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Purification of tubulin with controlled posttranslational modifications and isotypes from limited sources by polymerization-depolymerization cycles

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TITLE:

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

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Microtubules, in vitro reconstitution, tubulin, tubulin purification, polymerization, microtubule dynamics, tubulin code, tubulin isotypes, 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 isotypes 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 isotypes 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 isotypes 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 isotype composition. Hence, we developed this protocol to purify tubulin from different sources and with different isotype 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'[1-3]. The tubulin code includes two principal components: differential incorporation of α- and β-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[4] and treadmilling[5], and their other mechanisms and functions[6-15]. 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[16,17]. 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[18]. 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 α- and a β-tubulin genes is expressed in insect cells[19]. However, this method is very cumbersome and time-consuming and is therefore mostly used for studying the impact of tubulin mutations[20] and tubulin isotypes[21-23] 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[24]. 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[25-28]. 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**)[29].

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[30].

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(β-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 [$MgCl_2$]) solution by mixing 3.2 mL of 0.5 M PIPES, 40 µL of 0.5 M K-EGTA, and 20 µL of 1 M $MgCl_2$ 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 µL, 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 µL of 2-mercaptoethanol, 200 µL of 0.1 M PMSF, 100 µL of the protease inhibitors mix and, optionally for HEK-293 cells, 400 µL 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 µL, 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 µM 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 6×10^7 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 1.8×10^8 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 1×10^8 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 1×10^7cells 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 × *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 × *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 × *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 *ttll1*$^{-/-}$ mouse, lacking a major brain glutamylating enzyme, the tubulin tyrosine ligase-like 1 (TTLL1) protein[31].

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 ytheour 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 1/100th 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 1/100th 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 1/100th 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 × *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 × *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 1/100th 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 1/100th 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), 1/200th 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 × 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 1/200th 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 1/60th (200 µL), and for tubulin from brains, add 1/20th (600 µL) 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 × *g*, 4 °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 µL of SN3, and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analyses.

4.3.5. Resuspend the pellet P3 in BRB80 (in the same volume of SN3), take 1–4 µL, and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20 °C.

4.4. Second Polymerization (in High-Molarity Buffer)

4.4.1. Prepare the polymerization mix by combining 1 volume of SN3 (200 µL), 1 volume of pre-heated 1 M PIPES (200 µL, final concentration 0.5 M), 1/100th volume of 0.2-M GTP (2 µL, final concentration 1 mM), and 1 volume of pre-heated glycerol (200 µL) 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 °C, and incubate for 20 min.

4.4.4. Centrifuge the tubes at 150,000 × *g*, 30 °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 1/100th (120 µL), and for tubulin from brains, add 1/40th (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), 1/200th 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 1/100th (120 µL), and for tubulin from brains, add 1/40th (300 µL) 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 × *g*, 4 °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 µL and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analyses.

4.7.5. Resuspend the pellet P7 in BRB80 (same volume of SN7), take 1–4 µL, and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analyses.

4.7.6. Quantify the amount of tubulin (see **Representative Results**) and aliquot SN7 into small volumes, snap-freeze, and store at -80 °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[25]. They can also be used in microtubule-MAP co-pelleting assays and solid-state NMR spectroscopy[28].

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 α- and β-tubulins, which co-migrate as a single band in classical gels[32]. 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**)[24]. 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 µg – 1 µg – 2 µg – 4 µg) (**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. Please note that the same analysis can be done in Fiji, an upgraded version of ImageJ[33]. 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 µg 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 µg of the purified tubulin can be immunoblotted using respective antibodies[34,35]. 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-α-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, α- and β-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 µg, gray gradient line) and different volumes (0.5 and 1 µL, 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 α- and β-tubulin, TTLL7 overexpression specifically enriches β-tubulin glutamylation. **(B)** Tubulin purified from brain tissues of wild type and *ttll1*$^{-/-}$ mice were analyzed for patterns of glutamylation. Note the strong reduction of polyglutamylation of tubulin from *ttll1*$^{-/-}$ mice, which lack the major brain glutamylase TTLL1[36]. 'TUB' gels were used to separate α- and β-tubulin. An equal amount of tubulin load was confirmed by 12G10, an anti-α-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[16,17]. 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[18,19,32,37] 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[25]. 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[38-40] or tubulin PTMs[31,41].

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[24]. 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[18]. 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.

ACKNOWLEDGMENTS:

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The antibody 12G10, developed by J. Frankel and M. Nelson, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa.

