Purification of tubulins from porcine brains

Takashi Hotta, Hashimoto lab. NAIST, Aug. 2013

Purified tubulin is a powerful tool for a variety of in vitro microtubule (MT) experiments. It would be perfect if you could isolate and purify tubulins from your desired (plant) materials, however in general, it is not easy to prepare pure tubulin efficiently from those materials. Alternatively, tubulin has been purified from animal brains as they contain high concentration of tubulin. Here, we introduce a traditional method to purify tubulin from porcine (pig) brains. This method consists of three parts: 1, Isolation of tubulin with MT associated proteins (MAPs) based on assembly-disassembly cycles; 2, Further purification of tubulin using ion-exchange column chromatography; 3, Selection of assembly-competent tubulin and fluorescent labeling (optional). Whole process takes about three days, but it should not be so technically demanding. This protocol is based on Shelanski's protocol with some modifications, which is recommended to read as well. It is possible to modify this protocol and make it more simple and quick as some researchers actually do the purification within a day.

1. Isolation of tubulin with MT associated proteins (MAPs) based on assembly-disassembly cycles (1st day)

1.1 Outline

Porcine brain is the best source of tubulin at the present time. Due to the unavailability of historically popular bovine brains after the BSE problem, porcine brain is currently the most common source of vertebrate tubulin. Porcine brains can be purchased at local slaughterhouses at about 500 JPY per brain. Remember to inform your coworkers in advance that you will be handling porcine brains in the lab as they reek of fresh meet. The reversible property of assembly-disassembly of tubulin (and MT) combined with series of centrifugations enables the separation of tubulin from other unrelated proteins. In brief, MT assembly is induced by the addition of GTP and glycerol to the protein extracts prepared from the brain homogenates. MTs can be precipitated by centrifugation and separated from other proteins that remain in the supernatant. After MTs are disassembled by cold treatment on ice, tubulin is recovered in the supernatant after centrifugation. Through two rounds of this assembly-disassembly cycles, tubulin is partially purified. As the tubulin fraction still contains MAPs such as MT binding proteins or motor proteins, they have to be removed at a subsequent column-chromatography (See 2).

1.2 Preparations

- Four porcine brains freshly prepared in the morning of the procedure
- Wash buffer, 1L, 50 mM PIPES, 0.5 mM $MgCl_2$, 1 mM EGTA, pH 6.9
- Homogenization buffer, 1L, 100 mM PIPES, 1 mM $MgCl_2$, 2 mM EGTA, pH 6.9

Keep these buffers on ice

- A conventional kitchen blender (settled in a cold room)
- Clean forceps

1.3 Procedures

Preparation of the brain extracts

- Place the brains on ice and quickly remove the meninges (brain membranes) and the blood vessels with clean forceps (Fig. 1A). Avoid damaging the brain surface.
- Rinse the brains with total 1L of wash buffer and measure the weight. Usually 4 brains weigh about 380 g.
- Add roughly the same volume of ice-cold homogenization buffer (350 ml) and transfer the brain-buffer mixture to the kitchen blender in the cold room (Fig. 1B).
- Grind the brains. Pay attention not to increase the temperature of the homogenate.

First assembly-disassembly cycle

- Centrifuge the homogenate at 100,000 x g for 30 min at 4ºC (Fig. 1C).
- Collect the supernatant (bright red color) in a 500 ml measuring cylinder. It should be around 400 ml.
- Add 1/10 volume of glycerol (40 ml, if the supernatant is 400 ml) and mix them by inverting the cylinder sealed with parafilm several times.
- Transfer the solution to a 1L conical flask and add final 0.5 mM of GTP (We add the original powder of GTP). Incubate the solution at 33-37ºC on a water bath for 1 hour to induce MT assembly. You can tell the MT polymerization by the turbidity of the solution (Fig. 1D-F).
- In the meantime, warm up the centrifugation rotor for the upcoming centrifugation step.
- Centrifuge the turbid solution at 100,000 x g for 45 min at 33-37ºC and discard the supernatants. You can see the MT pellets with slight pinky color (Fig. 1G).
- Add ice-cold homogenize buffer (total 250 ml) onto the pellets and transfer them into a hand-held Teflon-glass homogenizer. Break the MT pellets gently with several strokes (Fig. 1H).
- Leave the solution for 1 hour on ice to allow sufficient disassembly of the MTs.
- In the meantime, refrigerate the centrifugation rotor to prepare for the next step.
- Centrifuge the solution at 100,000 x g for 30 min at 4ºC and collect the supernatants as tubulin fraction (Fig. 1I).

