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

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1 **TITLE:**

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

4  
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20  
21 **KEYWORDS:**

22 Microtubules, in vitro reconstitution, tubulin, tubulin purification, polymerization,  
23 microtubule dynamics, tubulin code, tubulin isoypes, suspension culture, tubulin  
24 posttranslational modifications

25  
26 **SUMMARY:**

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

31  
32 **ABSTRACT:**

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

41  
42 To date, this was technically challenging as brain tubulin, which is widely used in in vitro  
43 experiments, harbors many PTMs and has a defined isotype composition. Hence, we  
44 developed this protocol to purify tubulin from different sources and with different isotype  
45 compositions and controlled PTMs, using the classical approach of polymerization and  
46 depolymerization cycles. Compared to existing methods based on affinity purification, this

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

49

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

57

## 58 **INTRODUCTION:**

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

69

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

83

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

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

107  
108 In the protocol, we first describe the generation of the source material (cell mass or brain  
109 tissue), its lysis (**Figure 1A**), followed by the successive steps of tubulin polymerization and  
110 depolymerization to purify the tubulin (**Figure 1B**). We further describe the process to assess  
111 the purity (**Figure 2A, B**) and quantity (**Figure 3A, B**) of the purified tubulin. The method can  
112 be adapted to produce tubulin enriched with a selected PTM by overexpressing a modifying  
113 enzyme in cells prior to tubulin purification (**Figure 4B**). Alternatively, tubulin-modifying  
114 enzymes can be added to tubulin during the purification process. Finally, we can purify tubulin  
115 lacking specific isotypes or PTMs from the brains of mice deficient in the corresponding  
116 tubulin-modifying enzymes (**Figure 4B**)<sup>29</sup>.

117  
118 The method we describe here has two main advantages: (i) it allows the production of  
119 sufficiently large amounts of tubulin in a relatively short time, and (ii) it generates high-quality,  
120 pure tubulin, with either specific tubulin isotype composition or PTMs. In the associated video  
121 of this manuscript, we highlight some of the critical steps involved in this procedure.

## 122 **PROTOCOL:**

123  
124  
125 Animal care and use for this study were performed in accordance with the recommendations  
126 of the European Community (2010/63/UE). Experimental procedures were specifically  
127 approved by the ethics committee of the Institut Curie CEEA-IC #118 (authorization no.  
128 04395.03 given by National Authority) in compliance with the international guidelines.

### 129 **1. Preparation of Reagents for Tubulin Purification**

130  
131  
132 NOTE: All the buffers used for tubulin purification should contain potassium salts and NOT  
133 sodium salts<sup>30</sup>.

134  
135 1.1. Prepare 1 L of complete medium: Dulbecco's modified Eagle medium (DMEM), with  
136 10% fetal bovine serum (FBS, 100 mL), 200 mM L-glutamine (10 mL of 2 M stock), and 1x  
137 penicillin-streptomycin (10 mL of 100x stock). Store at 4 °C.