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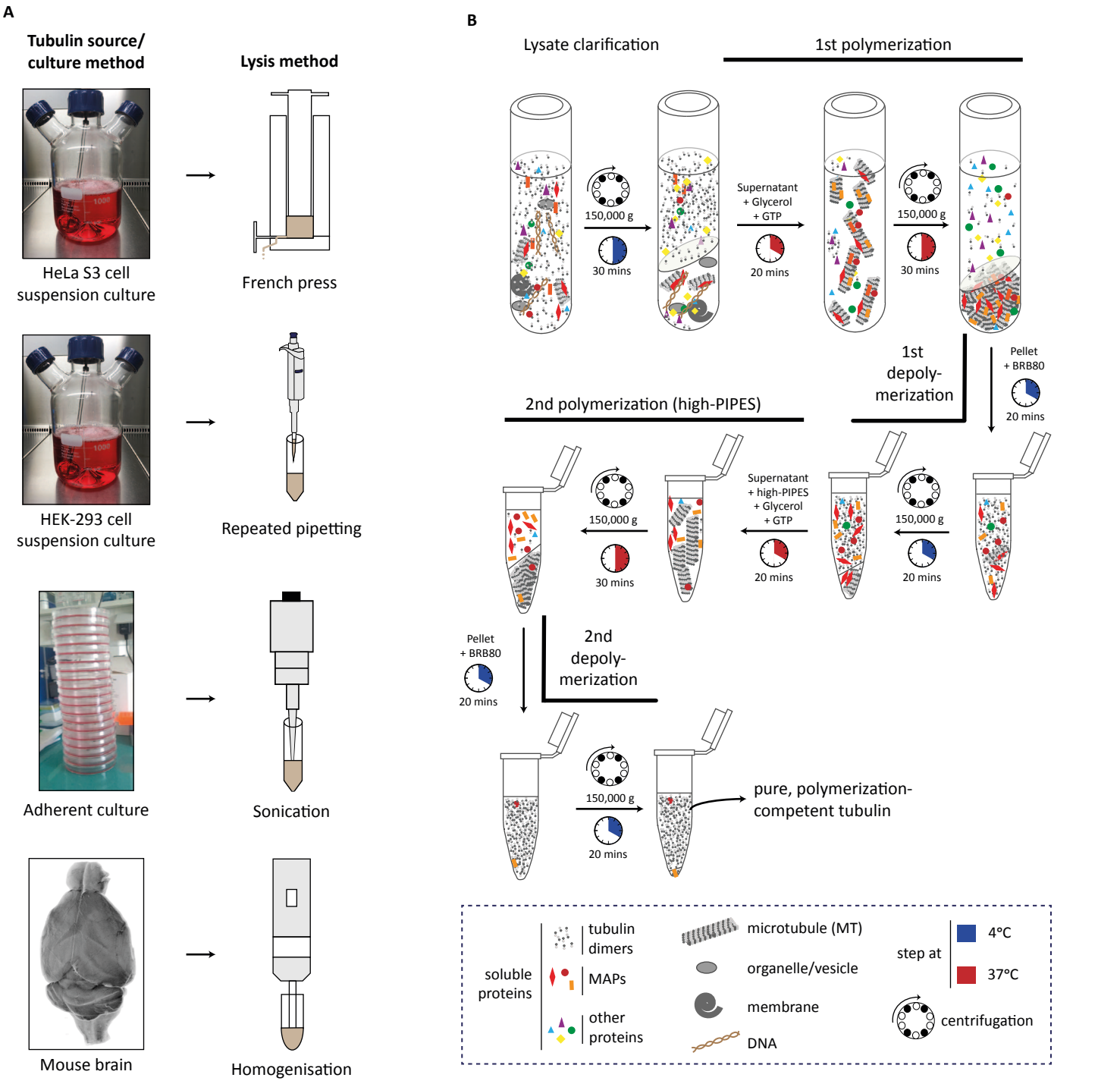

Figure 2
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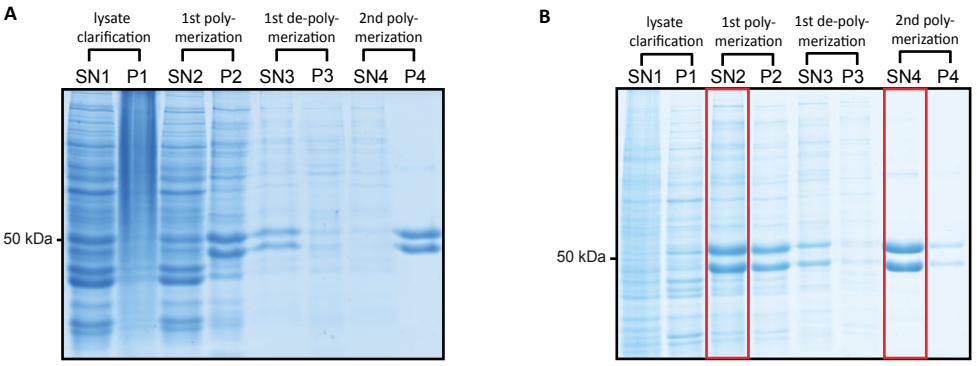