Second assembly-disassembly cycle

- Repeat the above cycle one more time. The tubulin solution should be now 250-300 ml. So, add 1/10 volume of glycerol (25-30 ml) and final 0.5 mM of GTP to initiate the MT assembly process for 1 hour at 33-37ºC (Fig. 1J, K).
- After the centrifuge at 100,000 x g for 45 min at 33-37ºC (Fig. 1L), the pellets are resuspended in 200 ml of ice-cold homogenization buffer.
- Solution of tubulin containing partially disassembled MTs is aliquoted in four 50 ml disposable tubes and frozen with liquid nitrogen (Fig. 1M).
- Tubulin solution can be stored at -80ºC for several years.

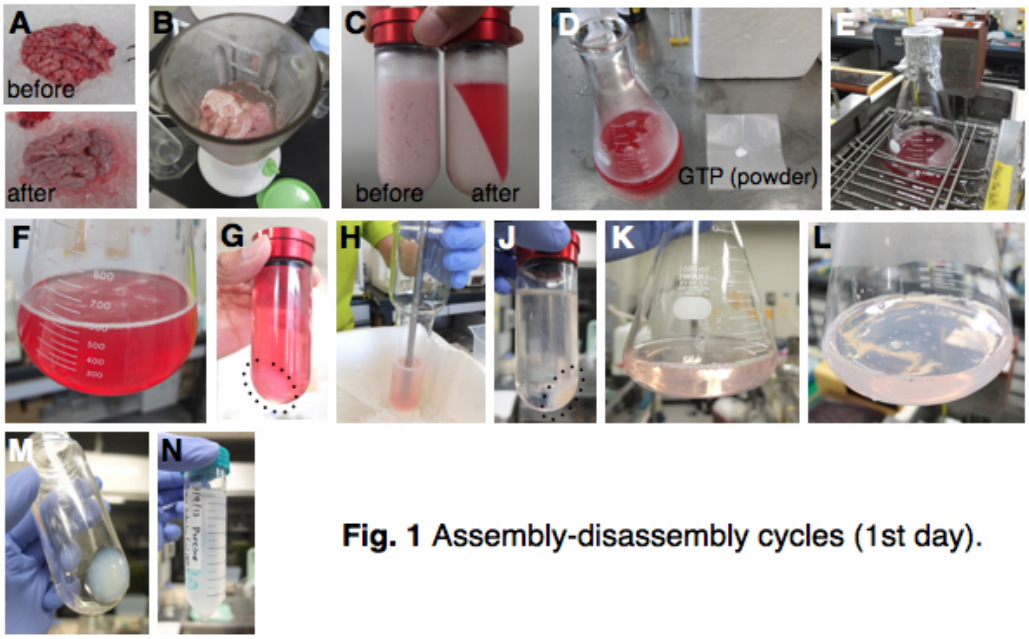

Fig. 1 Assembly-disassembly cycles (1st day).

2. Further purification of tubulin using ion-exchange column chromatography (2nd day)

2.1 Outline

Partially purified tubulin still contains some contaminants, most of which are thought to be tubulin (or MT) associating proteins. Separation of tubulin can be achieved by ion-exchange column chromatography. In this protocol, we adopted a traditional anion exchanger, diethylaminoethyl (DEAE)-Sephacel (GE healthcare). As most of the contaminants bind to the resin more weakly than tubulin does, low concentration of sodium chloride solution can efficiently remove those contaminants prior to the elution of tubulin with higher concentration of salt solution. Eluted tubulin is subjected to overnight dialysis for desalting. The entire process has to be carried out in the cold room. A peristaltic pump and an automatic fraction collector are needed for the chromatography, but gravity flow also works and the manual sample collection should be feasible in case you do not have those special equipments.

