Chapter 3

Purification and Fluorescent Labeling of Tubulin from *Xenopus laevis* Egg Extracts

Aaron C. Groen and Timothy J. Mitchison

Abstract

For many years, microtubule research has depended on tubulin purified from cow and pig brains, which may not be ideal for experiments using proteins or extracts from non-brain tissues and cold-blooded organisms. Here, we describe a method to purify functional tubulin from the eggs of the frog, *Xenopus laevis*. This tubulin has many benefits for the study of microtubules and microtubule based structures assembled in vitro at room temperature. Frog tubulin lacks many of the highly stabilizing posttranslational modifications present in pig brain-derived tubulin, and polymerizes efficiently at room temperature. In addition, fluorescently labeled frog egg tubulin incorporates into meiotic spindles assembled in egg extract more efficiently than brain tubulin, and is thus superior as a probe for Xenopus egg extract experiments. Frog egg tubulin will provide excellent opportunities to identify active nucleation complexes and revisit microtubule polymerization dynamics in vitro.

Key words Microtubules, Tubulin, Cell-free cytoplasm, *Xenopus laevis*

1 Introduction

Undiluted, cell-free cytoplasm prepared from meiotic *Xenopus laevis* eggs, commonly called "CSF extract" or "crude egg extract," reconstitutes many biological phenomena, including cell cycle progression, meiotic spindle assembly, and cytokinesis signaling [1–3]. This cytoplasm contains over 100 mg/ml of protein, including abundant ribosomes and organelles, and it is in a normal physiological state, making it a good source for biochemical purifications [4]. Here we describe a protocol for purifying frog egg tubulin from the cytoplasm isolated from the eggs of *Xenopus laevis*. The resulting "frog tubulin" has several advantages over bovine or porcine brain tubulin; it polymerizes at room temperature and does not contain posttranslational modifications that could interfere with in vitro polymerization dynamics [5, 6]. Labeled frog egg tubulin incorporates more efficiently than labeled bovine brain tubulin into meiotic spindles assembled in frog egg extract (Fig. 5).

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We suspect this is because the frog tubulin probe polymerizes more efficiently at room temperature, but posttranslational modification might also play a role. With such a relatively simple method for purification of native, unmodified tubulin, we anticipate future advances in understanding the biochemistry of microtubules.

Our purification method is based on conventional polymerization-depolymerization cycles using high salt (PIPES) to elute the contaminating microtubule associated proteins [7]. An alternative purification approach, based on affinity to a CH-TOG domain, was recently described [8]. The affinity approach is ideal for purification of tubulin from lysates where it is too dilute to polymerize efficiently. However, tubulin polymerizes efficiently in frog egg extract under the conditions we describe, which makes purification by polymerization easy and efficient.

In the protocol below, we provide a detailed description of how to prepare a conventional "CSF extract," using methods similar to those described previously [2]. We then describe our tubulin purification technique using the concentrated extract, and a method to label frog tubulin on lysine residues with fluorescent probes using NHS-ester chemistry. Depending on need, we typically label a portion of each frog egg tubulin preparation with several different dyes.

2 Materials

2.1 General Equipment

18G 1½ gauge needles.
Syringes (Model 1001 LT-Syringe 1 ml Luer-tip).
50 ml Falcon tube.
Amicon Ultra-concentrator 50 kDa cutoff (Amicon Ultra-15 Centrifugal Filter Unit Ultracel-50 membrane).
SP-Sepharose beads.

2.2 Reagents

PMSG (gonadotropin from pregnant mare serum).
HCG (chorionic gonadotropin human).
Cysteine (l-cysteine).
Leupeptin.
Pepstatin.
Chymostatin.
Cytochalasin D.
DMSO.

2.3 Extract Buffers

MMR (Marc's Modified Ringer's; 1×: 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.8, 0.1 mM EDTA).
Dejelly (1× XB salts; 2 % l-cysteine) *400 ml.*
XB Salts (20×: 2 M KCl, 20 mM $MgCl_2$, 1 mM $CaCl_2$).

