

These and other protocols can be found on the Mitchison lab homepage (<http://mitchison.med.harvard.edu/Protocols.htm>) and are adjusted to Hyman Lab user.

Useful Values

1 mg/ml tubulin = 10 μ M (assuming MW of $\alpha\beta$ -tubulin heterodimer is 100,000; in reality it is \sim 110,000 but almost all tubulin labs use this convenient conversion relationship);

1 μ m of a microtubule has \sim 1600 tubulin heterodimers;

Extinction coefficient of tubulin at 280 nm = 115,000 $M^{-1}cm^{-1}$ or 1.15 (mg/ml) $^{-1} cm^{-1}$; assuming that the MW of a tubulin heterodimer is 100,000 Daltons. This extinction coefficient was calculated from tubulin sequences and includes the contribution of the two bound guanine nucleotides to the absorbance at 280 nm.

Handling Tubulin

Tubulin is a labile molecule that converts to a non-polymerizing state within hours on ice. This lability demands a certain amount of care and discipline when handling tubulin. The following is what is recommended for general use of tubulin:

1. Tubulin is very stable at $-80^{\circ}C$ and is best stored in small aliquots at this temperature. Generally tubulin is prepared in large scale and stored as large aliquots at $-80^{\circ}C$ (PC-tubulin). You should recycle 1-4 of these large aliquots (please see protocol for cycling tubulin) and store small aliquots of the recycled tubulin at $-80^{\circ}C$ for daily use. One or more recycled aliquots is thawed for an experiment, and any leftover material discarded. Refreezing thawed aliquots of recycled tubulin is not recommended.
2. To use recycled tubulin, thaw the tubulin on ice - you can use liquid nitrogen to bring tubulin aliquots from the freezer to the bench. Use the thawed tubulin for polymerization preferably within 30min of thawing. When quantitative analysis of tubulin polymerization is necessary, careful and consistent handling of tubulin aliquots is essential for reproducible results.

Recycling Tubulin

Tubulin fractions from the PC-column are stored at $-80^{\circ}C$. The recycled tubulin is stored in small aliquots (5-20 μ l) in liquid nitrogen for day-to-day use. Generally, recycled tubulin is stored in Injection Buffer (IB) or 1x BRB80 without free GTP. This is done because the absence of free GTP makes polymerization with GMPCPP relatively straightforward. GMPCPP is a very useful, non-hydrolyzable, GTP analog that has \sim 5-10X lower affinity than GTP for tubulin.

Protocol for recycling already in old protocol book.

Calculate concentration of tubulin using an extinction coefficient at 280 nm of 115,000 $M^{-1}cm^{-1}$. Freeze in 10-50 μ l aliquots in liquid nitrogen and store at $-80^{\circ}C$. (Note: 1 mg/ml tubulin = 10 μ M)

Tubulin Polymerization with GTP/GMPCPP/Taxol

Solutions & Supplies

BRB80 (1X)

80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8 with KOH (generally made as a 5X stock and stored at $4^{\circ}C$)

100 mM GTP in ddH₂O or 1xBRB80

100 mM GMPCPP in ddH₂O

Taxol

10 mM stock; 100 μ M, 10 μ M and 1 μ M dilutions (and/or 200 μ M, 20 μ M and 2 μ M dilutions) all in anhydrous DMSO; Taxol is sold under the tradename "Paclitaxel" by Sigma, catalogue number T-7191.

Prepolymerization Clarification

Although not absolutely necessary, it is recommended mixing tubulin and nucleotides in 1X BRB80 for 5min on ice and then clarifying this mix using a TLA100 rotor for 5min at 90K rpm at 2°C. This clarification is especially recommended when polymerization includes GMPCPP and/or highly labeled fluorescent tubulins. A clarification spin is also recommended on thawed labeled tubulins prior to microinjection.

For the following sections, the "appropriate ratio" is hard to define. Ideally, you should aim for a ratio of 1 labeled tubulin dimer: 3 unlabeled. Your mileage may vary.

