Labeling Tubulin and Quantifying Labeling Stoichiometry

This is a general procedure for coupling moieties with reactive succinimidyl esters to tubulin. We have used it successfully to derivatize tubulin with succinimidyl esters of biotin, digoxigenin, and a wide range of fluorochromes such as tetramethylrhodamine, X-rhodamine, fluorescein, Oregon Green, Cy3, Cy5 and C2CF (bis-caged carboxyfluorescein). The procedure involves labeling polymeric tubulin, thereby protecting residues important for microtubule assembly. The labeling is performed at high pH to optimize the reaction with the succinimidyl esters and functional tubulin is selected after the labeling reaction by one or more cycles of polymerization and depolymerization.

I. Solutions & Supplies

phosphocellulose-purified tubulin (~50 mg = 2-4 3 ml aliquots of 5-10 mg/ml PC fractions)

Dye stock in anhydrous DMSO (20-100 mM)

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)

High pH Cushion: 0.1 M NaHEPES, pH 8.6, 1 mM $MgCl_2$, 1 mM EGTA, 60% (v/v) glycerol

Labeling Buffer: 0.1M NaHEPES, pH 8.6, 1 mM $MgCl_2$, 1 mM EGTA, 40% (v/v) glycerol

Quench: 2X BRB80, 100 mM K-Glutamate, 40% (v/v) glycerol

Low pH cushion: 60% (v/v) glycerol in 1X BRB80

10X IB (Injection Buffer): 500 mM K-Glutamate, 5 mM $MgCl_2$ (pH of 1X ~ 7.0)

50.2Ti rotor (warm = 37iC)

TLA100.4 or TLA100.3 and TLA100.2 rotors

Small Dounce (2 ml)

Note: 1M HEPES, pH to 8.6 with NaOH and store at -20iC

2M K-Glutamate - dissolve glutamic acid to 2M, carefully pH with KOH such that 50 mM has a pH ~7.0 and store at -20iC

(All buffers for labeling can be stored indefinitely at -20iC; dye stocks are best prepared fresh from powder that has been stored anhydrously at -20iC; residual dye solution can be stored at -20iC or -80iC under anhydrous conditions)

II. Labeling Protocol The procedure described below can be scaled down if desired. It is essential to perform all steps involving caged dyes under a safelight in a room well-shielded from light. A piece of red acetate sheet taped over a dimly lit lamp is adequate as a safelight. Other dye labelings can be done under room light, minimizing exposure during incubations by using foil.

1. Thaw 2-4 PC column fractions (30-60 mg tubulin) and add BRB80 to 0.5X, $MgCl_2$ to 3.5 mM , GTP to 1 mM and store on ice for 5'. Transfer to 37iC and add DMSO to 10% final in two steps, mixing gently but thoroughly and incubate at 37iC for 30 min. Alternatively, add half volume of glycerol to promote polymerization. In a side-by-side comparison, for reasons that are not clear, using DMSO instead of glycerol for the first polymerization step appears to increase the labeling stoichiometry by ~25% for C2CF-SNHS ester. For labeling with rhodamine (tetramethylrhodamine NHS ester) and X-rhodamine, we generally use glycerol polymerization.

2. Layer polymerized tubulin onto 20 ml warm High pH Cushion in two 50.2 Ti tubes. Pellet microtubules in a Beckman ultracentrifuge in a 50.2 Ti rotor at 40K for 45' at 35iC.

3. Aspirate the supernatant above the cushion and rinse the supernatant-cushion interface twice with warm (37iC) Labeling Buffer. Aspirate the cushion and resuspend the pellet using a cutoff large pipet tip in 1 - 2 ml of warm labeling buffer. Take care to keep the tubulin warm during the resuspension and continue resuspending till

no chunks of tubulin are visible. This is the most painful part of the labeling procedure.