Figure 3
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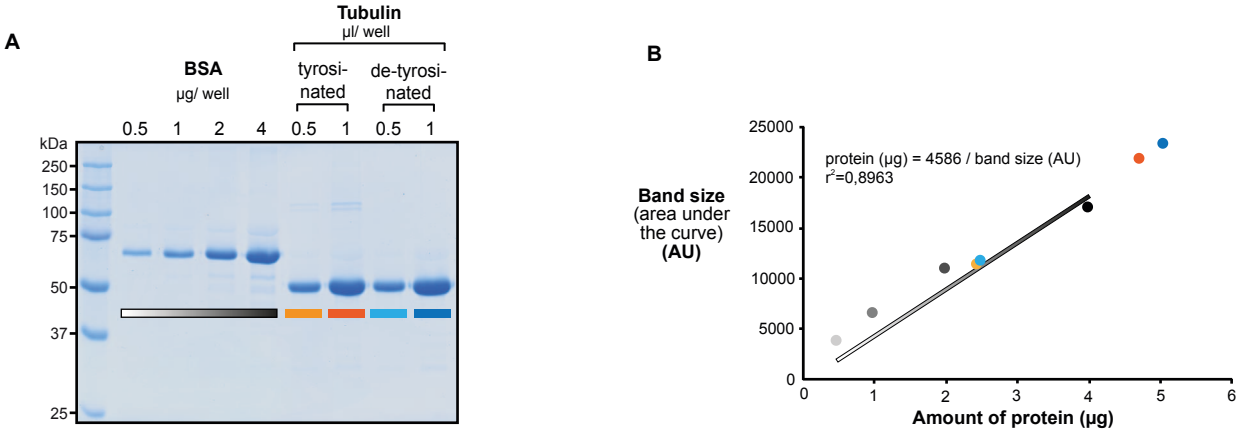

Figure 4

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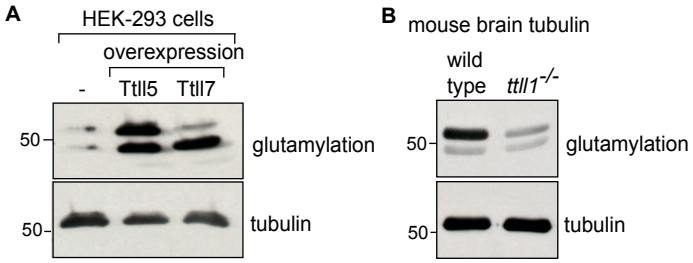

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1 M $MgCl_2$	Sigma	#M1028	
1- cell culture vessels	Techne F7610		Used for spinner cultures. Never stir the empty spinner bottles. When spinner bottles are in the cell culture incubator, always keep the lateral valves of spinner bottles slightly open to facilitate the equilibration of media with incubator's atmosphere. After use, fill the spinner bottles immediately with tap water to avoid drying of remaining cells on the bottle walls. Wash the bottles with deionised water, add app 200 ml of deionised water and autoclave. Under a sterile cell culture hood remove the water and allow the bottles to dry completely, still under the hood, for several hours. Never use detergents for cleaning the spinner bottles because any trace amounts of the detergent can be deleterious to the cells.
1.5 and 2 mL tubes			
14 mL round-bottom tubes			
15 cm diameter sterile culture dishes			
15 mL screw-cap tubes			
2-mercaptoethanol	Sigma	#M3148	2-mercaptoethanol is toxic and should be used under the hood.
4-(2-aminoethyl)-benzenesulfonyl fluor	Sigma	#A8456	
40% Acrylamide	Bio-Rad	#161-0140	
5-, 10- 20 mL syringes			
5 mL, 10 mL, 25 mL sterile pipettes			
50 mL screw-cap tubes			
Ammonium persulfate (APS)	Sigma	#A3678	
Anti-acetylated tubulin antibody, 6-11B	Sigma	#T6793	dilution: 1/2,000

Anti-alpha-tubulin antibody, 12G10	Developed by J. Frankel and M. Nelson, obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by the University of Iowa		dilution: 1/500
Anti-detyrosinated tubulin antibody	Merck	#AB3201	dilution: 1/1,000
Anti-glutamylated tubulin antibody, GT	AdipoGen	#AG-20B-0020	dilution: 1/20,000
Anti-polyglutamylated tubulin antibody	AdipoGen	# AG-25B-0030	dilution: 1/20,000
Anti-tyrosinated tubulin antibody, $YL^1/_2$	Abcam	#ab6160	dilution: 1/1,000
Aprotinin	Sigma	#A1153	
Balance (0.1 – 10 g)			
Beckman 1-l polypropylene bottles			For collecting spinner cultures
Beckman Avanti J-26 XP centrifuge			For collecting spinner cultures
Biological stirrer	Techne MCS-104L		Installed in the cell culture incubator (for spinner cultures), 25 rpm for Hela S3 and HEK 293 cells
Bis N,N'-Methylene-Bis-Acrylamide	Bio-Rad	#161-0201	
Blender IKA Ultra-Turrax			For lysing brain tissue, use 5-mm probe, with the machine set at power 6 or 7. Blend the brain tissue 2-3 times for 15 s on ice.
Bovine serum albumin (BSA)	Sigma	#A7906	
Bromophenol blue	Sigma	#1.08122	
Carboxypeptidase A (CPA)	Sigma	#C9268	Concentration: 1.7 U/µl
Cell culture hood			
Cell culture incubator set at 37 °C, 5% CO_2			
Dimethyl sulfoxide (DMSO)	Sigma	#D8418	DMSO can enhance cell and skin permeability of other compounds. Avoid contact and use skin and eye protection.
DMEM medium	Life Technologies	#41965062	
DTT, DL-Dithiothreitol	Sigma	#D9779	
EDTA	Euromedex	#EU0007-C	
EGTA	Sigma	#E3889	
Ethanol absolute	Fisher Chemical	#E/0650DF/15	