We usually apply only half of the tubulin stocks prepared on the first day to this column chromatography at one time. This means that tubulin from two porcine brains is purified in the following experimental scale.

2.2 Preparations

- DEAE-Sephacel suspension
- 0.2 M NaOH, 300 ml
- Equilibration buffer, 600 ml, 10 mM PIPES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.9
- Wash buffer, 400 ml, 10 mM PIPES, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.3 M NaCl, pH 6.9
- Elution buffer, 100 ml, 10 mM PIPES, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.8 M NaCl, pH 6.9

- Dialysis buffer, 100 mM PIPES, 1 mM EGTA, 0.5 mM $MgCl_2$, 80 mM NaCl, 33% glycerol, 0.5 mM DTT, 0.1 mM GTP (optional), pH 6.9
All buffers to be chilled on ice.
- A grass cylinder column
- Peristaltic pump and fraction collector
- Dialysis membrane

2.3 Procedures

Column packing

- Resuspend the DEAE-Sephacel solution and pour it into a 50 ml glass cylinder column set on a 200 ml measuring cylinder. Leave the resins precipitated and keep adding the suspension until the bed volume reaches 50 ml (Fig. 2A).
- Pour out the resin on a buchner funnel covered with a filter paper. Draw a gentle vacuum and rinse the resin with 1L of distilled water (DW) (Fig. 2B).
- Pour 300 ml of 0.2 M NaOH solution on the resin without vacuum. Once all the solution sinks in, rinse it with 1L of DW with gentle vacuum.
- Equilibrate the resin with 500 ml of equilibration buffer and transfer the mushy resin into the glass cylinder column.

Sample loading

- Take out two tubes of the frozen tubulin stock from -80ºC freezer and thaw them.
- To remove junks of MTs or denatured proteins, centrifuge the samples at 100,000 x g for 30 min at 4ºC
- Load the supernatant onto the DEAE-sephacel column (Fig. 2C). Gravity flow or pump slowly.

Wash

- Load 100 ml of the leftover of the equilibration buffer. Gravity flow or pump slowly.
- Load the wash buffer. Pump slowly. After about 50 ml, which is equivalent to the bed volume, is flowed, start taking the fractions with the fraction collector (Fig. 2D). We usually collect every 5 ml fraction at 3 ml/min of flow rate.
- To monitor the concentrations of the eluted proteins, test each eluted fraction with the Bradford protein assay. Mix 10 μl of each fraction with 40 μl of the Bradford solution on 96-well sample plate (Fig. 2E). Automatic plate readers can be used but we usually judge the blue color by eyes. Once the elution of MAPs plateaus, it is time to switch to the elution buffer. Usually, up to 200 ml of the wash buffer is used.

Elution and dialysis

- Switch to the elution buffer with exactly the same settings.
- Keep monitoring the protein concentration. After 40-45 ml of elution is done, peak fractions of tubulin can be obtained.
- Decide how many fractions to be taken for the dialysis. Usually 8 fractions (total 40 ml) are chosen.

- Transfer the sample into a dialysis membrane (Fig. 2F) and start overnight dialysis (Fig. 2G).

Evaluation of the eluates by SDS-PAGE (optional)
This evaluation is not necessary all the time. However, we recommend you to make sure if your procedures are successful at least upon your first trial. Each 5 μl of samples were taken from 1, thawed and centrifuged tubulin (initial); 2, flow through fraction at the column step; 3, all the collected fractions, and subjected to SDS-PAGE on 10% acrylamide gels. As shown in Fig. 2H, tubulin is efficiently purified in these fractions as judged on Bradford assay (Fig. 2E).