4. Add 10- to 20-fold molar excess of the dye to tubulin. Estimate the tubulin concentration assuming ~70% recovery of the starting tubulin. For dyes such as Cy5 and Cy3, use a 5-pack for labeling ~25 mg. For most dyes we label for 30'-40' at 37iC. For C2CF-SNHS (caged fluorescein), we have found it best to add the dye in two steps (20' apart) and label for 60' at 37iC. After adding the dye stock, gently vortex the mixture every 2'-3' during the course of the labeling.

5. At end of labeling incubation add an equal volume of Quench to the labeling reaction and mix well. Incubate for 5'.

6. Layer the quenched labeling reaction onto two TLA100.3 (or TLA100.4) tubes containing 1.5 ml of Low pH Cushion. Spin at 80K for 20 min at 35iC in a TLA100.3 or TLA100.4 rotor in a Beckman TLA100 ultracentrifuge.

7. Aspirate the supernatant above the cushion and rinse the supernatant-cushion interface twice with warm 1XBRB80. Aspirate the cushion and resuspend the pellet using a cutoff pipet tip in 1 ml of ice-cold 1X IB. Transfer resuspended chunks of the pellet to a small ice-cold dounce (1 or 2 ml volume) in an ice-water bath. Resuspend the pellet by gentle douncing till the suspension is uniform. Continue douncing intermittently for a total time of 30 min at 0iC.

Cold IB seems to promote more rapid depolymerization than BRB80; therefore, we use IB in the depolymerization step for all labeling procedures. For small scale labelings the pellet can be resuspended directly in the centrifuge tube and sonicated gently using a microtip sonicator to speed depolymerization.

8. Spin the depolymerized tubulin in a TLA100.2 (or TLA100.3) rotor at 80K for 10' at 2iC.

9. Recover the supernatant from the cold spin, add BRB80 to 1X (from a 5X stock), $MgCl_2$ to 4 mM, GTP to 1 mM and incubate on ice for 3'. Warm to 37iC for 2', add 1/2 volume of glycerol (33% v/v final), mix well and polymerize at 37iC for 30 min.

10. Layer the polymerization reaction on a 1 ml Low pH Cushion in a TLA100.3 tube and pellet the microtubules at 80K in a TLA100.3 rotor

for 20' at 37iC.

11. Aspirate the supernatant above the cushion and rinse the supernatant-cushion interface twice with warm IB. Aspirate the cushion and rinse the pellet twice with 1 ml warm IB to remove any residual glycerol. Resuspend the pellet using a cutoff pipet tip in 0.2-0.3 ml of ice cold IB. This pellet should resuspend easily. Incubate at 0iC for 20 to 30 min.

12. Spin the depolymerized tubulin in a TLA100 or TLA100.2 rotor at 80K for 10' at 2iC. Recover the supernatant, quickly estimate the tubulin concentration, adjust with IB if desired and freeze in 3 - 5 ul aliquots in liquid nitrogen. We generally aim for a final tubulin concentration of 5 - 15 mg/ml (50 - 150 µM). Careful determination of tubulin concentration and labeling stoichiometry can be performed as described below, after the tubulin has been aliquoted and frozen. C2CF-tubulin should be stored at -80iC in a foil-wrapped box.

III. Quantifying Tubulin Concentration and Labeling Stoichiometry To determine the tubulin concentration and stoichiometry of labeling, dilute the labeled tubulin 1/50 - 1/100 in IB and obtain a wavelength spectrum. Calculate the molar concentration of dye by using the absorbance at the peak wavelength and the extinction coefficient provided by the dye manufacturer. Determine the tubulin concentration by first subtracting out the contribution of the dye to the A_{280} and then using an extinction coefficient of 115,000 $M^{-1}cm^{-1}$. Section V provides a list of extinction coefficients and A_{280} absorbance (relative to absorbance at peak wavelength) for commonly used dyes. Note that the absorbance of fluorescein is pH-dependent and conjugates with fluorescein should either be diluted into a high pH buffer (~8.8-9.0) or the value measured at pH 7.0 multiplied by 1.2.