Fetal bovine serum (FBS)	Sigma	#F7524	
French pressure cell press	Thermo electron corporation	#FA-078A	with a #FA-032 cell; for lysing big amounts of cells. Set at medium ratio, and the gauge pressure of 1,000 psi (corresponds to 3,000 psi inside the disruption chamber).
Glycerol	VWR Chemicals	#24388.295	
Glycine	Sigma	#G8898	
GTP	Sigma	#G8877	
Heating block	Stuart	#SBH130D	
Hela cells		ATCC® CCL-2™	
Hela S3 cells	ATCC	ATCC® CCL-2.2™	
Hydrochloric acid (HCl)	VWR	#20252.290	
Inverted microscope			With fluorescence if cell transfection is to be verified
Isopropanol	VWR	#20842.298	
jetPEI	Polyplus	#101	
JLA-8.1000 rotor			For collecting spinner cultures
KOH	Sigma	#P1767	KOH is corrosive and causes burns; use eye and skin protection.
L-Glutamine	Life Technologies	#25030123	
Laboratory centrifuge for 50 mL tubes	Sigma	4-16 K	
Leupeptin	Sigma	#L2884	
Liquid nitrogen			
Micro-pipettes p2.5, p10, p20, p100, p200 and p1000 and corresponding tips			
Micropestles	Eppendorf	#0030 120.973	
Mouse brain tissue			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.

Needles 18G x 1 ½" (1.2 x 38 mm)	Terumo	#18G	
Needles 20G x 1 ½" (0.9 x 38 mm)	Terumo	#20G	
Needles 21G x 4 ¾" (0.8 x 120 mm)	B.Braun	#466 5643	
Parafilm			
PBS	Life Technologies	#14190169	
Penicillin-Streptomycin	Life Technologies	#15140130	
pH-meter			
Phenylmethanesulfonyl fluoride (PMSF)	Sigma	#P7626	PMSF powder is hazardous. Use skin and eye protection when preparing PMSF solutions.
PIPES	Sigma	#P6757	
Pipette-boy			
Rotors	Beckman 70.1 Ti; TLA-100.3; and TLA 55		
SDS-PAGE electrophoresis equipment	Bio-Rad	#1658001FC	
SDS, Sodium dodecyl sulphate	VWR	#442444H	For preparing Laemmeli buffer
SDS, Sodium dodecyl sulphate	Sigma	#L5750	For preparing 'TUB' SDS-PAGE gels
Sonicator	Branson	#101-148-070	Used for lysing cells grown as adherent cultures. Use 6.5 mm diameter probe, set the sonicator at "Output control" 1, "Duty cycle" 10% and time depending on the cell type used.
Tabletop centrifuge for 1.5 mL tubes	Eppendorf	5417R	
TEMED, N, N, N', N'-Tetramethylethylenediamine	Sigma	#9281	
Trichostatin A (TSA)	Sigma	#T8552	
Triton X-100	Sigma	#T9284	
Trizma base (Tris)	Sigma	#T1503	
Trypsin	Life Technologies	#15090046	
Ultracentrifuge rotors		TLA-55, TLA-100.3 and 70.1 Ti rotors	Set at 4°C or 30°C based on the need of the experim
Ultracentrifuge tubes	Beckman	#357448	for using with TLA-55 rotor
Ultracentrifuge tubes	Beckman	#349622	for using with TLA-100.3 rotor
Ultracentrifuge tubes	Beckman	#355631	for using with 70.1 Ti rotor

Ultracentrifuges	Beckman	Optima L80-XP (or equivalent) and Optima MAX-XP (or equivalent)	Set at 4°C or 30°C based on the need of the experim
Vortex mixer			
Water bath equipped with floaters or tube holders			Set at 30°C

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   OK

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

We believe this is already the case. In a few instances (in points 2.1, for example), the sentence starts with “For 2 L of suspension culture…”, but continues with imperative tense (“revive and grow…”). We hope this is acceptable.

7. The Protocol should contain only action items that direct the reader to do something.

   We have sometimes included information crucial to the successful outcome of a given action item, such as “The ratio of cell pellet volume to the lysis buffer is very important for a successful tubulin purification.” in 2.2.1.4. We hope this is OK.

8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

   We believe that this is now the case

9. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

   We believe that this is now the case

10. We do not have a separate pause paragraph. Please convert to note instead.

    This has been changed.