CSF-XB (1× XB salts, 10 mM K-HEPES, pH 7.7, 5 mM EGTA, pH 8, 50 mM sucrose) *2 l.*

CSF-XB plus protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin) *200 ml.*

2.4 Quick Buffer Recipe for Extract Buffers

2 l MMR: (80 ml 25× MMR).

800 ml Dejelly: (40 ml 20× XB salts, 8 g L cysteine, 3.6 ml 10 N KOH).

2 l CSF-XB: (100 ml 20× XB salts, 20 ml 0.5 M EGTA, pH 8.0, 20 ml 1 M K-HEPES, pH 7.7, 2 ml 1 M $MgCl_2$, 20 ml 2 M sucrose, 220 μl 10 N KOH).

2.5 Tubulin Prep Buffers

Dilution buffer (30 % glycerol in 1× BRB-80) *300 ml.*

Cushion buffer (40 % glycerol in 1× BRB-80) *100 ml.*

Depolymerization buffer (50 mM K-MES, 1 mM $CaCl_2$, pH 6.6) *50 ml.*

PIPES buffer (1 M K-PIPES, 10 mM $MgCl_2$, 20 mM EGTS, pH 6.9) *50 ml.*

BRB-80 (80 mM K-PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8) *50 ml.*

High pH cushion (0.1 K-HEPES, pH 8.6, 1 mM $MgCl_2$, 1 mM EGTA, 60 % glycerol).

Low pH cushion (1× BRB-80 in 60 % glycerol).

Labeling buffer (0.1 M K-HEPES, 1 mM $MgCl_2$, 1 mM EGTA, 40 % glycerol).

Quench (2× BRB-80, 100 mM K-glutamate, 40 % glycerol).

3 Methods

3.1 Preparation of Crude Xenopus laevis Egg Extracts Without Intact Actin

Maintain frogs and buffers and perform the entire preparation at 16 °C. Ten frogs should be sufficient to yield ~5 mg of frog egg tubulin from ~20 ml of crude extract generated (*see* Fig. 1). Prep time: ~1 h. (*See* Fig. 2 for flowchart of protocol.)

1. Inject each *Xenopus laevis* frog with 100 international units (IU) of pregnant mare gonadotropin (PMSG) at least 2 days before extract prep. (PMSG is diluted in sterilized ddH_2O).
2. One day prior to the preparation inject frogs with 500 IU of human chorionic gonadotropin (HCG) and placed in a single container containing 2 l MMR. (HCG is diluted in sterilized ddH_2O.)
3. Collect and pool eggs 24 h after HCG injection.
4. Remove poor quality eggs (activated or unequal animal and vegetal poles) throughout collection and washing procedure.
5. Eggs are washed 3× in MMR (~300 ml per wash) then 3× in dejelly solution (~200 ml per wash). For the last wash, incu-

