Labelling Porcine Tubulin

You need:

- Tubulin (~ 50 mg, 900 nmol)
- Dye stock, i.e. Cy3 Mono NHS Ester in anhydrous DMSO at 100 mM[1].
- GTP
- 1 M $MgCl_2$
- Glycerol
- 1xBRB8[2]
- High pH cushion[3]
- Labelling buffer[4]
- Quench[5]
- Low pH cushion[6]
- 2M K-Glutamate[7]

- 50.2 Ti BECKMAN rotor
- Bottle, with Cap Assembly, Polycarbonate, 26.3 mL, # 337901
- Beckman ultracentrifuge
- TLA100.3 or TLA100.4 or TLA120.2 BECKMAN rotor
- Tubes for TLA100.3 (thickwall polycarbonate tube, 3 ml, # 349622), TLA100.4 (thick-wall polycarbonate tube, 3.2 ml, # 362305) and TLA120.2 (thickwall polycarbonate tube, 1 ml, # 343778)
- Water bath at 37°C

1st Polymerisation

- 30-60 mg tubulin, thaw as fast as possible
- add GTP to 1 mM
- add $MgCl_2$ to 1 mM
- store on ice for 5 min
- add pre-warmed glycerol to 33% final concentration
- mix gently but thoroughly
- incubate at 37°C for 30 min
- layer polymerized tubulin onto 1 Vol warm high pH cushion,
- pellet microtubules in a 50.2 Ti rotor at 40K for 45 min at 35°C

Aspirate the supernatant above the cushion and rinse the supernatant-cushion interface twice with 37°C labelling buffer. Aspirate the cushion and resuspend the pellet using a cut-off large pipet tip in 1 - 2 ml of warm labelling buffer. Take care to keep the tubulin warm during the resuspension and continue resuspending till no chunks of tubulin are visible.

Labelling of the polymers

- Add 7- to 10-fold molar excess of the dye to tubulin. Estimate the tubulin concentration assuming ~70% recovery of the starting tubulin.
- Label for 30-40 min at 37°C. Gently vortex every 2-3 min during the course of labelling.
- Add an equal volume of Quench and mix well.
- Incubate for 15 min.
- Layer the quenched labelling reaction onto 1.5 ml of low pH cushion and spin at 80K for 20 min at 37°C in a TLA100.3 / TLA100.4 / TLA120.2 rotor.

Depolymerisation

- 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 cut-off pipet tip in an appropriate* Vol of ice-cold 1xBRB80. Resuspend the pellet by gentle pipetting till the suspension is uniform.
- Keep on ice for at least 60 min.
- Spin the depolymerized tubulin in a TLA100.2 / TLA100.3 / TLA120.2 rotor at 80K for 10 min at 2°C.
- Recover the supernatant from the cold spin.

*Generally aim for a final tubulin concentration of 5 - 15 mg/ml (50 - 150 µM).

Determine tubulin concentration and labelling stoichiometry

- Dilute the labelled tubulin 1/50 - 1/100 in BRB80.
- Determine A_{280} and correct for the contribution of the dye to the absorbance at A_{280}.
- Determine A_{max} (max emission of the fluorophore).

$$tubulin\ con. = \frac{A_{280} - (A_{max}\ x\ correction\ factor)}{Mol.Extinc.Coeff\ (tubulin)}\ x\ dilution\ factor$$

$$dye\ conc. = \frac{A_{max}\ x\ dilution\ factor}{Mol.Extinc.Coeff\ (dye)}$$

$$labelling\ stochiometry = \frac{dye\ conc.}{tubulin\ conc.}$$

Molecular Extinction Co-efficient ($M^{-1}cm^{-1}$) for tubulin 115 000

Monofunctional dye characteristics

	Amersham	**Invitrogen**	
	Cy3	TAMRA	Alex488
MW	765.95	527.53	643.41
A_{max}	550	555	495
E_{max}	570	580	519
Mol.Ext.Coeff.	150 000	65 000	71 000
Correction factor	0.08	0.11	0.30

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(1) Dyes.

- TAMRA, SE; 5-(and-6)-Carboxytetramethylrhodamine, Succinimidyl Ester (5(6)-TAMRA, SE), mixed isomers. Invitrogen, # C-1171.
- Alexa Fluor® 488 Carboxylic Acid, Succinimidyl Ester, mixed isomers. Invitrogen, # A-20100.

Dye stocks are best prepared fresh from powder that has been stored anhydrously at -20ºC; residual dye solution can be stored at -80ºC under anhydrous conditions.

(2) 1xBRB80
80mM Pipes
1mM $MgCl_2$
1mM EGTA

pH 6.8 with KOH

(3) High pH cushion
0.1M NaHEPES, pH 8.6
1mM $MgCl_2$
1mM EGTA
60% v/v Glycerol

(4) Labelling buffer
0.1M NaHEPES, pH 8.6
1mM $MgCl_2$
1mM EGTA
40% v/v Glycerol

(5) Quench
2x BRB80,
100mM K-Glutamate
40% v/v Glycerol

(6) Low pH cushion
1x BRB80
60% Glycerol

(7) 2M K-Glutamate
Dissolve glutamic acid to 2M, carefully pH with KOH such that 50 mM has a pH~7

All solutions can be stored at -20°C.