11. Only one note can follow one step. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

    This has now been adapted to JOVE standards.

12. 2.2.3: Age, sex, strain of the mouse used for study.

    This information has now been added to the step 2.2.3.

13. 4: Rationale for polymerization/depolymerization in purification?

    This has now been added in the introduction:

"In this procedure, tubulin is cycled between the soluble (tubulin dimer, at 4°C) and polymerized form (microtubule, at 30°C and in the presence of GTP). Each form is separated through successive steps of centrifugation: tubulin dimers will remain in the supernatant after a cold (4°C) spin, while microtubules will be pelleted at 30°C. Furthermore, one polymerization step is carried out at high PIPES concentration, which allows to remove microtubule-associated proteins from the microtubules, and thus, from the finally purified tubulin."

14. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

    We have now made a separate file with all the protocol steps we think should be filmed. The same steps have been also highlighted in the manuscript file. The filmable content is less than 3 pages and it identifies all the necessary details for making the video.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

    We believe that this is the case.

16. Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

    OK

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

    Not relevant to this manuscript

18. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

OK

19. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.

OK

20. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

We believe that this has been taken into consideration.

21. Please do not abbreviate the journal titles in the references section.

This has been changed in the current version of the manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes in detail the purification of tubulin from various cell lines and mouse brain using cycles of microtubule (MT) polymerization, centrifugation, isolation of the pellet fraction, and MT depolymerization. It resembles a Nature Protocols manuscript by the same group (Souphron, J., Bodakuntla, S., Jijumon, A.S., Lakisic, G., Gautreau, A.M., Janke, C., and Magiera, M.M. (2019). Purification of tubulin with controlled post-translational modifications by polymerization-depolymerization cycles. Nature protocols 14, 1634-1660). The procedures desribed here are based on classic approaches using pig or cow brain, which contain massive amounts of tubulin. Protocols have been adapted to working with smaller quantities of tubulin.

This is a very clear description of an important procedure in the microtubule field. I have a few comments that hopefully help to further clarify the manuscript.

We thank the referee for the supportive comments on our manuscript and for corroborating with us on the importance of this procedure for the microtubule field. In fact, we were invited by the JOVE editors to prepare this publication following our publication in Nature Protocols. For this reason, the written part is of course very similar to the one you can find in Nature Protocols, but we believe that being able to film some of the crucial steps of this procedure will help fellow scientists performing this protocol successfully.

Below, we have addressed all the concerns of this reviewer with point by point responses.

Major Concerns:

None

Minor Concerns:

1) In the protocol the authors start out with a warning (NOTE: All the buffers used for tubulin purification should contain potassium salts and NOT sodium salts). It would be handy if the reason(s) for this warning were

explained, also because other groups do use sodium salts for MT polymerization.

We thank the referee for pointing this out. The rationale behind this advice can be found here: Wolff *et al.,* Protein Science 1996:

"Our results with taxol-polymerized tubulin and organic cations (Wolff et al., 1996), and the present findings with alkali metal cations, show that there is marked cation selectivity for increasing the rate of polymerization. Three components appear to contribute to this selectivity: (I) a background influence of the anion depending on its chaotropic potential; (2) promotion of polymerization with cation selectivity Na+ > K+ > Li+ > Cs+; and (3) an apparent inhibitory cation effect at high concentrations with a reverse rank order to that promoting polymerization, i.e., Cs+ > Li+ > K+ > Na+."

Thus, using potassium rather than sodium salts in the protocol is preferred, as it makes tubulin polymerisation into microtubules less dependent on high tubulin concentration. We have now added the Wolff et al reference to the protocol.

2) On page 7 the authors state : "On day -10, plate these cells on six 15-cm-diameter dishes at 10 million cells per plate and grow them to confluence. This is the recommended number of cells to successfully purify tubulin from 2 L of suspension cultures (see Table of Materials)". What is actually the recommended number, the 10 million cells on day -10 or the number of cells after reaching confluence ? What is the number of cells after reaching confluence ?

We thank the referee for this question. This information has been now added to the protocol text.

"2.1.1.3. On day -7, trypsinize and collect the cells grown to 80-90% confluence (approximately 1.8X108 cells). Collect cells from 3 dishes at a time, pellet them down (200Xg, 5 min, room temperature) and re-suspend all cells in 10 mL of DMEM medium."

3) Point 2.2.1.4 states "Add 10 mL of lysis buffer". A bit later on page 9 the authors state : "PAUSE: Cells resuspended in lysis buffer can be snap-frozen in liquid nitrogen and stored at -80°C for two months". However, resuspension is not mentioned in point 2.2.1.4, only the addition of lysis buffer is mentioned. So what should exactly be done if one wants to pause

at this stage ? I am asking this because the next step (3.1.1) is cell lysis and it is important to know what the difference is between cell resuspension and cell lysis, and whether lysed cells can be stored.

We thank the referee for pointing this. We have now made it clear in point

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

This step does not induce cell lysis. However, once the cell suspension is frozen and thawed, cell lysis will occur and will be completed by different steps of cell lysis (see section 3).