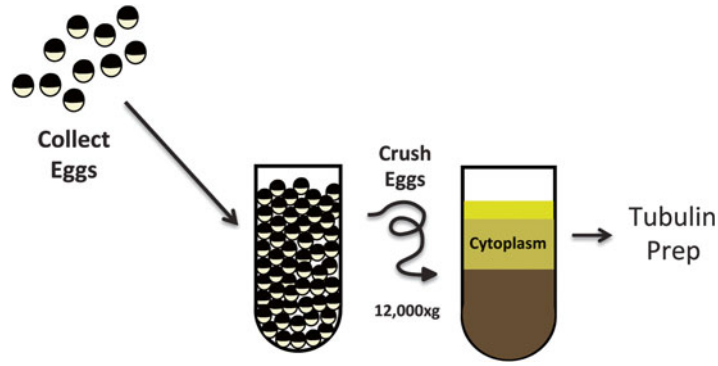

Fig. 1 *Xenopus laevis* egg extract preparation

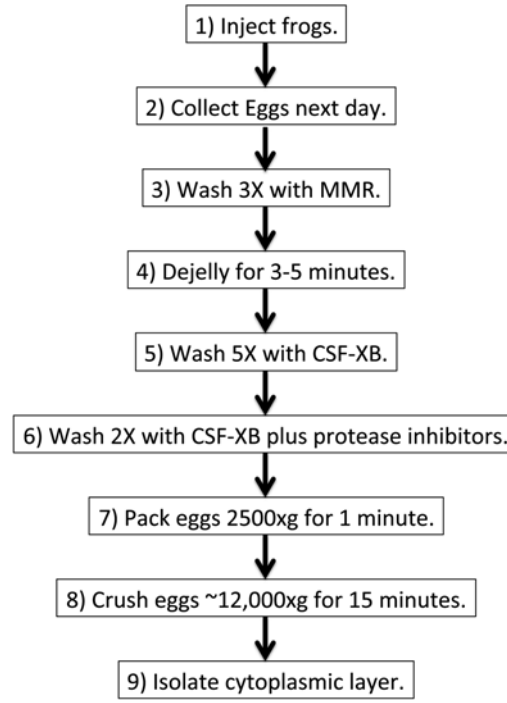

Fig. 2 Flow diagram of extract protocol

bate eggs in dejelly solution for 3–5 min or until eggs are tightly packed at the bottom of the dish. Gently swirling eggs helps to remove jelly and decreases incubation time.

6. Wash dejellied eggs 5× in CSF-XB (~400 ml per wash). Gently swirl eggs during each wash to prevent lysis.
7. Wash eggs 2× in CSF-XB plus protease inhibitors (~100 ml per wash).

8. Using a transfer pipet with the tip cut off, transfer eggs to Beckman Ultra-Clear 13×51 mm tubes containing 1 ml of CSF-XB plus protease inhibitors and cytochalasin D (10 μg/ml).
9. Remove extra buffer by packing eggs via centrifugation (2500×*g* for 1 min). Remove buffer using vacuum aspirator.
10. Eggs are crushed at ~12,000×*g* in a swinging bucket rotor for 15 min (SW50.1 at 10,000 rpm or JS13.1 at 9700 rpm). The rotor is kept at room temperature, while the centrifuge is maintained at 16 °C.
11. Extract layer (straw colored layer) is isolated with a 18G 1½ gauge needle attached to a 1 ml syringe (*see* **Note 1**; Fig. 2). Collect the dense straw colored layer above the black egg shell layer by rotating the bevel of needle in this area as you remove the extract with the syringe.)
12. Pool extract from each tube and add 1 μg/ml LPC, 1 μg/ml cytochalasin D, and sucrose to 50 mM.
13. Extract is now ready for tubulin purification (*see* **Note 2**).

3.2 Purification of Xenopus laevis Tubulin

Tubulin polymerization steps are performed at room temperature (18–24 °C), whereas depolymerization steps occur at 0–4 °C. Prep time: ~5–6 h (Modified from Refs. [2, 7, 9]; *See* Fig. 3 for flowchart of protocol.)

1. Place crude extract (this prep is for 20 ml) in a 50 ml Falcon tube and add 10 % DMSO (v/v). Cap Falcon tube and slowly invert three to four times. Incubate for 30 min at room temperature. This induces efficient but reversible polymerization of the egg tubulin.
2. Aliquot 5 ml of polymerization reaction to new Falcon tubes and dilute tenfold in dilution buffer resulting in 50 ml extract solution. Glycerol in the dilution buffer prevents microtubule depolymerization.
3. Layer each 50 ml reaction over 2 ml of cushion buffer and spin for 30 min at 27,000×*g* at 23 °C. (We use Beckman JA-17 for this spin.). The cushion prevents pelleting of the abundant organelles in the extract.
4. Keep tubes at room temperature. Remove 50 ml above the cushion layer and wash cushion interface 3× with 1× BRB-80.
5. Remove cushion carefully without disturbing the pellet.
6. Resuspend each pellet in 0.5–1 ml cold depolymerization buffer (Pellet 1; Fig. 4a). The calcium in the buffer greatly enhances depolymerization compared to cold temperature alone.
7. Place on ice for 20 min and triturate every 2 min.