GTP Polymerization

1. On ice, mix unlabeled and labeled tubulin at an appropriate ratio in 1X BRB80 with 1 mM DTT and 1 mM GTP. Incubate at 0°C for 5min.
2. Clarify the mix in TLA100 rotor at 90K rpm for 5min at 2°C.
3. Collect the supernatant and incubate at 37°C. If the tubulin concentration is 2 mg/ml or higher, assembly will proceed rapidly to steady state (~30min). If the concentration is lower, nucleation can be limiting and the precise kinetics of approach to steady state is difficult to predict and will depend on the amount of active tubulin in your mix.

For many experiments, seeds are added to surmount the nucleation barrier, thereby specifically assaying elongation - for this, make GMPCPP seeds, wash out any free GMPCPP and add a small volume of the seeds (~1/20-1/50 vol) after the polymerization mix has been at 37°C for 1min.

Axonemes or centrosomes can also be used as nucleating structures. If the purpose is labeling or recycling the tubulin, polymerization is promoted by addition of DMSO (10% v/v) or glycerol (33% (v/v) final concentration).

Taxol or DMSO Polymerization

1. On ice, mix unlabeled and labeled tubulin at an appropriate ratio in 1X BRB80 with 1 mM DTT and 1 mM GTP. Incubate at 0°C for 5min.
2. Clarify the mix in TLA100 rotor at 90K rpm for 5min at 2°C. Incubate the supernatant at 37°C for 1-2min.

Now there are two options:

- a. Add taxol stepwise (equimolar ratios below) as follows (for 1 mg/ml tubulin):
 - (Pipet in the taxol and immediately flick the tube to mix it in)
 - Add 1/10 vol 1 μ M taxol; Incubate at 37°C for 5-10min
 - Add 1/10 vol 10 μ M taxol; Incubate at 37°C for 5-10min
 - Add 1/10 vol 100 μ M taxol; Incubate at 37°C for 15min

Go to step 3!

- b. Add 10%(v/v) DMSO and incubate at 37°C for 20-30min

Continue with step 3!

3. Pellet microtubules over a warm 40% glycerol in BRB80 cushion in a TLA100, 100.2 or 100.3 rotor, 12min at cca. 200,000xg (cca 70krpm for any of them).
4. Discard the supernatant.
5. Rinse the pellet with warm (37°C) water (preferably with 0.5% Triton X 100).
6. Resuspend in warm BRB80 + 1 mM DTT + 10-20 μ M taxol (taxol should be at least equimolar and preferably in excess to the tubulin)

Notes: If taxol is added all at once it will cause tubulin precipitation! If polymerizing 2 mg/ml tubulin, use 2 μ M, 20 μ M and 200 μ M steps.

For DMSO polymerization it is best to have high tubulin concentrations (5-10 mg/ml) in the original mix before adding DMSO. However, MTs must be resuspended after pelleting with equimolar taxol.

A "quick-and-dirty" taxol polymerization method:

1. Thaw recycled tubulin stored in IB/BRB80 (generally 5-20 mg/ml)
2. Add equal volume 2X BRB80, 2 mM DTT, 2 mM GTP, 10 μ M Taxol
3. Incubate at 37°C for 20-30min
4. Pellet microtubules and resuspend as described above (steps 3-6)

If there is labeled tubulin in the mix, dilute the microtubules to 1-10 μ g/ml and check under a fluorescent microscope. Taxol-stabilized microtubules can be sheared by diluting them to \sim 100 μ g/ml and then passing them through a 27g needle \sim 5-6 times. All dilutions of taxol-stabilized microtubule should be done into buffers containing 10-20 μ M taxol.

GMPCPP Polymerization

Some considerations:

GMPCPP is the best current GTP analog for tubulin polymerization. You can buy it from Jena Bioscience (www.jenabioscience.com) or steal it from the Howard lab.

In the presence of potassium as counterion, GMPCPP is very slowly hydrolyzed within the microtubule lattice, and is essentially non-hydrolyzable within the time course of most experiments.