138

- 139 1.2. Prepare 10 M potassium hydroxide (KOH) by dissolving 140 g of KOH in water, adjust  
140 the final volume to 250 mL, and store at room temperature.  
141
- 142 1.3. Prepare 0.5 M ethylenediamine tetraacetic acid (EDTA), pH 8, by dissolving 36.5 g of  
143 EDTA in water, adjust the pH to 8.0 using KOH (otherwise EDTA will not dissolve) and the final  
144 volume to 250 mL, filter-sterilize, and store at room temperature.  
145
- 146 1.4. Prepare 5 mM phosphate-buffered saline (PBS)-EDTA by adding 5 mL of 0.5 M EDTA  
147 to 500 mL of PBS, filter-sterilize, and store at room temperature.  
148
- 149 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  
150 with KOH (otherwise PIPES will not dissolve) and the final volume to 500 mL, filter-sterilize,  
151 and store at 4 °C.  
152
- 153 1.6. Prepare 1 M K-PIPES, pH 6.8, by dissolving 15.1 g of PIPES in water, adjust to pH 6.8  
154 with KOH and the final volume to 50 mL, filter-sterilize, and store at 4 °C.  
155
- 156 1.7. Prepare 0.5 M potassium-ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-  
157 tetraacetic acid (K-EGTA, pH 7.7) by dissolving 47.5 g of EGTA in water, adjust to pH 7.7 with  
158 KOH and the final volume to 250 mL, filter-sterilize, and store at room temperature.  
159
- 160 1.8. Prepare BRB80 (80 mM K-PIPES, pH 6.8; 1 mM K-EGTA; 1 mM magnesium chloride  
161 [ $\text{MgCl}_2$ ]) solution by mixing 3.2 mL of 0.5 M PIPES, 40  $\mu\text{L}$  of 0.5 M K-EGTA, and 20  $\mu\text{L}$  of 1 M  
162  $\text{MgCl}_2$  and adjust the final volume to 20 mL. Store at 4 °C.  
163
- 164 1.9. Prepare 0.1 M phenylmethanesulfonyl fluoride (PMSF) by dissolving 435 mg of PMSF  
165 in isopropanol to obtain a final volume of 25 mL and store at -20 °C.  
166
- 167 1.10. Prepare protease inhibitors mix (200x) by dissolving 10 mg of aprotinin, 10 mg of  
168 leupeptin, and 10 mg of 4-(2-aminoethyl)-benzenesulfonyl fluoride in water to obtain a final  
169 volume of 2.5 mL, make aliquots of 100  $\mu\text{L}$ , and store at -20 °C.  
170
- 171 1.11. Prepare 10% Triton X-100 by mixing 5 mL of Triton X-100 in 45 mL of water, filter-  
172 sterilize, and store at room temperature.  
173
- 174 1.12. Prepare Lysis buffer (BRB80 supplemented with 1 mM 2-mercaptoethanol, 1 mM  
175 PMSF, 1x protease inhibitors mix and, optionally for HEK-293 cells, 0.2% Triton X-100) on the  
176 day of tubulin purification by mixing 20 mL of BRB80 with 1.5  $\mu\text{L}$  of 2-mercaptoethanol, 200  $\mu\text{L}$   
177 of 0.1 M PMSF, 100  $\mu\text{L}$  of the protease inhibitors mix and, optionally for HEK-293 cells, 400  $\mu\text{L}$   
178 of 10% Triton X-100.  
179
- 180 NOTE: 2-mercaptoethanol is toxic and should be used in the fume-hood.  
181
- 182 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  
183 using KOH, make aliquots of 20  $\mu\text{L}$ , and store at -20 °C. Avoid repeated freeze-thaw cycles.  
184

185 1.14. Prepare 1 M tris(hydroxymethyl) aminomethane-hydrochloride (Tris-HCl) by dissolving  
186 60.56 g of Tris in water, adjust to pH 6.8 with HCl, complete to a final volume of 500 mL, filter-  
187 sterilize, and store at room temperature.

188

189 1.15. Prepare 5x Laemmli sample buffer (450 mM dithiothreitol (DTT); 10% sodium  
190 dodecylsulfate (SDS); 400  $\mu$ M Tris-HCl, pH 6.8; 50% glycerol;  $\sim$ 0.006% bromophenol blue) by  
191 adding 4 g of SDS to 16 mL of preheated 1 M Tris-HCl, pH 6.8, and mix the solution gently. Add  
192 2.6 g of DTT and 20 mL of 100% glycerol to the mix and stir until the solution becomes  
193 homogenous. Add the desired amount of bromophenol blue (2.5 mg) to reach the required  
194 color intensity. Make 5 mL aliquots and store at  $-20$  °C. Prepare the 2x working solution of  
195 Laemmli sample buffer by diluting the 5x stock in distilled water.

196

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

198

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

203

### 204 **2.1. Amplification of Cells**

205

#### 206 **2.1.1. Cells Grown as Suspension Cultures**

207

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

210

211 2.1.1.1. For 2 L of suspension culture, revive and grow the preferred cell type to obtain  
212  $6 \times 10^7$  cells 10 days before the day of preparation. On day -10, plate cells on six 15 cm  
213 diameter dishes at  $10^7$  cells per plate.

214

215 2.1.1.2. On day -8, preheat the required amount of complete medium to 37 °C. Add 1 L  
216 of pre-heated medium to each spinner bottle under sterile conditions. Place the spinners on  
217 a stirrer table set at 20–25 rpm inside the cell culture incubator, slightly open the lateral  
218 spinner caps to allow the medium to equilibrate to the incubator's atmosphere.

219

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

222

223 2.1.1.3. On day -7, trypsinize and collect the cells grown to 80–90% confluence  
224 (approximately  $1.8 \times 10^8$  cells). Collect cells from 3 dishes at a time, spin down ( $200 \times g$ , 5 min,  
225 room temperature), and re-suspend all cells in 10 mL of DMEM.