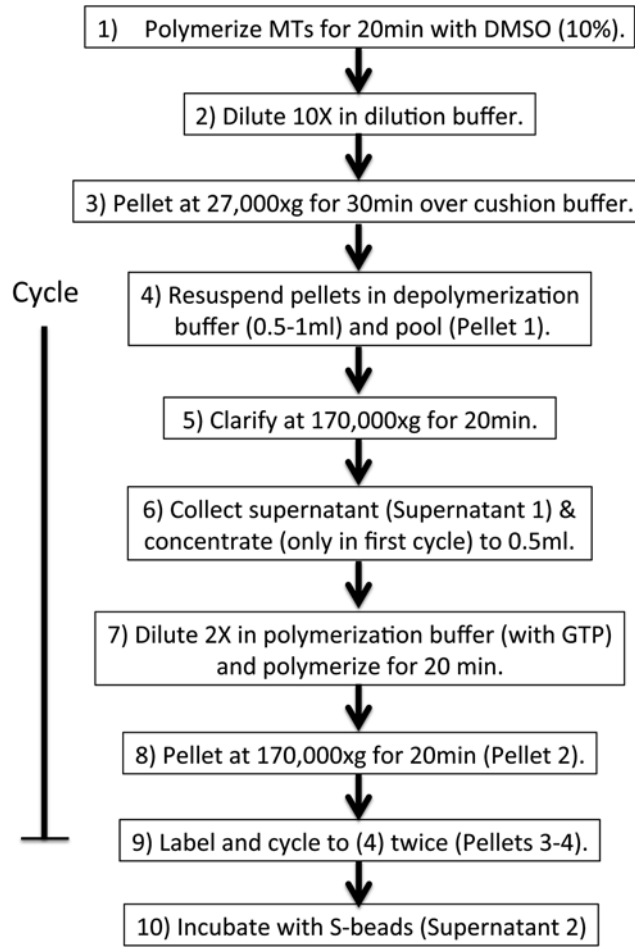

Fig. 3 Flow diagram of the purification of tubulin from *Xenopus laevis* egg extracts

8. Pool all pellets. Spin at 170,000 × *g* for 20 min at 4 °C. (We use an MLA-80.). This spin pellets ribosomes, organelles, keratin, and actin and intermediate filaments that co-pelleted with the microtubules.
9. Collect supernatant (Supernatant 1; Fig. 4a) and concentrate to 0.5 ml in an Amicon Ultra-concentrator 50 kDa cutoff. The final protein concentration should be in the range of 30–50 mg/ml. Concentrating at this step makes the subsequent polymerization more efficient.
10. Dilute 2× in polymerization buffer and add ATP (1 mM), GTP (5 mM), DMSO (10 %), and glycerol (10 %). Incubate for 20 min at room temperature to polymerize.

