CHO Centrosome Prep:

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Cells:

We grow our CHOs with MEM[[alpha]] (without nucleosides) + 10% Bovine Calf Serum and penn/strep/glutamine. For a prep it is best to grow twenty large plates (150 mM) and the cells should be grown to overconfluence - till they start piling up on each other (you will need to feed them frequently to keep them happy)

Protocol Rationale:

The protocol is identical to Tim's published in Vol 134 of Methods in Enzymology. The key step is the lysis which solubilizes centrosomes away from nuclei by very low ionic strength lysis after treatment of cells with nocodazole and cytochalasin B. The released centrosomes are then centrifuged onto a Ficoll cushion (to avoid pelleting) and the interface between the lysate and the Ficoll is collected and the centrosomes are concentrated on a sucrose gradient. Fractions are assayed by spindown and double IF with 5051 serum and anti-tubulin and the pooled fractions are frozen in liq N2.

What you need:

Equipment:

- refractometer
- Sorvall with cold HB-4
- Ultra with cold SW27
- Some sort of gradient fractionator with a fraction collector
- 16 30 ml corex tubes (cold)

Solutions:

A lot of these solutions are w/w; Tim says that to make these weigh out sucrose and then add buffer till weight is 100g.

Wash & Lysis:

PE: 10 mM PIPES, 1mM EDTA, 8 mM BME
Make a 50X stock and pH to 7.2 with KOH
LB: 1mM Tris-HCl, 8 mM BME
Make Tris as 2M stock and pH to 8.0 with HCl
LB + 0.5% NP-40 :
(warm for 30' to 37 deg.C to ensure NP-40 has dissolved)
PBS: 130 mM NaCl, 2 mM KCl. 8 mM Na2HPO4, 2 mM KH2PO4
Make a 10X stock

Night before:

Make 600 ml of

- 1X PBS
- 0.1X PBS
- 0.1X PBS, 8% (w/w) ultrapure sucrose
- 8% (w/w) ultrapure sucrose
- LB (w/o BME): add 280 ul BME before use
- LB + 0.5% NP-40 (w/o BME): add 280 ul BME before use

and put all these buffers in coldroom

Sucrose Gradients :

Use ultrapure sucrose

20% (w/w) and 62.5% (w/w) sucrose in 1x PE + 0.1% TX-100. Make 100 grams of each as follows:
Weigh out sucrose and then on the balance add 1X PE + 0.1% TX-100 till weight is 100 grams.
Just before pouring gradients add 28 ul BME/50 g

Pour gradients night before or during drug treatment (see later). I find it easiest to pour night before and store in cold.

To pour gradients - first put a 5 ml heavy sucrose pad and then pour gradient on top of that. For the prep outlined below I pour 2 gradients in SW27 tubes:

4 ml heavy sucrose pad (use 62.5% or higher).
16 ml gradient.
The centrosomes are very close to the bottom of this gradient. The pad eliminates them from entering the curve of the tube and also gives a little leeway in setting up the fractionation.

Ficoll cushion:

20% (w/w) Ficoll (MW 400,000) in 1X PE + 0.1% NP-40

1. Make up PE + 0.1% NP-40 (no BME).
2. Weigh out 10g Ficoll.
3. Add PE + 0.1 % NP-40 till total weight = 50 grams.
4. Stir at RT for several hours to dissolve and store cold.
5. Before use, add 28 ul BME

Protocol:

1. Warm up 300 ml of CHO medium to 37 deg.C and add cytochalasin B (150 ul of 10 mg/ml) and nocodazole (300 ul of 10 mg/ml)
2. Make sure all buffers are in coldroom, there is rocker in the coldroom and there is a good aspirator in the coldroom. Hook up a sawed off pipet to the aspirator and make sure there is a LARGE trap (at least 4L)
3. Add medium with drugs to 10 plates of cells. Add medium with drugs to the other 10 plates 45' later.
4. After 90' in drug medium process first 10 plates: bring to coldroom and wash with the following buffers: 1X PBS 0.1X PBS, 8% (w/w) sucrose 8% (w/w) sucrose LB and then pipet on LB + 0.5% NP-40 (10 mls/plate) (These washes must be done rapidly - all washes should be under 1' per plate. The way I do them is to pour on the wash buffer from a beaker or grad cylinder (~ 30 mls), immediately rock back and forth and aspirate ASAP before pouring on the next wash. It is critical to do this quickly to get good lysis or you will lose most of the centrosomes in the nuclear pellet). After adding the LB + 0.5% NP-40 transfer the plate onto a rocker in the coldroom.
5. After 10' pipet off the lysate into a 30 ml corex tube. Add 1/50 vol of 50X PE (0.5 ml for 25 mls; with BME)

6. Spin tubes in HB-4 for 3' at 3000 rpm at 4 deg.C
7. Transfer supe to a fresh corex tube and underlay with 2 ml of Ficoll cushion (I use a 6cc syringe with 16 gauge needle with a thin piece of tygon tubing which I can slide to the bottom of the tube).
8. Spin at 12,700 rpm for 15 at 4 deg.C for 15'. As soon as spin is started, process the next set of plates through steps 4-7.
9. Aspirate supe till approx. 2 ml above cushion and then collect interface with a pasteur - see Tim's protocol. Collect ~2 ml / tube and pool. Check Ficoll concentration by refractometry and dilute to 10% (w/w) or lower - necessary to make sure it layers onto the sucrose gradient and doesn't sink.
10. Finish collecting interfaces from second set of plates and then pool all collected interfaces, ensure Ficoll is < 10% (w/w) and load onto 1 gradient. Spin 1 hr - 1hr 30', SW 27 at 2 deg.C.
11. Fractionate gradient from bottom - 0.3 - 0.5 ml fractions and read sucrose concentration by refractometry. Assay fractions between 48 and 60 % (w/w) sucrose. 5 ul of fraction + 5 ml PE - mix well and pellet onto coverslips: 12,500 rpm for 15' at 4 deg.C in HB-4. Post fix in methanol (-20 deg.C) for 5' and rehydrate and do 5051 + antitubulin followed by anti-mouse, anti-human secondaries. Assess peak by concentration of double staining dots, pool and freeze in liq N2 in 10 ul aliquots.