single mouse brains. As an increasing number of mouse models of tubulin mutations and modifications are being generated, this protocol allows direct analysis of the properties and interactions of microtubules with altered tubulin isotype composition<sup>38-40</sup> or tubulin PTMs<sup>31,41</sup>.

The approach is based on cycles of polymerization and depolymerization. Thus, specific tubulin isotypes or tubulin with particular PTMs that affect the assembly and disassembly properties of microtubules could result in a disproportionate loss or reduction of such tubulin forms during the purification process. Nevertheless, we have shown that major tubulin PTMs, such as acetylation, detyrosination, glutamylation, and glycylation, are retained on the microtubules throughout the tubulin purification process<sup>24</sup>. However, it should be noted that for quantitative analyses of the tubulin composition in cells or tissues, the TOG-column-based tubulin purification approach is more appropriate as it would allow an unbiased, polymerization-independent tubulin purification<sup>18</sup>. Despite its limitation, our protocol offers a great advantage in generating large amounts of high-quality tubulin that can be used in meticulous in vitro reconstitution experiments. In particular, it facilitates the use of PTM-rich brain tubulin in routine experiments.

#### **DISCLOSURES:**

The authors have nothing to disclose.

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## REFERENCES:

- 1 Verhey, K. J., Gaertig, J. The tubulin code. *Cell Cycle*. **6** (17), 2152-2160 (2007).
- 2 Janke, C. The tubulin code: Molecular components, readout mechanisms, and functions. *Journal of Cell Biology*. **206** (4), 461-472 (2014).
- 3 Janke, C., Magiera, M. M. The tubulin code and its role in controlling microtubule properties and functions. *Nature Reviews: Molecular Cell Biology*. **21** (6), 307-326 (2020).
- 4 Mitchison, T., Kirschner, M. Dynamic instability of microtubule growth. *Nature*. **312** (5991), 237-242 (1984).
- 5 Margolis, R. L., Wilson, L. Opposite end assembly and disassembly of microtubules at steady state in vitro. *Cell*. **13** (1), 1-8 (1978).
- 6 Borisy, G. G., Olmsted, J. B. Nucleated assembly of microtubules in porcine brain extracts. *Science*. **177** (55), 1196-1197 (1972).
- 7 Kirschner, M. W., Williams, R. C. The mechanism of microtubule assembly in vitro. *Journal of Supramolecular Structure*. **2** (2-4), 412-428 (1974).
- 8 Baas, P. W., Lin, S. Hooks and comets: The story of microtubule polarity orientation in the neuron. *Developmental Neurobiology*. **71** (6), 403-418 (2011).
- 9 Stepanova, T. et al. Visualization of microtubule growth in cultured neurons via the use of EB3-GFP (end-binding protein 3-green fluorescent protein). *Journal of Neuroscience*. **23** (7), 2655-2664 (2003).
- 10 Nedelec, F. J., Surrey, T., Maggs, A. C., Leibler, S. Self-organization of microtubules and motors. *Nature*. **389** (6648), 305-308 (1997).
- 11 Bieling, P., Telley, I. A., Surrey, T. A minimal midzone protein module controls formation and length of antiparallel microtubule overlaps. *Cell*. **142** (3), 420-432 (2010).
- 12 Roostalu, J. et al. Directional switching of the kinesin Cin8 through motor coupling. *Science*. **332** (6025), 94-99 (2011).
- 13 Schaedel, L. et al. Microtubules self-repair in response to mechanical stress. *Nature Materials*. **14** (11), 1156-1163 (2015).
- 14 Hendricks, A. G., Goldman, Y. E., Holzbaur, E. L. F. Reconstituting the motility of isolated intracellular cargoes. *Methods in Enzymology*. **540** 249-262 (2014).
- 15 Dogterom, M., Surrey, T. Microtubule organization in vitro. *Current Opinion in Cell Biology*. **25** (1), 23-29 (2013).
- 16 Vallee, R. B. Reversible assembly purification of microtubules without assembly-promoting agents and further purification of tubulin, microtubule-associated proteins, and MAP fragments. *Methods in Enzymology*. **134** 89-104 (1986).
- 17 Castoldi, M., Popov, A. V. Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. *Protein Expression and Purification*. **32** (1), 83-88 (2003).
- 18 Widlund, P. O. et al. One-step purification of assembly-competent tubulin from diverse eukaryotic sources. *Molecular Biology of the Cell*. **23** (22), 4393-4401 (2012).
- 19 Minoura, I. et al. Overexpression, purification, and functional analysis of recombinant human tubulin dimer. *FEBS Letters*. **587** (21), 3450-3455 (2013).
- 20 Uchimura, S. et al. A flipped ion pair at the dynein-microtubule interface is critical for dynein motility and ATPase activation. *Journal of Cell Biology*. **208** (2), 211-222 (2015).
- 21 Pamula, M. C., Ti, S.-C., Kapoor, T. M. The structured core of human beta tubulin confers isotype-specific polymerization properties. *Journal of Cell Biology*. **213** (4), 425-433 (2016).