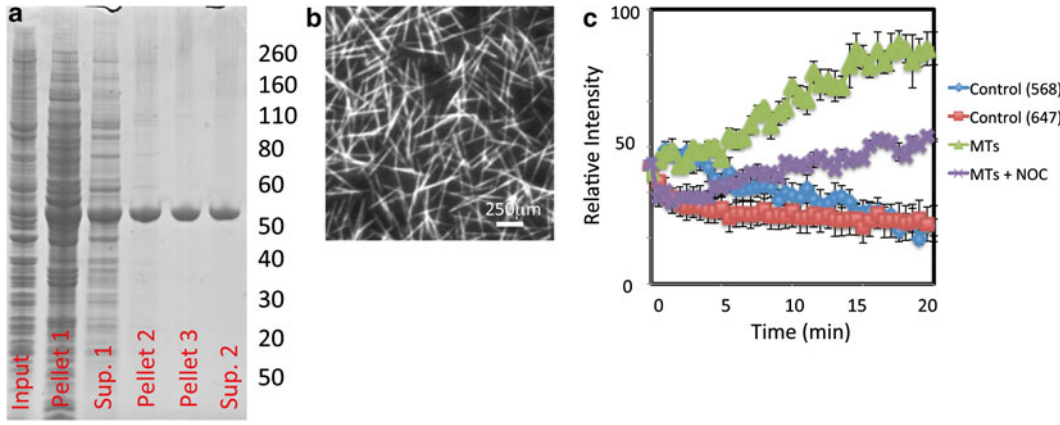

Fig. 4 Purification of tubulin from cytoplasm. (**a**) Coomassie stained SDS-PAGE showing isolated fractions during tubulin prep. (**b**) 60× TIRF image of *Xenopus laevis* tubulin polymerized in vitro on plain glass. Scale Bar = 250 mm. (**c**) Analysis of microtubule polymerization using FRET between A568/A647 on a fluorescence spectrophotometer. Control (568) and Control (647) are in vitro microtubule polymerization reactions containing frog tubulin labeled with either Alexa 568 or Alexa 647. MTs and MTs + NOC (+10 μm nocodazole) are microtubule polymerization reactions containing frog tubulin with tubulin labeled with both A568 and A647. Relative intensity of fluorescence observed with 578Ex/665Em. $N = 3$. Error bars = Standard error

11. Spin at 170,000 × *g* for 20 min at 23 °C over a 3 ml high-pH cushion to make fluorochrome-labeled tubulin, or use a pH 6.8 cushion buffer to make unlabeled tubulin; Pellet 2. Elevated pH is required for efficient microtubule labeling using NHS-ester chemistry.
12. Keep at room temperature. Remove 1 ml above cushion layer and wash interface 3× with labeling buffer. For unlabeled tubulin wash interface with 1× BRB-80; for labeled tubulin skip to Subheading 3.3).
13. Resuspend the pellet in 200 μl of cold depolymerization buffer. Incubate on ice for 20 min and triturate every 2 min.
14. Spin at 170,000 × *g* for 20 min at 4 °C. (We use TLA-100.1.)
15. Collect supernatant and dilute 2× in polymerization buffer then add ATP (1 mM), GTP (5 mM), DMSO (10 %), and glycerol (10 %). Incubate for 20 min at room temperature to polymerize.
16. Place polymerized microtubules over 1 ml of cushion buffer and spin at 170,000 × *g* for 20 min at 23 °C. (Pellet 3; We use TLA-100.3.)
17. Resuspend in 200–300 μl cold 1× BRB-80 in a 1.5 ml eppendorf tube. Incubate on ice for 20 min.
18. Wash 100 μl of 50 % slurry of SP-Sepharose Fast Flow beads with 1× BRB-80 three times. Completely dry beads by picking

a small hole near the bottom of the eppendorf tube with a 27-gauge needle and spinning 2000 × *g* for 2 min into a 2 ml eppendorf (to collect the excess BRB-80) using a tabletop microcentrifuge. SP-Sepharose efficiently binds and removes the microtubule binding proteins and motors that co-purify with microtubules. It serves the same purpose as phosophocellulose in a conventional brain tubulin purification protocol. It removes some of the residual microtubule associated proteins present after the high salt wash.

19. Add dry beads with a spatula to the depolymerized tubulin and incubate for 1 h on ice. Flick tube every 10 min to prevent beads from settling to the bottom of the tube.
20. Spin mixture at 4 °C for 1 min at 10,000 × *g* and collect as much of the supernatant as possible without collecting beads.
21. To collect remaining tubulin from the beads, pick a small hole near the bottom of the eppendorf tube with a 27-gauge needle and spin into a 2 ml eppendorf using a tabletop microcentrifuge. Pool both samples.
22. Spin tubulin at 170,000 × *g* for 20 min at 4 °C for a final clarifying spin (Supernatant 2).
23. Determine tubulin concentration and stoichiometry of labeling (*see* **Note 3**).
24. Flash-freeze in liquid nitrogen in 1–10 μl aliquots and store at −80 °C. In our experience tubulin is stable for at least 1 year at −80 °C although warming to higher temperatures during storage can inactivate it.