226

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

230

231 2.1.1.4. Add 5 mL of the cell suspension to each spinner bottle containing 1 L of DMEM,  
232 return the spinners to the stirrer table in the cell culture incubator, and allow cells to grow for  
233 one week.

234

235 2.1.2. Cells Grown as Adherent Cultures

236

237 NOTE: To successfully purify tubulin from adherent cells, use a minimum of 10 dishes of 80–  
238 90% confluence.

239

240 2.1.2.1. Revive and amplify the desired cell type to obtain  $1 \times 10^8$  cells three days before  
241 the day of the tubulin preparation.

242

243 2.1.2.2. On day -3, plate these cells on ten 15 cm dishes at  $1 \times 10^7$  cells per dish and  
244 allow them to grow 80–90% confluence.

245

246 2.1.2.3. On day -1, if required, transfect cells with a plasmid to express a tubulin-  
247 modifying enzyme or a particular tubulin isotype.

248

249 2.2. Harvesting the Cells/Brain Tissue

250

251 2.2.1. Cells Grown as Suspension Cultures

252

253 2.2.1.1. Transfer the cell suspension from spinners into 1 L centrifuge bottles (**Table of**  
254 **Materials**) and pellet cells at  $250 \times g$ , 15 min, room temperature. For immediately starting  
255 another culture of HeLa S3 cells in the spinner bottles, leave 100 mL of cell suspension in the  
256 spinners, and add 1 L of complete, pre-heated DMEM to the spinner bottle.

257

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

259

260 2.2.1.2. Resuspend pelleted cells from each centrifuge bottle in 10 mL of ice-cold PBS,  
261 and transfer all the cells into 50 mL screw-cap tubes. During re-suspension, keep the cells on  
262 ice. Pellet the cells at  $250 \times g$ , 15 min, 4 °C.

263

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

266

267 2.2.1.3. Discard the supernatant and determine the volume of the cell pellet. From 2 L  
268 of suspension culture (two spinner bottles), expect a cell pellet of 5–6 mL.

269

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

272

273 2.2.1.4. Add 1 volume (10 mL) of lysis buffer and re-suspend the cell pellet.

274

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

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

279  
280 NOTE: Cells resuspended in lysis buffer can be snap-frozen in liquid nitrogen and stored at -80  
281 °C for two months.

## 282 283 2.2.2. Cells Grown as Adherent Cultures

284  
285 NOTE: Cells from adherent cultures must be harvested very quickly for successful tubulin  
286 purification (approximately 15 mins for harvesting ten 15 cm dishes). Three people  
287 participated in this step of the protocol.

288  
289 2.2.2.1. Remove the medium from 15 cm dishes by inclining the dishes, and then gently  
290 wash the cells with 7 mL of PBS-EDTA at room temperature (person 1). Work only with three  
291 15 cm dishes at a time to avoid leaving the cells without medium or buffer.

292  
293 2.2.2.2. Add 5 mL of PBS-EDTA to the cells and incubate them for 5 min at room  
294 temperature.

295  
296 2.2.2.3. Use a cell lifter to gently detach the cells by shoveling them to one edge of the  
297 dish (person 2), and collect all the cells in a 50 mL screw-cap tube (person 3). Rinse each plate  
298 with an additional 2 mL of PBS-EDTA to collect any remaining cells from the dishes. During this  
299 step, keep the 50 mL screw-cap tube containing the cell suspension on ice.

300  
301 2.2.2.4. Pellet the cells at  $250 \times g$ , 10 min, 4 °C. Discard the supernatant and determine  
302 the volume of the cell pellet. Expect a volume of ~1 mL from ten 15 cm dishes.

303  
304 NOTE: In the protocol described below, the cell pellet volume is assumed to be 10 mL. Adjust  
305 the experiments according to the pellet volumes.

306  
307 2.2.2.5. Resuspend the cells in 1 volume (10 mL) of lysis buffer.

308  
309 NOTE: Cells resuspended in lysis buffer can be stored at -80 °C for up to two months.

## 310 311 2.2.3. Brain Tissue

312  
313 NOTE: Mice of any age, sex, or genetic background can be used. The choice of the transgenic  
314 mouse strain will depend on the scientific question to be addressed. In this manuscript, we  
315 show the example of tubulin purified from the *ttl1*<sup>-/-</sup> mouse, lacking a major brain  
316 glutamylating enzyme, the tubulin tyrosine ligase-like 1 (TTL1) protein<sup>31</sup>.