744 22 Vemu, A. et al. Structure and dynamics of single-isoform recombinant neuronal Human  
745 tubulin. *Journal of Biological Chemistry*. **291** (25), 12907-12915 (2016).

746 23 Ti, S.-C., Alushin, G. M., Kapoor, T. M. Human beta-tubulin isotypes can regulate  
747 microtubule protofilament number and stability. *Developmental Cell*. **47** (2), 175-190 e175  
748 (2018).

749 24 Souphron, J. et al. Purification of tubulin with controlled post-translational  
750 modifications by polymerization–depolymerization cycles. *Nature Protocols*. **14** 1634–1660  
751 (2019).

752 25 Barisic, M. et al. Microtubule detyrosination guides chromosomes during mitosis.  
753 *Science*. **348** (6236), 799-803 (2015).

754 26 Nirschl, J. J., Magiera, M. M., Lazarus, J. E., Janke, C., Holzbaur, E. L. F. alpha-Tubulin  
755 tyrosination and CLIP-170 phosphorylation regulate the initiation of dynein-driven transport  
756 in neurons. *Cell Reports*. **14** (11), 2637-2652 (2016).

757 27 Guedes-Dias, P. et al. Kinesin-3 responds to local microtubule dynamics to target  
758 synaptic cargo delivery to the presynapse. *Current Biology*. **29** (2), 268-282 e268 (2019).

759 28 Luo, Y. et al. Direct observation of dynamic protein interactions involving human  
760 microtubules using solid-state NMR spectroscopy. *Nature Communications*. **11** (1), 18 (2020).

761 29 Even, A. et al. ATAT1-enriched vesicles promote microtubule acetylation via axonal  
762 transport. *Science Advances*. **5** (12), eaax2705 (2019).

763 30 Wolff, J., Sackett, D. L., Knipling, L. Cation selective promotion of tubulin  
764 polymerization by alkali metal chlorides. *Protein Science* **5** (10), 2020-2028 (1996).

765 31 Magiera, M. M. et al. Excessive tubulin polyglutamylation causes neurodegeneration  
766 and perturbs neuronal transport. *EMBO Journal*. **37** (23), e100440 (2018).

767 32 Lacroix, B., Janke, C. Generation of differentially polyglutamylated microtubules.  
768 *Methods in Molecular Biology*. **777** 57-69 (2011).

769 33 Schneider, C. A., Rasband, W. S., Eliceiri, K. W. NIH Image to ImageJ: 25 years of image  
770 analysis. *Nature Methods*. **9** (7), 671-675 (2012).

771 34 Magiera, M. M., Janke, C. in *Methods in Cell Biology* Vol. 115 *Microtubules*, in vitro eds  
772 John J. Correia & Leslie Wilson) 247-267 (Academic Press, 2013).

773 35 Hausrat, T. J., Radwitz, J., Lombino, F. L., Breiden, P., Kneussel, M. Alpha- and beta-  
774 tubulin isotypes are differentially expressed during brain development. *Developmental*  
775 *Neurobiology*. 10.1002/dneu.22745 (2020).

776 36 Janke, C. et al. Tubulin polyglutamylase enzymes are members of the TTL domain  
777 protein family. *Science*. **308** (5729), 1758-1762 (2005).

778 37 Newton, C. N. et al. Intrinsically slow dynamic instability of HeLa cell microtubules in  
779 vitro. *Journal of Biological Chemistry*. **277** (45), 42456-42462 (2002).

780 38 Belvindrah, R. et al. Mutation of the alpha-tubulin Tuba1a leads to straighter  
781 microtubules and perturbs neuronal migration. *Journal of Cell Biology*. **216** (8), 2443-2461  
782 (2017).

783 39 Breuss, M. et al. Mutations in the murine homologue of TUBB5 cause microcephaly by  
784 perturbing cell cycle progression and inducing p53 associated apoptosis. *Development*.  
785 dev.131516 [pii] 10.1242/dev.131516 (2016).

786 40 Latremoliere, A. et al. Neuronal-specific TUBB3 is not required for normal neuronal  
787 function but is essential for timely axon regeneration. *Cell Reports*. **24** (7), 1865-1879 e1869  
788 (2018).

789 41 Morley, S. J. et al. Acetylated tubulin is essential for touch sensation in mice. *Elife*. **5**  
790 (2016).



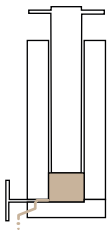
A

Tubulin source/  
culture method



HeLa S3 cell  
suspension culture

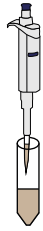
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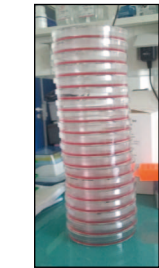
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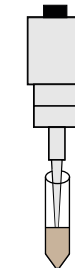
HEK-293 cell  
suspension culture



Repeated pipetting



Adherent culture



Sonication



Mouse brain



Homogenisation

B

Lysate clarification

1st polymerization

