

This protocol was developed and authored by John G. Peloquin. Protocol taken from <http://www.borisylab.northwestern.edu/pages/protocols/rhotub.html>

Preparation of tubulin for conjugation

DEAE - or phosphocellulose-purified tubulin may be used for derivitizations. However, I find it faster and more efficient to remove MAPs from cycled microtubule protein with 0.5 M PIPES by the following procedure:

1) 2X cycled microtubule protein (10-20 mg/ml) in PEM buffer (0.1M PIPES, 1.0mM EGTA, 0.5mM MgCl₂, pH 6.9) is adjusted to 0.5M PIPES, 1mM MgCl₂, 1mM GTP, 10% DMSO by addition of an equal volume of 1M PIPES, pH 6.9, 1/60 volume 100mM MgCl₂, 1/50 volume 100mM GTP, and 1/5 volume DMSO.

2) Incubate 37 C, 10 min - it should become very turbid and viscous.

3) Pellet MTs at 20,000 x g, 20 min, 37 C (18,000 rpm in an SS-34 rotor).

4) Remove the supernatant which contains the MAPs (although there are some residual MAPs, they do not survive the conjugation and cycling procedure).

5) Estimate the pellet volume.

6) Resuspend the pellet in 2-4 volumes of cold PEM by up and down pipetting with a cut off yellow Eppendorf tip.

7) Incubate at 0 C, 10 min.

8) Add 1mM GTP and 10% DMSO.

9) Incubate 37 C, 10 min.

Polymerized MTs are ready for derivitization.

Preparation of rhodamine labeled tubulin.

X-Rhodamine or Tetramethylrhodamine succinimidyl esters obtained from Molecular Probes.

1) Prepare a 10 mg/ml stock solution of Rhodamine-NHS in anhydrous DMSO (the stock can be stored at -80 C and freeze-thawed; it will last indefinitely if kept dry).

2) Add Rhodamine-NHS stock (add reagent while vortexing to allow thorough mixing) to polymerized tubulin from 9) to yield a molar ratio of 20 to 1, assuming the tubulin concentration in

the MT pellet measured in 5) is 80 mg/ml. (e.g. 1 mg X-Rhodamine-NHS per 0.1 ml MT pellet is approximately 20:1, since $0.1 \text{ ml} * 80 \text{ mg/ml} = 8 \text{ mg tubulin}$ and $MW(\text{Tubulin})/MW(\text{X-Rhodamine-NHS}) = 100,000/632 = 158$; therefore $[1 \text{ mg X-Rhodamine-NHS}/8 \text{ mg Tubulin}] * 158 = 20$.

3) Incubate 37 C, 10 min. The reaction can be terminated by adding 5mM Potassium Glutamate, but omission of this step has little effect on labeling stoichiometry.

4) Pellet MTs at 37 C.

5) Resuspend pellet in 5x volume of cold PEM.

6) Incubate 0 C, 10 min.

7) Sediment at 20,000 x g, 20 min, 4 C (In 1.5mL Eppendorf tubes at 18,000 rpm in an SS-34 rotor) to remove denatured protein and unreacted reagent.

8) Adjust the supernatant to 10% DMSO, 1mM GTP.

9) Incubate 37 C, 10 min to polymerize Mts.

10) Pellet MTs at 37 C.

11) Repeat steps 5)-10) above.

12) Resuspend MT pellet in cold PEM or chosen injection buffer.

13) Incubate 0 C, 10 min.

14) Sediment at 4 C to clarify.

15) Freeze aliquots in liquid nitrogen.

To calculate Dye to Protein Ratio, I use Extinction coefficient(X-Rhodamine)= 70,000 at Lamda=584 and Extinction coefficient(TMR)=50,000 at Lamda=556 and determine protein by BCA or Bradford using a BSA standard. For X-Rhodamine tubulin D/P= 0.5-0.8. For TMR tubulin D/P=0.8-1.2. Yields are typically 30-50%. Increasing the initial reaction stoichiometry will increase the D/P, but significantly lower yields. Yields are typically 30-50%.

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