In the presence of sodium as counterion, GMPCPP is hydrolyzed slightly faster in the lattice -- this hydrolysis is accelerated tremendously by treatment with glycerol. Given this information on the effect of buffer counterions on GMPCPP stability within microtubule lattices, it is better to always use potassium counterion buffers for all our microtubule work.

GMPCPP is a potent nucleator of microtubules. Therefore, at tubulin concentrations of 1 mg/ml or higher, very numerous and short microtubules are formed in the presence of GMPCPP. If longer GMPCPP microtubules are desired, nucleation can be limited by diluting the tubulin to \sim 2-3 μ M (0.2 - 0.3 mg/ml).

You can generally make a 1-3 mg/ml CPP tubulin mix and store it at -80°C in small aliquots. Directly polymerizing this mix results in short GMPCPP seeds. Diluting the mix to roughly 2-3 μ M final tubulin while thawing it, results in formation of longer CPP microtubules.

Protocol

1. On ice mix unlabeled tubulin and labeled tubulin (1-3 mg/ml final) at an appropriate ratio in 1X BRB80 with 1 mM DTT and 0.5-1 mM GMPCPP. Incubate at 0°C for 5-10min.
2. Clarify mix in TLA100 rotor at 90K for 5min at 2°C. Freeze supernatant in 5-10 μ l aliquots in liquid nitrogen and store at -80°C.

To form short GMPCPP seeds

3. To form short GMPCPP seeds, transfer a tube from the freezer to a 37°C bath. Incubate 15-20min at 37°C. Dilute to 150-200 μ l with warm BRB80 + 1 mM DTT, pellet the seeds in a TLA100 rotor (90K 5min at 25-30°C), discard supernatant and resuspend pellet in 1-2X the starting volume of BRB80 + 1 mM DTT. This process removes free CPP and any unpolymerized tubulin. Seeds are generally added at 1/20-1/50 vol. (1x10⁶ seeds/ml for spin down experiments) to a polymerization mix containing tubulin and 1 mM GTP.

Tubulin Protocols (Mitchison Lab)

Given the ~10X higher affinity of tubulin for GTP versus GMPCPP, the amount of GMPCPP added from a seed mix at these dilutions is insignificant. Therefore, you can add seeds directly into a polymerization mix without dilution/sedimentation/resuspension.

To form long GMPCPP microtubules

3. To form long GMPCPP microtubules, thaw a CPP mix tube by adding in enough warm BRB80 + 1 mM DTT such that the final tubulin concentration is 2-3 μM (pipet in 37°C BRB80 + 1 mM DTT, mix by gently pipeting up and down until the frozen seed mix pellet is thawed, then place in 37°C bath). Incubate at 37°C for 30min or longer. Free CPP can be removed by spinning the mix through a cushion or the CPP microtubules can be used directly for assays.

Determining Concentration of GMPCPP/Taxol MTs

To accurately estimate the concentration of tubulin polymer in GMPCPP/Taxol polymerizations, MTs are pelleted and resuspended in buffer without free nucleotide. A small amount of the resuspended MTs are then diluted into a buffer containing CaCl_2 on ice to induce depolymerization and tubulin concentration determined by the OD280nm.

Here is a protocol for GMPCPP MTs (identical for taxol MTs, except that the resuspension buffer will have 10-20 μM taxol):

1. Pellet polymerization mixture (2 mg/ml) 90K 5min in TLA100 at 35°C.
2. Remove supernatant as thoroughly as possible.
3. Resuspend pellet in 80% of starting volume using 37°C BRB80 + 1 mM DTT until homogenous (require quite a lot of pipeting up and down)
4. Dilute 10 μl resuspended MTs into 90 μl of BRB80 + 50 mM KCl + 5 mM CaCl_2 and incubate on ice for 10min (dilute 10 μl BRB80 + 1 mM DTT similarly as a blank).
5. After 10min at 0°C, read A280 against blank and calculate concentration using an extinction coefficient of 115,000 $\text{M}^{-1}\text{cm}^{-1}$.