317  
318 2.2.3.1. Sacrifice the mouse by cervical dislocation, quickly decapitate, and collect the  
319 brain into a round-bottom tube. If there is excess blood on the brain, quickly wash with lysis  
320 buffer. Collect the brain as soon as the mouse is sacrificed as a post-mortem delay can affect  
321 the success of tubulin purification. Use round-bottom tubes to accommodate the width of the  
322 probe used for homogenization.

323

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

326

327 2.2.3.2. Add 500 µL of lysis buffer to a single brain extracted from an adult mouse. For  
328 the rest of the protocol, the volume of lysis buffer added is assumed to be 10 mL. Adjust for  
329 ytheour experiment according to the number of brains to be used.

330

### 331 **3. Lysis of Cells or Brain Tissue**

332

#### 333 3.1. Cells Grown as Suspension Cultures

334

335 3.1.1. For HEK-293, lyse the cells on ice by repetitively pipetting up and down using pipette  
336 tips of different widths. First, attach a p1000 tip to a 10 mL pipette, and pipette the cell  
337 suspension up and down every 5 min, for 10 min (three cycles of pipetting). Second, attach a  
338 p200 tip to a p1000 tip and further pipette every 5 min, for 20 min (five cycles of pipetting).

339

340 3.1.2. For HeLa S3, lyse the cells using a French press (see **Table of Materials** for settings).

341

342 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  
343 volume of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analysis.

344

#### 345 3.2. Cells Grown as Adherent Cultures

346

347 3.2.1. Transfer the cells into a 14 mL round-bottom tube whose height has been reduced to  
348 accommodate the sonicator probe (see **Table of Materials** for settings). Sonicate the cells for  
349 ~45 pulses, and confirm cell lysis by sampling a drop of the lysis mix under a microscope.

350

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

354

355 3.2.2. Pipette the cells up and down on ice every 5 min for 20 min (five cycles of pipetting),  
356 using a p200 tip.

357

358 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  
359 of 2x Laemmli buffer, boil for 5 min and store at -20 °C for further analysis.

360

#### 361 **3.3. Brain Tissue**

362

363 3.3.1. Lyse the brain tissue using a tissue blender (see **Table of Materials** for settings).  
364 Alternatively, lyse the tissue using a microtube pestle or an equivalent equipment and pipette  
365 up and down on ice with a 1 mL syringe with an 18 G needle.

366

367 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  
368 of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analysis.

369

### 370 **4. Purification of Tubulin**

371

372 4.1. Lysate Clarification

373

374 4.1.1. Clear the lysate (separating pellet and soluble fraction of the lysis mix) by  
375 centrifugation at  $150,000 \times g$ ,  $4\text{ }^{\circ}\text{C}$ , 30 min. See **Table of Materials** for details about  
376 ultracentrifuge rotors and tubes. For cell extracts, a white floating layer is often formed after  
377 centrifugation. Do not transfer this floating layer along with the supernatant, as it interferes  
378 with tubulin polymerization. Use a syringe of appropriate volume attached to a long 20 G or  
379 21 G needle to gently remove the supernatant without disturbing the floating layer. If the  
380 supernatant is still cloudy, centrifuge at  $5,000 \times g$ ,  $4\text{ }^{\circ}\text{C}$  for 10 min.

381

382 4.1.2. Transfer the supernatant (SN1) to an ultracentrifuge tube and note its volume. For a  
383 10 mL cell pellet, expect a volume of  $\sim 12$  mL for SN1.

384

385 4.1.3. Take  $1/100^{\text{th}}$  volume of SN1 ( $120\text{ }\mu\text{L}$  for 12 mL of SN1), and add the same volume of 2x  
386 Laemmli buffer, boil for 5 min, and store at  $-20\text{ }^{\circ}\text{C}$  for further analysis.

387

388 4.1.4. Resuspend the pellet (P1) in BRB80 (**Table of Materials**) using the same volume as SN1.  
389 Take  $1/100^{\text{th}}$  volume of P1 ( $200\text{ }\mu\text{L}$  for 20 mL of P1), and add the same volume of 2x Laemmli  
390 buffer, boil for 5 min, and store at  $-20\text{ }^{\circ}\text{C}$  for further analysis.

391

392 4.2. **First Polymerization in Low-Molarity Buffer**

393

394 4.2.1. Prepare the polymerization mix by combining 1 volume of SN1 (12 mL),  $1/200^{\text{th}}$  volume  
395 of 0.2 M GTP ( $60\text{ }\mu\text{L}$ ; final concentration 1 mM), and 0.5 volume of pre-heated glycerol (6 mL)  
396 in a screw-cap tube of the appropriate volume.

