Labeling Kinesin with Benzylguanine (BG)-oligonucleotide

Here, microtubules are used to affinity purify kinesin labeled with benzylguanine (BG)-oligonucleotides from excess label. This also results in the removal of dead kinesin motors.

Preparing Microtubules (MTs):

1) Thaw 20ul aliquot of tubulin (20 mg/ml) at 37°C.
2) Immediately place on ice.
3) Polymerize tubulin by mixing together:
   - 20 ul 13 mg/ml tubulin
   - 20 ul 2X polymerization mix
4) Incubate at 37°C for 20 min.

From this point on, the MTs should never be placed ice as microtubules depolymerize in the cold.

5) Taxol stabilize the MTs. The final concentration of taxol should be equimolar to the tubulin concentration.
   - Taxol binds to tubulin in a 1:1 complex. Taxol (sold by Sigma as paclitaxel) is made to a 10mM stock in DMSO.
   - Adding taxol directly before the MTs have polymerized causes tubulin to precipitate (see Mitchison lab website for more details: http://mitchison.med.harvard.edu/protocols.html)
6) Mix together:
   - 40 ul MTs polymerization mix from previous step
   - 40 ul 1X BRB80 containing the appropriate amount of taxol.
7) Incubate at 37°C for at least 10 minutes.

Store this at room temperature! All of the following steps should be done at room temperature and contain taxol.

8) Separate tubulin dimer from polymerized MTs. Pipette MTs from step 7 onto a 60% glycerol (or 40% sucrose) cushion
   - The glycerol cushion should be an = or great volume than the MT volume.
9) Spin 50,000 rpm for 15 minutes at room temp in a TLA100.
10) Carefully remove glycerol cushion and then resuspend MT pellet in BRB80 (or desired dynein buffer) + 20uM taxol, 1mM DTT.
11) Measure OD280 to determine MT concentration (5ul MTs into 95ul 6M guanidine HCl).

Buffers

2X Polymerization Mix:

- 100 ul 5X BRB80
- 0.5 ul 1M DTT
- 50 ul 100% DMSO
- 5 ul 100 mM GTP
- 5 ul 100 mM MgCl2
- 89.5 ul filter sterilized ddH_2O

Glycerol Cushion

- 60% glycerol
- 1X BRB12
- 1mM DTT
- 20uM Taxol

BRB80 Buffer

- 1X BRB80
- 1mM DTT
- 20uM Taxol

BRB12 Buffer

- 1X BRB12
- 1mM DTT
- 20uM Taxol

5X BRB80

- 400mM PIPES (pH6.8)
- 10mM $MgCl_2$
- 5mM EGTA

5X BRB12

- 60mM PIPES (pH 6.8)
- 10mM $MgCl_2$
- 5mM EGTA

Labeling the motor by binding and releasing from microtubules:

1) Mix the following adjusting for molar ratios accordingly (molar ratios):
   - 50ul of 3.5uM K560 (1x)
   - 22ul of ~240uM BG-oligo (20-30x)
2) Incubate at room temperature then add the following in this order:
   - 0.85ul of 0.1M AMPPNP (1mM)
   - 0.17ul of 10mM taxol (20uM)
   - 0.85ul of 100x Apyrase (1x ~ 6.6 units/ml)
   - 11.5 ul of 50uM MTs (3-4x) (Taxol should be in the buffer before MTs are added).
3) Volume is ~85ul making final concentrations of all the components
   - ~2uM K560.
   - ~60uM BG-oligo
   - ~1mM AMPPNP
   - ~20uM Taxol
   - ~6.6 units/ml Apyrase
   - ~6.7uM MTs
     i) The more concentrated the components are, the more efficient the labeling reaction.
4) Incubate at room temperature for 15 min after mixing the solution by flicking the tube gently.
5) Centrifuge through a cushion in TLA100, 50krpm, 25C, 15min in a TLA 100.
6) Remove the top layer (S1) carefully taking a small amount of cushion.
   - Take 4ul of S1 for a gel sample.
7) Wash the tube and above the cushion by adding BRB12 to the top and removing.

Repeat 3X total removing a bit of cushion each time but leaving the pellet completely covered in cushion.

8) Remove the cushion carefully without disturbing pellet.
9) Wash the pellet 3X with BRB12 avoiding disturbing the pellet.
10) Resuspend the pellet in 50ul of Release buffer
    - take 3ul for a gel as P1.
11) Incubate release reaction for 5 min at room temperature.
12) Pellet MTs at 80krpm, 6 min, 25C with the TLA 100.
13) Remove supernatent and at sucrose to a final concentration of 10%
    - take 3ul for a gel as S2.
14) The supernatant supplemented with sucrose can be aliquoted, flash frozen and stored at -80C. This contains the purified kinesin motor labeled with oligo with minimal free BG-oligo. There will be a mixture of labeled and unlabeled kinesin if the reaction has not gone to completion.
15) Resuspend the pellet in 50ul of buffer and take a sample for P2.
16) Run gel of all samples with an appropriate standard to measure labeling efficiency (gel shift from ~8kda oligo) and yield (i.e. final concentration of S2 sample).

Buffers

Release Buffer

- 30ul 2M KCl
- 40ul 5x BRB80
- 0.2ul 1M DTT
- 10ul 100mM ATP*MgCl2
- 0.4ul 10mM Taxol
- 119.4 ul ddH2O

BRB12 Cushion

- 60% Glycerol
- 1x BRB12
- 1mM DTT
- 20uM Taxol

BRB12 Buffer

- 1xBRB12
- 1mM DTT
- 20uM Taxol