3.3 Fluorescent Labeling of Frog Egg Tubulin

1. Resuspend pellet in 0.4 ml of labeling buffer and add the fluorescent probe NHS ester to 1 mM final concentration, in two steps separated by 15 min. We use the Alexa conjugated NHS esters which are stored at −20 °C as 50 mM DMSO stock solutions. Other NHS esters, including long-chain biotin derivatives, can also be used. Incubate for 30 min at room temperature and triturate every 2 min for the lysine labeling reaction to proceed.
2. Add 100 μl of quench buffer. This inactivates remaining NHS esters, which protects the tubulin from further lysine modification after depolymerization.
3. Spin at 170,000 × *g* for 20 min at 23 °C over a 3 ml low-pH cushion. There should be a small, intensely colored pellet, and a much large volume of dye solution over the cushion.
4. Keep at room temperature. Remove 0.5 ml above cushion and wash interface 3× with BRB-80 to remove as much unbound dye as possible. Proceed to **step 13** in Subheading 3.2.

3.4 Confirmation of Polymerization Activity of Purified Frog Tubulin

3.4.1 Microscopy: Confirming Microtubule Polymerization Activity

1. Make tubulin polymerization reaction (1× BRB80, 1 mM GTP, 1 mM DTT, tubulin) above the critical concentration for polymerization (~1 mg/ml) and incubate on ice for 5 min (inspired by Refs. [10, 11].
2. Incubate at 23 °C for 20 min. Do not dilute microtubules below the critical concentration unless Taxol is added in a equimolar concentration to tubulin [12].
3. Directly image microtubules using light microscopy—wide-field, TIRF, DIC microscopy (Fig. 4b). Microtubules should appear as single long filaments.

3.4.2 Fluorescence Spectrophotometry: Measuring Polymerization Kinetics

1. Make two different tubulin polymerization reactions (1× BRB80, 1 mM GTP, 1 mM DTT, tubulin) labeled with different fluorophores capable of FRET (i.e., A647 and A568). The stoichiometry of label must be close to 1 to measure FRET and the polymerization reaction must contain an equal concentration of each labeled tubulin.
2. Combine both tubulin polymerization reactions and measure on a fluorescence spectrophotometer.
3. Polymerization kinetics should include a brief lag phase followed by an increase in FRET signal, reaching a maximum in approximately 20 min. Any deviations suggest tubulin purification was not successful.

3.4.3 Physiological Confirmation of Microtubule Polymerization Activity

1. Assemble meiotic Xenopus egg extract spindles as previously described [2].
2. Add directly labeled frog egg tubulin to meiotic spindles (~50 μg/ml for general viewing or 10 ng/μl for single molecule speckle microscopy).
3. Image using light microcopy (wide-field or spinning disc confocal microscopy; Fig. 5). Note that frog egg tubulin incorporates (relative to background) to a greater extent than bovine tubulin (Fig. 5).

4 Notes

1. The straw colored layer is fragmented into many parts. Collect everything, including the denser yellow layer right above the black layer.
2. For larger tubulin preps, extract can be frozen for storage. Freeze in liquid nitrogen in 1 ml aliquots and store in −80 °C.