397

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

400

401 4.2.2. Pipette the mix up and down, gently avoiding the formation of air bubbles and transfer  
402 it to the appropriate ultracentrifuge tubes.

403

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

407

408 4.2.3. Cover the tubes with parafilm, transfer to a water bath set at  $30\text{ }^{\circ}\text{C}$ , and incubate for  
409 20 min.

410

411 4.2.4. Centrifuge the tubes at  $150,000 \times g$ ,  $30\text{ }^{\circ}\text{C}$  for 30 min. Remove the supernatant (SN2),  
412 and keep the pellet of polymerized microtubules (P2).

413

414 NOTE: Microtubule pellet can be snap-frozen and stored at  $-80\text{ }^{\circ}\text{C}$  for up to 1 year.

415

416 4.2.5. Take  $1/200^{\text{th}}$  volume of SN2 ( $90\text{ }\mu\text{L}$  for 18 mL SN2) and add the same volume of 2x  
417 Laemmli buffer, boil for 5 min, and store at  $-20\text{ }^{\circ}\text{C}$  for further analysis.

418

### 419 4.3. First Depolymerization

420

421 4.3.1. Depolymerize microtubules by adding ice-cold BRB80 to the pellet P2, and leave on ice  
422 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>  
423 (600  $\mu$ L) of the volume of the SN1.

424

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

427

428 4.3.2. Resuspend the microtubule pellet gently, avoiding air bubbles, until the solution is  
429 completely homogeneous. Use a p1000 tip for a couple of cycles of pipetting followed by a  
430 p200 tip every 5 min, for 20 min (five cycles of pipetting). This is a crucial step for the success  
431 of the tubulin purification.

432

433 4.3.3. Transfer the solution to appropriate ultracentrifuge tubes, and spin down at 150,000  
434  $\times g$ , 4  $^{\circ}$ C for 20 min. Transfer the SN3 to a new 1.5 mL ultracentrifuge tube. The pellet formed  
435 after this centrifugation step (P3) contains precipitated proteins (microtubule-associated  
436 proteins or MAPs) and non-depolymerized microtubules. The supernatant (SN3) contains  
437 soluble components: depolymerized tubulin dimers and MAPs, which have detached from the  
438 depolymerized microtubules.

439

440 4.3.4. Take 1–4  $\mu$ L of SN3, and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store  
441 at -20  $^{\circ}$ C for further analyses.

442

443 4.3.5. Resuspend the pellet P3 in BRB80 (in the same volume of SN3), take 1–4  $\mu$ L, and add  
444 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20  $^{\circ}$ C.

445

### 446 4.4. Second Polymerization (in High-Molarity Buffer)

447

448 4.4.1. Prepare the polymerization mix by combining 1 volume of SN3 (200  $\mu$ L), 1 volume of  
449 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,  
450 final concentration 1 mM), and 1 volume of pre-heated glycerol (200  $\mu$ L) in a tube of the  
451 appropriate volume.

452

453 4.4.2. Pipette the mix up and down, avoiding the formation of air bubbles, and transfer it to  
454 ultracentrifuge tubes.

455

456 4.4.3. Cover the tubes with parafilm, transfer them to a water bath set at 30  $^{\circ}$ C, and incubate  
457 for 20 min.

458

459 4.4.4. Centrifuge the tubes at 150,000  $\times g$ , 30  $^{\circ}$ C for 30 min. Remove the supernatant (SN4),  
460 and keep the pellet of polymerized microtubules (P4). The pellet P4 contains the polymerized  
461 microtubules, and the supernatant SN4 contains unpolymerized tubulin, MAPs, and other  
462 soluble proteins.

463

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

466

467 4.4.5. Take 1–4 µL and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20  
468 °C for further analyses.

469

470 4.5. Second Depolymerization

471

472 4.5.1. Depolymerize microtubules by adding ice-cold BRB80 to the pellet P4, and leave on ice  
473 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>  
474 (300 µL) of the volume of the SN1.

475

476 4.5.2. Pipette up and down with a p200 tip every 5 min, for 20 min (five cycles of pipetting).

477

478 4.5.3. Transfer the solution to a 1.5 mL ultracentrifuge tube, and spin down at 150,000 × *g*,  
479 4 °C for 20 min. Transfer the SN5 to a new 1.5 mL ultracentrifuge tube. The pellet formed after  
480 this centrifugation step (P5) contains non-depolymerized microtubules. The supernatant  
481 (SN5) contains the soluble tubulin.