4) In step 4.4.1 the authors state : "Prepare the polymerization mix by combining 1 volume of SN3 (200 µl), 1 volume of pre-heated 1 M PIPES (200 µl, final concentration 0.5 M), 1/100th volume of 0.2-M GTP (2 µL, final concentration 1 mM), 0.5 volume of pre-heated glycerol (6 mL) in a screw-cap tube of the appropriate volume". The 0.5 volume of glycerol seems off, is it really 6 ml? And is the volume GTP (1/100th) correct?

We thank the referee for noticing this mistake. We have now corrected the values.

5) On page 20 in the Discussion section the authors state "Tubulin purified from cell lines lacks all tubulin PTMs including detyrosination". This is a rather bold statement. Many papers have used cell lines to analyze specific PTMs on MTs/tubulin, including, for example, the original observation on detyrosination (Gundersen, G.G., Kalnoski, M.H., and Bulinski, J.C. (1984). Distinct populations of microtubules: tyrosinated and nontyrosinated alpha tubulin are distributed differently in vivo. Cell 38, 779-789) was made in cell lines. Perhaps the authors can rephrase ?

In the cell lines, tubulin PTMs, including detyrosination have been so far shown to be localised only on specialised microtubule structures (such as mitotic spindles) and not on interphase microtubules (Gundersen et al Cell, 1984; Lacroix et al JCB, 2010). As in an asynchronous culture of cells the percentage of cells undergoing mitosis is around 5% (assuming a doubling time of app. 24 h and mitosis lasting app. 30 min; Schori and Sedivy, Methods, 2007), it is safe to assume that only a very small percentage of microtubules are modified.

However, we do understand the reviewer's concern and hence we modified the sentence: "Tubulin purified from cell lines is virtually free of tubulin PTMs."

Typos:

1) on page 3 : Remove . in "which. allows".
2) In the Table of materials : Remove) at the end of the phrase "Developed by J. Frankel and M. Nelson, obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by the University of Iowa)"

Those have now been corrected.

Reviewer #2:

Manuscript Summary:

The manuscript describes the purification of tubulin from adherent or suspension cell lines and from single mouse brains. The lab has a lot of expertise in using the described technique and studying purified tubulin with different modifications.

Major Concerns:

None.

Minor Concerns:

There are a number of points that should be clarified to make the protocol easy to implement for other researchers.

1.1. Assuming concentrations are given as final concentration, then either leave out volumes of stock solutions added or give also the concentration of stock solutions, e.g. 200mM L-Glut (10ml 2M stock) otherwise reader might think they are asked to add 10ml of 200mM.

Following reviewer's suggestion, we have now explicitly stated the stock concentrations to avoid any misunderstanding.

1.4. As KOH is already used to make solution in 1.2, it might make sense to change order. Make sure to rephrase so that base is added to water rather than the other way round as the reaction is exothermic.

Preparation of KOH step has been moved up.

1.10. Suggest suitable sizes for aliquots. 100µl might make sense here based on what is used for 1.12.

Aliquot sizes have been added.

1.12. Clarify that a fume hood is meant as hood can also refer to microbiological safety hood.

This has been clarified.

1.13. Recommend sizes of aliquots.

Aliquot sizes have been added.

1.15. 5x Laemmli is described, but 2x Laemmli used throughout method

We have now added a sentence about the preparation of 2x buffer.

“Prepare the 2x working solution of Laemmli sample buffer by diluting the 5x stock in distilled water.”

2.1.1. Make clear how number of cells relates to number of spinner flasks and total volume of cells grown from the start. This way reader doesn't need to work backwards. As the method is here described for 2 spinner flasks, state that. Readers can then upscale as needed if they wish. However, given that the volumes are pretty small at the end assuming a 10ml pellet volume, which needs at least 3 spinner bottles of 1L each, the choice of describing growth of 2L is slightly odd. You might want to consider describing what is needed for a standard prep with 10ml pellet, describe the purification with the 6ml expected pellet volume from the two spinner bottles, or describe the amplification section per unit - i.e. 1 spinner bottle. Mention the incubator settings in the note.

We thank the reviewer for this suggestion. In fact, we described a protocol that could be adapted to tubulin preparation from different sources (cells grown as adherent or liquid cultures, or mouse brains). In these different preparations, the cell pellet and brain weights/volumes are quite different. Thus, it is not possible to choose an imaginary cell volume that suits all three different approaches. Hence, we assumed the cell pellet as 10 ml which is mathematically easy to adapt to other volumes.

3.1.1. The technique for resuspending pellets during depolymerisation steps needs further clarification, especially as this step is so crucial for the success of the prep. Pipet up and down every 5 minutes for 10 minutes can mean different things. I assume it means three cycles of pipetting several times up and down (please specify how many times) with 5 minute breaks in-between.

We thank the reviewer for this remark. We have clarified this part by writing how many times the pipetting needs to be repeated.