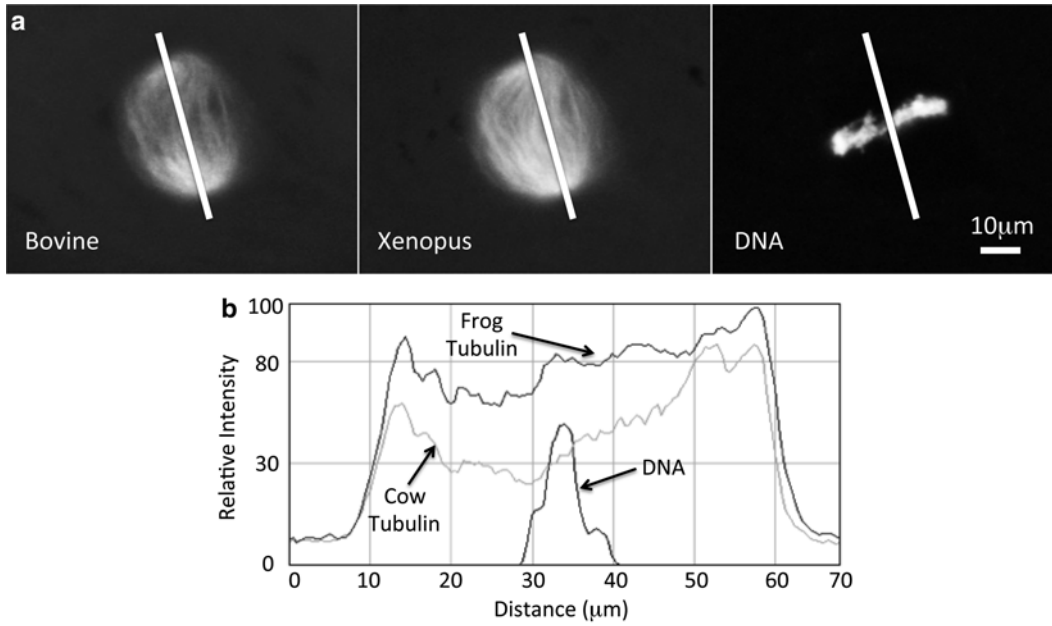

Fig. 5 Frog tubulin incorporation in meiotic extract spindles is greater than bovine tubulin. (**a**) Meiotic extract spindles incorporating A568 bovine tubulin (*left*), A647 Xenopus tubulin (*middle*), and DNA (DAPI). (**b**) Intensity profile of line shown in (**a**). Note: more Xenopus tubulin (relative to the background) incorporates into the microtubules within the spindle than bovine tubulin

3. To determine the concentration of tubulin and stoichiometry of label: (a) *Stoichiometry of dye* = (*Concentration of dye*)/(*Concentration of tubulin*). (b) *Concentration of Tubulin* = [Absorbance (at 280 nm) − (Absorbance at excitation wavelength of dye) × (Absorbance of dye at 280 as fraction of peak absorbance or CF)] × Dilution Factor/Extinction Coefficient of tubulin (115,000). (c) *Concentration of fluorophore* = Absorbance (at excitation wavelength of dye) × dilution factor/extinction coefficient of dye.

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References

1. Lustig KD, Stukenberg PT, McGarry TJ et al (1997) Small pool expression screening: identification of genes involved in cell cycle control, apoptosis, and early development. Methods Enzymol 283:83–99
2. Desai A, Murray A, Mitchison TJ, Walczak CE (1999) The use of Xenopus egg extracts to study mitotic spindle assembly and function in vitro. Methods Cell Biol 61:385–412
3. Nguyen PA, Groen AC, Loose M et al (2014) Spatial organization of cytokinesis signaling reconstituted in a cell-free system. Science 346:244–247. doi:10.1126/science.1256773
4. Groen AC, Ngyuen PA, Field CM et al (2014) Glycogen-supplemented mitotic cytosol for analyzing Xenopus egg microtubule organization. Methods Enzymol 540:417–433. doi:10.1016/B978-0-12-397924-7.00023-6
5. Song Y, Brady ST (2014) Post-translational modifications of tubulin: pathways to functional diversity of microtubules. Trends Cell Biol. doi:10.1016/j.tcb.2014.10.004
6. Podolski M, Mahamdeh M, Howard J (2014) Stu2, the budding yeast XMAP215/Dis1 homolog, promotes assembly of yeast microtubules by increasing growth rate and decreasing catastrophe frequency. J Biol Chem 289:28087–28093. doi:10.1074/jbc.M114.584300
7. Castoldi M, Popov AV (2003) Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. Protein Expr Purif 32:83–88. doi:10.1016/S1046-5928(03)00218-3
8. Widlund PO, Podolski M, Reber S et al (2012) One-step purification of assembly-competent tubulin from diverse eukaryotic sources. Mol Biol Cell 23:4393–4401. doi:10.1091/mbc.E12-06-0444
9. Hyman A, Drechsel D, Kellogg D et al (1991) Preparation of modified tubulins. Methods Enzymol 196:478–485
10. Correia JJ, Wilson L (2013) Microtubules, in vitro. Academic Press, Amsterdam
11. Cammack R, Atwood T, Campbell P et al (2006) Oxford dictionary of biochemistry and molecular biology, 2nd edn. Oxford University Press, Oxford
12. Parness J, Horwitz SB (1981) Taxol binds to polymerized tubulin in vitro. J Cell Biol 91:479–487