482

483 4.5.4. Take 1–4 µL and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20  
484 °C for further analyses.

485

486 4.5.5. Resuspend the pellet P5 in BRB80 (same volume of SN5), take 1–4 µL, and add  
487 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20 °C for further analyses.

488

489 4.6. Third Polymerization (in Low-Molarity Buffer)

490

491 4.6.1. Prepare the polymerization mix: 1 volume of SN5 (120 µL), 1/200<sup>th</sup> volume of 0.2-M  
492 GTP (0.6 µL, final concentration is 1 mM), and 0.5 volume of pre-heated glycerol (60 µL) in a  
493 tube of the appropriate volume.

494

495 4.6.2. Pipette the mix up and down, gently avoiding formation of air bubbles, and transfer it  
496 to the appropriate ultracentrifuge tubes.

497

498 4.6.3. Cover the tubes with parafilm, transfer them to a water bath set at 30 °C, and incubate  
499 for 20 min.

500

501 4.6.4. Centrifuge the tubes at 150,000 × *g*, 30 °C for 30 min. The pellet (P6) contains  
502 polymerized microtubules and the supernatant SN6 contains small amounts of non-  
503 polymerized tubulin.

504

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

506

507 4.6.5. Take 1–4 µL and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20  
508 °C for further analyses.

509

510 4.7. Third Depolymerization

- 511  
512 4.7.1. Depolymerize microtubules by adding ice-cold BRB80 to the pellet P6, and leave on ice  
513 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>  
514 (300  $\mu$ L) of the volume of the SN1.  
515  
516 4.7.2. Pipette up and down with a p200 tip every 5 min, for 20 min (five cycles of pipetting).  
517  
518 4.7.3. Transfer the solution to the appropriate ultracentrifuge tubes, and spin down at  
519 150,000  $\times g$ , 4  $^{\circ}$ C for 20 min. Transfer SN7 to a new 1.5 mL ultracentrifuge tube. The pellet  
520 (P7) contains small amounts of non-depolymerized microtubules. The supernatant (SN7)  
521 contains exclusively depolymerized microtubules (soluble tubulin).  
522  
523 4.7.4. Take 1–4  $\mu$ L and add 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20  
524  $^{\circ}$ C for further analyses.  
525  
526 4.7.5. Resuspend the pellet P7 in BRB80 (same volume of SN7), take 1–4  $\mu$ L, and add  
527 9 volumes of 2x Laemmli buffer, boil for 5 min, and store at -20  $^{\circ}$ C for further analyses.  
528  
529 4.7.6. Quantify the amount of tubulin (see **Representative Results**) and aliquot SN7 into  
530 small volumes, snap-freeze, and store at -80  $^{\circ}$ C.  
531

#### 532 **REPRESENTATIVE RESULTS:**

533 The main goal of this method is to produce high-quality, assembly-competent tubulin in  
534 quantities sufficient to perform repeated in vitro experiments with the purified components.  
535 Microtubules assembled from this tubulin can be used in reconstitution assays based on the  
536 total internal reflection fluorescence (TIRF) microscopy technique with either dynamic or  
537 stable microtubules, in experiments testing microtubule dynamics, interactions with MAPs or  
538 molecular motors, and force generation by the motors<sup>25</sup>. They can also be used in  
539 microtubule-MAP co-pelleting assays and solid-state NMR spectroscopy<sup>28</sup>.  
540

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

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

559  
560 For the quantification of purified tubulin, run the samples along with the known quantities of  
561 bovine serum albumin (BSA, 0.5  $\mu\text{g}$  – 1  $\mu\text{g}$  – 2  $\mu\text{g}$  – 4  $\mu\text{g}$ ) (**Figure 3A**) on SDS-PAGE. Gels are  
562 stained with Coomassie brilliant blue, scanned, and the intensities of BSA and tubulin bands  
563 are measured by quantitative densitometry (**Figure 3B**) as described at  
564 [https://openwetware.org/wiki/Protein\\_Quantification\\_Using\\_ImageJ](https://openwetware.org/wiki/Protein_Quantification_Using_ImageJ). Please note that the  
565 same analysis can be done in Fiji, an upgraded version of ImageJ<sup>33</sup>. Values from the BSA bands  
566 were used to determine the linear regression equation, which was used to calculate the  
567 amount of protein in the tubulin bands. Only tubulin band intensities within the range of the  
568 BSA curve are used to determine tubulin concentration. Based on the calculated tubulin  
569 concentration, aliquots of desired volumes of tubulin are prepared, snap-frozen in liquid  
570 nitrogen, and stored at -80 °C. We usually obtain about ~2 mg of tubulin from four spinner  
571 bottles of HeLa S3 suspension cultures (~15 g of cells), ~250  $\mu\text{g}$  of tubulin from ten 15-cm  
572 diameter dishes (~1.2 g of cells), and ~1 mg of tubulin from 1 g of mouse brain tissue.