“Resuspend the microtubule pellet gently, avoid air bubbles, until the solution is completely homogeneous. Use a p1000 tip for a couple of times 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.”

Such clarifications have been made throughout the manuscript.

3.2.2. see 3.1.1.

This has been clarified.

3.3.1. I think pestle might be a more common name for a potter?

We thank the reviewer for this suggestion. This has been now changed.

4.2.5. Based on the volumes suggested, these are relative to volumes of SN2, not SN1.

We thank the referee for pointing this out. We have now corrected this typo.

4.3.2. see 3.1.1.

This has been clarified.

4.4.1. Volume of glycerol should probably be 100 µl here, or maybe 200 µl, but not 6ml

We thank the reviewer for spotting this mistake, this has now been corrected.

4.5.1. Highlight better that depolymerisation volumes are all relative to SN1 rather than supernatant volumes of that step.

We have now added a note about this.

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

4.5.2. see 3.1.1.

This has been clarified.

4.7.1. see 4.5.1.

This has been clarified.

4.7.2. see 3.1.1.

This has been clarified.

Page18: "TUB" SDS-PAGE gels are mentioned to monitor the purification and gels like that are shown, but the method isn't described. It would make sense if this would be included here. Alternatively, if a detailed method has been described elsewhere, this should be cited and referred to here. There

seems to be a reference to a next section, but this wasn't included in the reviewer's files, although chemicals needed for SDS-PAGE were listed.

We have now added the reference where these gels ('TUB' SDS-PAGE) are explained in detail.

Figure 2: Samples are only shown until SN4 and P4, but the method describes a third polymerisation/depolymerisation cycle with samples until SN7. Is that not routinely done? Ideally the chapter would be accompanied with a gel showing a typical experiment with all samples.

In fact, we do not routinely analyse all the collected samples during the tubulin purification. The final tubulin fraction (SN7) is very precious and thus, we first use it for determining its concentration. If we notice any anomaly in the concentration of tubulin (in comparision to our previous purifications), then we load all the samples until SN7 for assessing the quality of purification.

Besides, the success of the tubulin purification is determined in the first two polymerizations steps (low and high-salt conditions). In the third (and further) polymerization steps the tubulin can be easily cycled in low salt conditions.

We have now added a sentence pointing this out in the representative Result section:

"The final tubulin sample, which is very precious, is only loaded on the gel allowing to determine tubulin concentration (see next paragraph)"

Figure 3: The gel is very nice, but extrapolating to read the concentration of protein beyond the BSA standards isn't good practice as one cannot sure that the staining continues to be linear in that range. Ideally the figure would be replaced with an example where samples lie within the range of the standards.

We thank the reviewer for pointing this out and we agree that extrapolating the curve to determine the concentration is not a good idea. We suggest that the reader loads at least two amounts of purified tubulin for quantification, and only uses the amount that falls within the BSA standard curve to determine the concentration. We have adapted the text accordingly.

Thus, we would like to still keep the same gel which has different amounts of tubulin (one amount falling in the range of the curve and the other amount outside the range of the curve) and we have explicitly mentioned that the bands

outside of the BSA standard curve should not be considered for concentration determination.

The manuscript contains a small number of typos and grammatical errors that should be corrected during copy-editing.

Manuscript has been proof-read and the typos have been corrected.

Dear Lindsay, dear Vineeta,

Thank you again for your invitation to publish our protocol in JOVE. We also thank you for the helpful comments on our manuscript from your side.

We have now revised the manuscript by considering all of the referees' and editorial comments, as explained in detail by our point-by-point response. As suggested, we kept all the changes to the manuscript in the 'Track changes' mode. We believe that the current version of the manuscript addresses all of the earlier concerns.

In addition, we prepared a separate file with the script that needs to be filmed. Please come back to us if further clarifications are needed.

Best regards,

Magda Magiera.

Video script

Color code: Suspension cultures, Adherent cultures, Brain tissue, applies to all three sources

Before we start with the video,
***Text**: Revive the cells and amplify them.*
Video: On day -8, prepare the required amount of DMEM complete medium to 37°C. Add 1 L of pre-heated medium to each spinner bottle under a laminar flow cabinet. Place the spinners on a stirrer table set at 20-25 rpm inside the cell culture incubator, open slightly the lateral spinner caps to allow the medium to equilibrate to the incubator's atmosphere.

Video: On day -7, trypsinize and collect the cells grown to 80-90% confluence in 10 mL of DMEM. Add 5 mL of the cell suspension to each spinner bottle containing 1 L of DMEM medium, return the spinners to the stirrer table in the cell culture incubator and allow cells to grow for one week.

Video: On day 0, transfer the cell suspension from spinners into 1 L centrifuge bottles, pellet cells at 250×g, 15 min, room temperature.

Video: Resuspend pelleted cells from each centrifuge bottle in 10 mL of ice-cold PBS

Video: Discard the supernatant and show the volume of the cell pellet. From 2 L of suspension culture (two spinner bottles), expect a cell pellet of app. 6 mL.