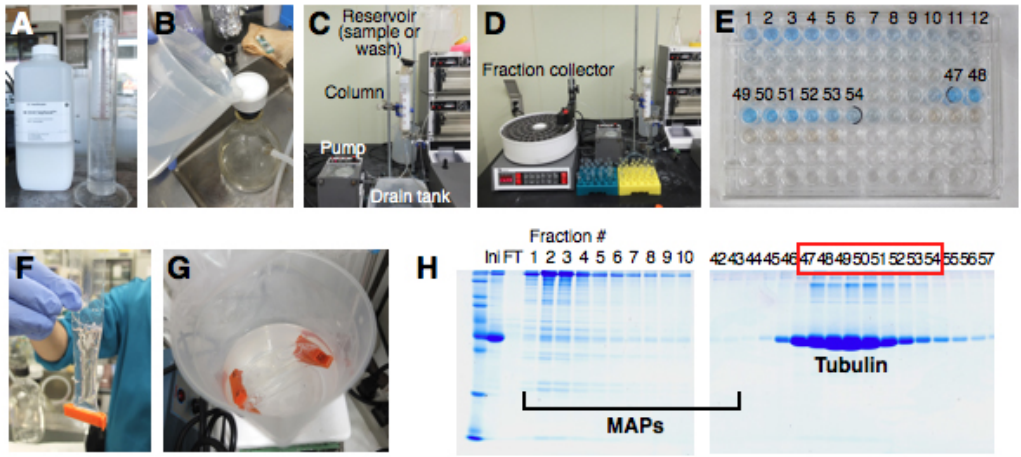

Fig. 2 Ion-exchange column chromatography (2nd day).

3. Selection of assembly-competent tubulin and fluorescent labeling (optional) (3rd day)

3.1 Outline
It is almost done. After all the processes above, tubulin sample is quite pure. On the third day, one more assembly-disassembly cycle is performed to select assembly-competent tubulins. Also during this step, ADP treatment is carried out, which is to remove possible contaminated kinesin motors. Kinesin dissociated from MTs when ADP is added instead of ATP. As an optional step, fluorescent labeling can be done to the assembled MTs during this final cycle. Fluorescently labeled tubulin is useful for many in vitro assays that are performed under fluorescent microscopes. As fluorescent groups are introduced mainly onto the lattice (outer surface) of the MTs, in theory, labels do not interfere the polymerization ability of tubulin dimers. After the final depolymerization followed by a centrifugation, tubulin is finally purified. Purified tubulin samples have to be snap frozen in liquid nitrogen so that they can be stored in -80ºC for years. In the following protocol, half

of the tubulin is labeled with rhodamine and the other half is prepared as non-labeled tubulin.

3.2 Preparations
- MT cushion, 10 ml, 100 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, 0.1 mM GTP, 60% glycerol, pH 6.9.
- Labeling buffer, 2 ml, 50 mM PIPES, 1 mM EGTA, 1 mM MgCl2, 0.5 mM GTP, 40% glycerol, pH 8.0.
Both of above solutions have to be pre-warmed at 37ºC and supplemented with GTP shortly before use.
- Tetramethylrhodamine solution (optional), 15 μl, 50 mg/ml 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (Molecular Probes, USA). Dissolved in DMSO.
- BRB80, 5 ml, 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8. Keep on ice.

3.3 Procedures
MT-assembly and ADP treatment
- Recover dialyzed tubulin solution. The volume should be now about 25% of original (if the starting volume was 40 ml, it should be around 12-14 ml).
- Centrifuge at 100,000 x g for 30 min at 4ºC to remove any denatured proteins.
- Add GTP to the supernatant at 0.5 or 1 mM and incubate for 1 hour at 33-37ºC (Fig. 3A). The transparent tubulin solution turns turbid over time, indicating MT polymerization.
- Add ADP at 1 mM and continue incubation for additional 15 min to remove kinesin from MTs
- Overlay each 3 ml of the assembled MT solution onto 1 ml of MT cushion in 5 ml ultracentrifuge tubes (total 4 or 6 tubes) and centrifuge at 100,000 x g for 40 min at 33-37ºC (Fig. 3B upper). Do not forget to pre-warm the centrifugation rotor.
- Remove the supernatants and obtain MTs as pellet (Fig. 3B lower).