573  
574 To confirm the enrichment of a particular tubulin isotype or modification, ~0.1  $\mu\text{g}$  of the  
575 purified tubulin can be immunoblotted using respective antibodies<sup>34,35</sup>. The control tubulin  
576 will vary depending on the tubulin of interest. For tubulin modified in vitro with a modifying  
577 enzyme, use non-treated tubulin as control. For tubulin modified in cellulo by the  
578 overexpression of a modifying enzyme, use tubulin purified from cells that do not express the  
579 enzyme as control (**Figure 4A**). Control tubulin for tubulin purified from knockout-mouse  
580 brains will be tubulin from wild type mice (**Figure 4B**). In all immunoblot analyses, an equal  
581 load of tubulin is verified by using a PTM-independent anti- $\alpha$ -tubulin antibody (12G10).

582  
583 **FIGURE LEGENDS:**

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

595  
596 **Figure 2: Evaluating the success of the tubulin purification.** Samples collected at different  
597 steps of the tubulin purification protocol were run on a 'TUB' sodium dodecylsulfate-  
598 polyacrylamide gel electrophoresis (SDS-PAGE) gel (see protocol for details) and stained with  
599 Coomassie brilliant blue. **(A)** In a successful tubulin purification,  $\alpha$ - and  $\beta$ -tubulins are  
600 progressively enriched throughout the process. After the second polymerization, the  
601 microtubule pellet (P4) is virtually free of contamination from other proteins or microtubule-  
602 associated proteins (MAPs). Note that it is normal to lose some tubulin during the procedure.  
603 **(B)** In an unsuccessful tubulin purification, the final tubulin yield is low, and tubulin remains

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

607

608 **Figure 3: Quantification of the purified tubulin using Coomassie-stained sodium**  
609 **dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and densitometry. (A)**

610 Coomassie-stained SDS-PAGE gel with known quantities of bovine serum albumin (BSA; 0.5,  
611 1, 2 and 4  $\mu\text{g}$ , gray gradient line) and different volumes (0.5 and 1  $\mu\text{L}$ , light and dark colors,  
612 respectively) of purified tubulin. In the example shown, tyrosinated tubulin (HeLa S3 tubulin,  
613 light and dark orange) and detyrosinated tubulin (HeLa S3 tubulin treated with  
614 carboxypeptidase A, light and dark blue) were loaded on the gel. **(B)** BSA bands from (A) were  
615 quantified using ImageJ (in arbitrary units, AU) and plotted against the amount of protein  
616 loaded (gray to black points). Those points were used to calculate the linear regression line  
617 (the gray gradient line) and equation, which were used to calculate the amounts of protein in  
618 the tubulin samples (light and dark orange and blue points) loaded on the gel. This facilitated  
619 the calculation of the concentration of the tubulin samples. Note that the points that lie  
620 beyond the BSA standard curve should not be used to determine concentration (dark orange  
621 and blue points).

622

623 **Figure 4: Immunoblot analysis of purified tubulin with different PTMs. (A)** Tubulins purified  
624 from HEK-293 cells: wild type, or cells overexpressing TLL5 or TLL7 were analyzed for the  
625 specific enrichment of polyglutamylation using the GT335 antibody. While TLL5  
626 overexpression increases polyglutamylation on  $\alpha$ - and  $\beta$ -tubulin, TLL7 overexpression  
627 specifically enriches  $\beta$ -tubulin glutamylation. **(B)** Tubulin purified from brain tissues of wild  
628 type and *ttl1*<sup>-/-</sup> mice were analyzed for patterns of glutamylation. Note the strong reduction  
629 of polyglutamylation of tubulin from *ttl1*<sup>-/-</sup> mice, which lack the major brain glutamylase  
630 TLL1<sup>36</sup>. 'TUB' gels were used to separate  $\alpha$ - and  $\beta$ -tubulin. An equal amount of tubulin load  
631 was confirmed by 12G10, an anti- $\alpha$ -tubulin antibody.