***Text**: For purification from adherent cell lines, revive and grow cells in ten 15-cm dishes to confluence.*

Video: Remove the medium from 15-cm dishes by inclining the dishes and then gently wash the cells with 7 mL of room-temperature PBS-EDTA (person 1).

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

Video: 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 to 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 cell suspension on ice.

Video: Pellet the cells at 250×g, 10 min, at 4°C. Discard the supernatant and determine the volume of the cell pellet. Expect a volume of approx. 1 mL from ten 15-cm dishes. For the rest of the protocol, the cell pellet is assumed to be 10 mL.

***Text**: For purification from mouse brain, sacrifice the mouse by cervical dislocation and dissect the brain.*
Video: 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.

Video: Add 500 µL of lysis buffer to a single brain extracted from an adult mouse.

***Text**: Cell lysis of suspension cultures*
For HEK-293, Video: 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).

Video: For HeLa S3, lyse the cells using a French press

Video: For HeLa S3, often a white floating layer is formed after centrifugation. Do not transfer this floating layer along with the supernatant, as it interferes with tubulin polymerization efficiency. Use a syringe of appropriate volume attached to a 20G or 21G long needle to gently remove the supernatant without disturbing the floating layer.
***Text:** White floating layer formation after centrifugation is more for HEK-293 cells comparing HeLa S3 cells.*

***Text**: For lysis of adherent cultures*

Video: Transfer the cells into a 14-mL round-bottom tube that has been cut in height to accommodate the sonicator probe (see **Table of Materials** for settings). Sonicate the cells for about 45 pulses and ensure the cell lysis by sampling a drop of the lysis mix under a microscope.

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

***Text**: For lysis of brain tissue*
*Video: Lyse the brain tissue using a tissue blender (see **Table of Materials** for settings). Alternatively, lyse the tissue using a microtube potter or an equivalent equipment and pipet up and down on ice with a 1-mL syringe with an 18G needle.*

***Text**: The rest of the protocol is very similar for the different sources with the exception of volumes. So, we show here the example of tubulin purification from suspension cultures for the rest of the video.*

***Text**: After the cell lysis, take sample for quality analysis*
Video: Take 1/100th volume of the lysis mix (L) (200 µL for 20 mL of L) and add the same volume of 2× Laemmli buffer, boil for 5 min and store at -20°C for further analysis.

Video: Clear the lysate (separating pellet and soluble fraction of the lysis mix) by centrifugation at 150,000×g, 4°C, 30 min.

Video: Transfer the supernatant (SN1) to ultracentrifuge tubes and note its volume. For a 10 mL cell pellet, expect a volume of app. 12 mL for SN1.
Video: Resuspend the pellet (P1) in BRB80 using the same volume as SN1.
***Text**: Take the samples for analysis*

Polymerization 1: Video: Prepare the polymerization mix by combining 1 volume of SN1 (12 mL), 1/200th volume of 0.2-M GTP (60 µL, final concentration 1 mM), 0.5 volume of pre-heated glycerol (6 mL) in a tube of the appropriate volume.
Video: Pipet the mix up and down gently avoiding the formation of air bubbles and transfer it to the appropriate ultracentrifuge tubes.

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

Video: Centrifuge the tubes at 150,000×g, 30°C for 30 min. Remove the supernatant (SN2) and keep the pellet of polymerized microtubules (P2).

Depolymerization 1: Video: Depolymerize microtubules by adding ice-cold BRB80 to the pellet P2 and leave on ice for 5 min.

Video: Resuspend the microtubule pellet gently, avoid air bubbles, until the solution is completely homogeneous. Use a p1000 tip for a couple of times followed by a p200 tip every 5 min, for 20 min (five cycles of pipetting).

Video: Transfer the solution to appropriate ultracentrifuge tubes and spin down at 150,000×g, 4°C for 20 min.

Polymerization 2: Video: Prepare the polymerization mix by combining 1 volume of SN3 (200 µl), 1 volume of pre-heated 1 M PIPES (200 µl, final concentration 0.5 M), 1/100th volume of 0.2-M GTP (2 µL, final concentration 1 mM), 1 volume of pre-heated glycerol (200 µL) in a tube of the appropriate volume.

Video: Centrifuge the tubes at 150,000×g, 30°C for 30 min. Remove the supernatant (SN4) and keep the pellet of polymerized microtubules (P4).

***Text**: **Depolymerization 2:** Repeat the depolymerization steps like depolymerization 1, see text for volumes*

Video: Show depolymerized tubulin solution tube

***Text**: **Polymerization 3:** Repeat the polymerization steps like polymerization 1, see text for volumes*

Video: Show polymerized pellet tube

***Text**: **depolymerization 3:** Repeat the depolymerization steps like depolymerization 1, see text for volumes*

Video: Show depolymerized tubulin solution tube. Aliquot, snap-freeze and store at -80C.

***Video and text:** Representative results: Show gel and explain the quality of tubulin.*