Tubulin samples are divided into two here and half is used for rhodamine-labeling. For the other half, skip the labeling process and go ahead with the immediate disassembly process.

Fluorescent labeling of MTs
- Add total 900 μl of pre-warmed labeling buffer to the half of MT pellets obtained above and combine them in one of the tubes.
- Add 15 μl of tetramethylrhodamine solution and vortex the MT-dye mixture (Fig. 3C).
- Cover the tube with aluminum foil to avoid photobleach and incubate for 15 min at 33-37ºC.
- Centrifuge at 100,000 x g for 40 min at 33-37ºC.

Disassembly of MTs
- Add total 500 μl (for rhodamine-tubulin) or 1.8 ml (for non-labeled tubulin) of ice-cold BRB80 to the MT pellets.

- Dissociate the pellets in a 5 ml Teflon-glass homogenizer and leave on ice for 1 hour for the sufficient depolymerization of the MTs (Fig. 3D).
- Centrifuge at 100,000 x g for 30 min at 4ºC and take the supernatant as purified tubulin.
- Add glycerol at final 33% and mix well. Final volume should be around 750 μl (rhodamine-tubulin) or 2.7 ml (non-labeled tubulin).
- Aliquot every 10-30 μl in small tubes such as PCR tubes and snap freeze with liquid nitrogen (Fig. 3E).

Quality evaluation and densitometric analysis
- Run the purified tubulin samples on SDS-PAGE gel.
- Load approximately 1.5 μg of tubulin per lane that is roughly estimated by Bradford protein assay, and several different amount of BSA for references (such as 0.375, 0.75, 1.5 and 3 μg per lanes).
- Stain the gel with Coomassie and quantify the intensity of each band using imageJ.
As shown in Fig. 3F, tubulin samples do not have any noticeable contamination and their concentration was calculated as 10.9 mg/ml (rhodamine-tubulin) and 4.0 mg/ml (Non-labeled tubulin) by the densitometric analysis.

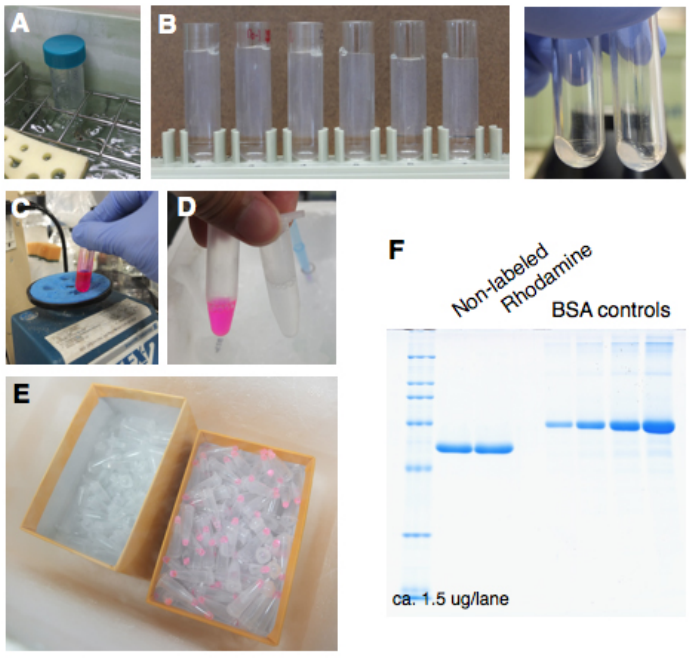

Fig. 3 Selection of assembly-competent tubulin and fluorescent labeling (3rd day).

Reference
Shelanski, M.L., Gaskin, F. and Cantor, C.R. 1973. Microtubule assembly in the absence of added nucleotides. *Pro. Natl. Acad. Sci. USA*, **70:** 765–768