Tubulin Polymerization with GTP/GMPCPP/Taxol

I. 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 4iC)

100 mM GTP

100 mM GMPCPP

Taxol: 10 mM stock; 100 µM, 10 µM and 1 µM dilutions (and/or 200 µM, 20 µM and 2 µM dilutions) all in anhydrous DMSO; Taxol is sold under the tradename "Paclitaxel" by Sigma

Recycled Tubulin

Labeled Tubulin (optional)

II. Prepolymerization Clarification

Although not absolutely necessary, we recommend mixing tubulin and nucleotides in 1X BRB80 for 5' on ice and then clarifying this mix using a TLA100 rotor for 5' at 90K at 2iC. We especially recommend this clarification when polymerization includes GMPCPP and/or highly labeled fluorescent tubulins. We also recommend a clarification spin on thawed labeled tubulins prior to microinjection.

III. GTP Polymerization

1. On ice mix unlabeled tubulin and labeled tubulin at an appropriate ratio in 1X BRB80 with 1 mM DTT and 1 mM GTP. Incubate at 0iC for 5'.

2. Clarify mix in TLA100 rotor at 90K for 5' at 2iC.

3. Collect supernatant and incubate at 37iC. If the tubulin concentration is 2 mg/ml or higher, assembly will proceed rapidly to

steady state (~30'). 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, we add seeds to surmount the nucleation barrier, thereby specifically assaying elongation -- for this we routinely 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 37iC for 1'. 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 or glycerol as described above.

IV. Taxol Polymerization

1. On ice mix unlabeled tubulin and labeled tubulin at an appropriate ratio in 1X BRB80 with 1 mM DTT and 1 mM GTP. Incubate at 0iC for 5'.

2. Clarify mix in TLA100 rotor at 90K for 5' at 2iC. Incubate supernatant at 37iC for 1'-2'.

Now there are two options :

I. Add taxol stepwise to equimolar 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 µM taxol; Incubate at 37iC for 5'-10'

Add 1/10 vol 10 µM taxol; Incubate at 37iC for 5'-10'

Add 1/10 vol 100 µM taxol; Incubate at 37iC for 15'

Pellet microtubules over a warm 40% glycerol in BRB80 cushion in a TLA100, 100.2 or 100.3 rotor, aspirate and wash sample/cushion interface, rinse pellet and resuspend in warm BRB80 + 1 mM DTT + 10-20 µM taxol (taxol should be at least equimolar and preferably in excess to the tubulin)

Note: If taxol is added all at once it will cause tubulin precipitation! If

polymerizing 2 mg/ml tubulin, use 2 µM, 20 µM and 200 µM steps

OR II. Add 10%(v/v) DMSO and incubate at 37iC for 20'-30'

Pellet and resuspend microtubules as described above

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 (popular in motor labs where taxol microtubules are used as substrates for motility/ATPase assays):

1. Thaw recycled tubulin stored in IB (generally 5-20 mg/ml)

2. Add equal volume 2X BRB80 + 2 mM DTT + 2 mM GTP + 20% DMSO

3. Incubate at 37iC for 20'-30'

4. Pellet microtubules and resuspend as described above

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

V. GMPCPP Polymerization

GMPCPP is the best current GTP analog for tubulin polymerization. Its major limitation is lack of commercial availability. 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, we 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 ~2-3 μM (0.2 - 0.3 mg/ml). We generally make a 1-3 mg/ml CPP tubulin mix and store it at -80iC in small aliquots. Directly polymerizing this mix results in short GMPCPP seeds. Diluting the mix while thawing it results in formation of longer CPP microtubules.

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 0iC for 5'-10'.

2. Clarify mix in TLA100 rotor at 90K for 5' at 2iC. Freeze supernatant in 5-10 μl aliquots in liquid nitrogen and store at -80iC.

3A. To form short GMPCPP seeds, transfer a tube from the freezer to a 37iC bath. Incubate 15'-20' at 37iC. Dilute to 150-200 μl with warm BRB80 + 1 mM DTT, pellet the seeds in a TLA100 rotor (90K 5' at 25-30iC), 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 to a polymerization mix containing tubulin and 1 mM GTP. 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, we often add seeds directly into a polymerization mix without dilution/sedimentation/resuspension.

3B. 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 37iC BRB80 + 1 mM DTT, mix by gently pipeting up and down until the frozen seed mix pellet is thawed, then place in 37iC bath). Incubate at 37iC for 30' or longer. Free CPP can be removed as described in 3A or the CPP microtubules can be used directly for assays.

VI. 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 A_{280}. 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 5' in TLA100 at 35iC.

2. Remove supe as thoroughly as possible.

3. Resuspend pellet in 80% of starting volume using 37iC 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 10' (dilute 10 µl BRB80 + 1 mM DTT similarly as a blank).

5. After 10' at 0iC, read A_{280} against blank and calculate concentration using an extinction coefficient of 115,000 $M^{-1}cm^{-1}$.