

This protocol was developed and authored by John G. Peloquin. Protocol taken from:
<http://www.borisylab.northwestern.edu/pages/protocols/cy3tub.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 Cy3 labeled tubulin.

Cy3 succinimidyl ester (Research Organics) -- Cy3 is sold in 5-packs of vials each containing sufficient dye to label 1 mg of IgG. I have found 1 vial is sufficient to label 3-4 mg of tubulin or 40-50 microliters of pelleted MTs.

1) Dissolve 1 vial of Cy3 in 20 microliters of dry DMSO

2) Add to polymerized MTs from 9) above.

- 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 of Cy3=130,000 at $\lambda=554$ and determine protein by BCA or Bradford using BSA as a standard. Dye/Protein Ratio is about 0.5. Yields are typically 30-50%.

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