An example of calculating concentration and stoichiometry for tubulin labeled with tetramethylrhodamine (TMR) NHS ester:

Tubulin concentration = [(A_{280} - Contribution of dye to A_{280}) x Dilution Factor] / Extinction

coefficient of tubulin at 280 nm

TMR concentration = (A_{555} x Dilution Factor) / Extinction Coefficient of

TMR at 555 nm

Labeling Stoichiometry = TMR concentration / Tubulin concentration

A wavelength spectrum of 1/100 dilution of the final labeled tubulin product gave the following absorbance values:

A_{280} = 0.23; A_{555} = 0.20

Therefore, Tubulin concentration = [{0.23 - (0.2 x 0.2)} x 100]/115000 = 165 µM

TMR concentration = [0.20 x 100]/95000 = 210 µM

Labeling Stoichiometry = 210/165 = 1.3

To determine the concentration and labeling stoichiometry of C2CF-tubulin, the C2CF must be first uncaged to fluorescein. To do this, dilute the labeled tubulin 1/50 to 1/100 in IB + 2 mM DTT in an eppendorf tube. Put the eppendorf tube on a hand held UV lamp, cover with foil (shiny side down) and expose to long wavelength UV for 30'. Obtain a wavelength spectrum from 200 to 600 nm after the 30' activation, using IB + 2 mM DTT exposed to UV in parallel as a blank. Assuming a 100% efficiency for the uncaging reaction, the concentration of C2CF can be calculated from the spectrum after activation as follows:

Concentration of C2CF = (A_{495} x Dilution Factor x 1.2)/74000

(The factor of 1.2 corrects for the pH dependence of the absorption spectrum of fluorescein)

IV. Using Labeled Tubulins a) Microinjection into cells/addition to extracts: For microinjections, we dilute the tubulin in IB to 2-5 mg/ml, clarify by centrifugation and inject ~1/10 of cell volume. For frog extract studies, we add labeled tubulin to 1/40 - 1/200th of the extract tubulin pool (~20 µM).

b) Preparation of fluorescent microtubule substrates or for monitoring polymerization and dynamics of pure tubulin: We use a mixture of labeled and unlabeled tubulin for polymerization. The ratio of labeled

to unlabeled will depend on the particular application and on the brightness of the labeled tubulin. Labeled tubulins, especially those labeled to high stoichiometry, exhibit very different properties from unlabeled tubulin. Therefore, we use the highest ratio of unlabeled to labeled tubulin that provides signal intensity sufficient for a particular experiment. **V. Properties of Fluorescent Dyes Used for Tubulin Labeling**

Dye	Excitation (nm)	Emission (nm)	emax (M-1cm-1)	e280/
Fluorescein	**495**	**519**	**74,000**	**0.19**
Oregon Green 488	**495**	**521**	**76,000**	**0.19**
Cy3	**550**	**570**	**150,000**	**0.08**
Tetramethylrhodamine	**550**	**576**	**95,000**	**0.21**
X-Rhodamine	**574**	**602**	**78,000**	**0.20**
Texas Red	**583**	**603**	**116,000**	**0.15**
Cy5	**649**	**670**	**250,000**	**0.05**

emax = Extinction coefficient of dye at its peak wavelength

e280/emax = Absorbance of dye at 280 nm as a fraction of its absorbance at its peak wavelength

5 (and-6) carboxyfluorescein succinimidyl ester (Molecular Probes C-1311)

Oregon Green 488 carboxylic acid, succinimidyl ester 5-isomer (Molecular Probes O-6147)

5 (and-6) carboxytetramethylrhodamine succinimidyl ester (Molecular Probes C-1171)

5 (and-6) carboxy-X-rhodamine succinimidyl ester (Molecular Probes C-1309)

Texas Red-X succinimidyl ester, mixed isomers (Molecular Probes T-6134)

Cy3-OSu monofunctional reactive fluorophore (Amersham PA13100)

Cy5-OSu monofunctional reactive fluorophore (Amersham PA13600)