632

### 633 **DISCUSSION:**

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

647

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

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

656  
657 Finally, our protocol facilitates the purification of tubulin from single mouse brains. As an  
658 increasing number of mouse models of tubulin mutations and modifications are being  
659 generated, this protocol allows direct analysis of the properties and interactions of  
660 microtubules with altered tubulin isotype composition<sup>38-40</sup> or tubulin PTMs<sup>31,41</sup>.

661  
662 The approach is based on cycles of polymerization and depolymerization. Thus, specific  
663 tubulin isoforms or tubulin with particular PTMs that affect the assembly and disassembly  
664 properties of microtubules could result in a disproportionate loss or reduction of such tubulin  
665 forms during the purification process. Nevertheless, we have shown that major tubulin PTMs,  
666 such as acetylation, detyrosination, glutamylation, and glycylation, are retained on the  
667 microtubules throughout the tubulin purification process<sup>24</sup>. However, it should be noted that  
668 for quantitative analyses of the tubulin composition in cells or tissues, the TOG-column-based  
669 tubulin purification approach is more appropriate as it would allow an unbiased,  
670 polymerization-independent tubulin purification<sup>18</sup>. Despite its limitation, our protocol offers  
671 a great advantage in generating large amounts of high-quality tubulin that can be used in  
672 meticulous in vitro reconstitution experiments. In particular, it facilitates the use of PTM-rich  
673 brain tubulin in routine experiments.

674

#### 675 **DISCLOSURES:**

676 The authors have nothing to disclose.

677

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688

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693

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**A**

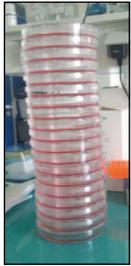
**Tubulin source/  
culture method**



HeLa S3 cell  
suspension culture



HEK-293 cell  
suspension culture

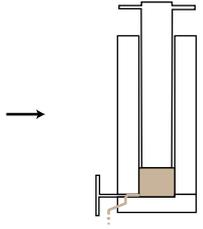


Adherent culture

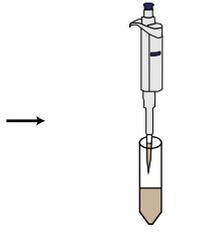


Mouse brain

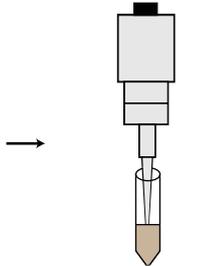
**Lysis method**



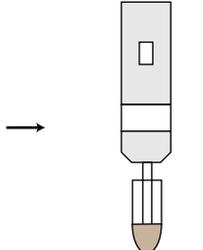
French press



Repeated pipetting



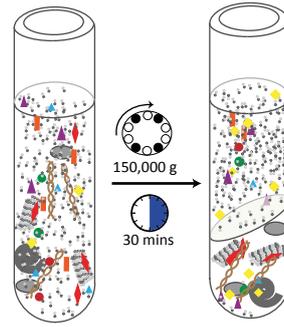
Sonication



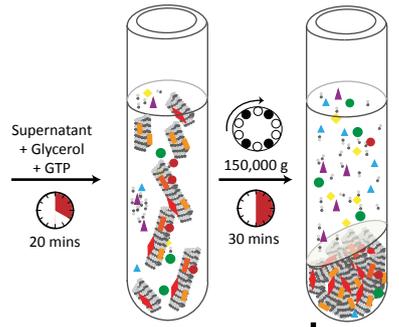
Homogenisation

**B**

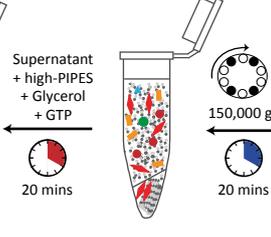
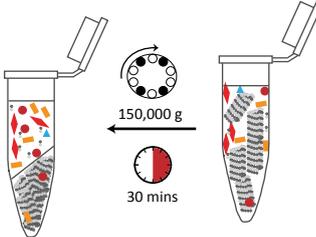
**Lysate clarification**



**1st polymerization**

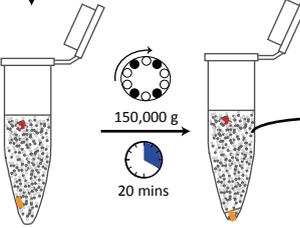


**2nd polymerization (high-PIPES)**



Pellet  
+ BRB80  
20 mins

**2nd depoly-  
merization**



pure, polymerization-  
competent tubulin

