MT Spindowns from Extracts

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Notes:

The key variable in MT spindown experiments is ATP. Under high ATP conditions,conventional MAPs are selectively co-sedimented with microtubules. In theabsence of ATP (or in presence of AMPPNP which induces rigor binding of motorsto MTs), both motors and MAPs will bind to MTs and pellet with themicrotubules. For MAP and motor analysis, I often supplement the extract with exogenous taxol-stabilized MTs to ensure that binding sites are not limitingand I am not seeing competition effects. For a unknown protein, it is best totry a variety of conditions. Below is a protocol for MAP pelleting under high ATP conditions. I have listed the modifications for MAP/motor protocolafterwards.

MAP pelleting:

1. Prespin extract in TLA100.3 at 70K for 20' at 4 deg.C. Transfer supe to tube on ice.
2. Extracts must have a source of GTP. For Xenopus egg extracts, we simply add an energy regeneration mix to extracts and the extract maintains physiologicalGTP levels. For dilute tissue culture extracts, it is best to supplement theextract with 0.5 mM MgGTP. This can be done before or after the prespin.
3. Add 2 mM MgATP to extracts, warm to RT and add taxol to 5 uM. Mix well andincubate for 2'-3' before adding an additional 15 uM taxol (final is 20 uMtaxol). Incubate for 20'-30' at RT - 37 deg.C. (We use Xenopus extracts forwhich physiological temperature is RT; for tissue culture cells, tubulin willpolymerize better at higher temperatures. 30 - 33 deg.C is a good compromiserange to balance polymerization and proteolysis. Extracts must be supplementedwith a peptide protease inhibitor cocktail just before warming up to preventexcessive proteolysis).
4. Layer the polymerized mixture onto a 1M sucrose cushion in BRB80 containing 0.5 mM ATP, 10 uM taxol and protease inhibitors and pellet in a TLS55 at 40K for 20' at 22 deg.C. You can also use a fixed angle rotor such as a TLA100.3 or TLA100.4. I like to pellet

MTs at 100-150,000 g for 20 - 30' in TLA100 rotors over 30-40% glycerol/sucrose cushions. For larger ultra rotors, increase the speed and/or time to reach an equivalent clearing factor.

5. Save supe for gel/blot, aspirate cushion while washing 2-3x with BRB80 and remove as much of the cushion as possible (MTs form a clear, gelatinous pellet).
6. Boil pellet in sample buffer and analyze.

MAP and motor pelleting:

1. do not add extra ATP.
2. add 2 -5 mM MgAMPPNP and/or supplement the extract with 15 U/ml hexokinaseand 20 mM glucose (should try all three conditions - only AMPPNP, only glucose-hexokinase, both).
3. supplement extract with taxol-stabilized MTs to 0.2-0.3 mg/ml finalconcentration after adding taxol to warmed up extract as above. Thisconcentration is for concentrated Xenopus extracts - could use 0.1 mg/ml fortissue culture cell extracts.

Note on Buffers:

I have done this with extracts prepared using BRB80 and XB (10 mM HEPES, pH7.7; 100 mM KCl, 50 mM sucrose and 1 mM MgCl2 - a classically MT-unfriendly buffer) and find it works with both. I think it is best to try **Y**our**F**avorite **B**uffer and BRB80 side-by-side to maximize chances ofsuccess. I ended up using XB since I was trying to relate MAP profiles to cell cycle-dependent MT dynamics in XB extracts. The buffer in the cushion is notvery important.

Note on Taxol stabilized MTs:

1. Dilute tubulin to 2 mg/ml tubulin in BRB80 + 1 mM GTP + 1 mM DTT on ice; incubate 5'.
2. Warm to 37 deg.C, add 1/100 vol of 0.02 mM taxol in DMSO; 5' at 37 deg.C
3. Add 1/100 vol of 0.2 mM taxol; 5' at 37 deg.C
4. Add 1/100 vol of 2 mM taxol; 10-15'' at 37 deg.C. These MTs can be stored at RT for upto 1 week.