Isolation of mitotic spindles and/or midbodies

Adapted from Kuriyama and Ensrud, 1999; Mullins & McIntosh, 1982
by Ahna Skop (2000-2004)

CHO cells cultured in a DMEM/F-12 Medium or OPTI-MEM (Gibco)
HeLa (MEM + NEAA + 10%FBS + 15mM Hepes)
+ 1X antibiotics (of a 100X pen/strep stock) (Sigma #4668)
10% FBS (FCS,HyClone)
15mM HEPES, pH 7.2
incubate in a 5-10% CO2 incubator at 37C

100mM thymidine in ddH2O, sterilized	2.4g/100mls	10g/416mls
5mg/ml nocodazole in DMSO		
5mg/ml taxol in DMSO		
5mg/ml phalloidin		
50mM MES, pH 6.3		
50mM MES, pH 6.3;0.35% sarcosyl (from 30% stock)		
1X PBS (Ca2+, Mg2+ Free)		

**I use Fisher triple flasks (cell apartments as I call them-flasks that have 3 levels for growing). For midbody isolations I use 10 cell apartments for one mass spec run. You need a LOT since during mitotic shake off you lose quite a bit of cells. I use 50mls of culture medium per triple flask.

Cell synchronization:
1. Add thymidine to a final concentration of 2-5 mM onto a monolayer of cells at about 60-70% confluency on a 175cm2 Falcon flask. 1/20 dilution of 100mM thymidine. **2.5mls thymidine/50mls media or 5mls thymidine/100mls.**

WARNING: Do NOT overgrow your cells, otherwise you will get dead cells in your mitotic shake off, which you do not want.

2. Culture cells for 12-16 hr.

3. Remove thymidine and wash cells once with **serum-free** DMEM/F-12 or OPTI-MEM medium.
-wash with Ca^{2+} Free PBS

4. Culture cells for an additional 4-5 hrs in fresh culture medium. You can omit this step as Mullins did not do.

5. Add nocodazole to a final conc of 0.1ug/ml, and further culture cells for 4-4.5hrs. **1ul noco** [5mg/ml stock] in **50mls**

6. Harvest round cells at M phase by gentle shaking and centrifuge them at 1000rpm for 2min at 37C in a clinical centrifuge.

NEXT STEPS MUST BE CARRIED OUT AT 37C. PREWARM CULTURE MEDIA, ddH20, etc!!!

7. Resuspend the cell pellet with **10ml prewarmed culture medium** and transfer into a 15ml conical tube. Wash 1x (1000g, 2min) Set time zero when nocodazole has been washed away.

8. Resuspend the cell pellet with **2ml prewarmed culture medium** and allow to stand for **15min (for spindles), 30-35min for midbody isolation** at 37C. (May take 60min)

9. Prepare the spindle isolation buffer by addition of Pipes, Triton X-100, and taxol stock solutions to PREWARMED (37C) ddH2O. **PREPARE FRESH!!!!**

Spindle isolation buffer	**12mls**
ddH2O (tissue grade)	11mls
2mM Pipes, pH 6.9	25ul 1M PIPES, pH 6.9
0.25% Triton X-100	21ul Triton X-100
20ug/ml taxol	48ul taxol [5mg/ml stock]

10. Add taxol & phalloidin to the cell suspension to a final concentration of 5ug/ml and stabilize spindle microtubules and actin *in vivo*. Allow to stand for 30sec. Note that prolonged incubation of mitotic cells with taxol causes reorganization of MTs arrays. **2ul taxol** (5mg/ml taxol) + **2ul phalloidin** (5mg/ml) in 2mls (from step 8).

11. Spin down cells at 1000rpm for 1min and discard the supernatant.

12. Carefully wash the tube by dropping prewarmed ddH2O down the wall of the tube without disturbing the pellet.

13.After removing the washing liquid carefully (by aspiration), resuspend the cell pellet in 100 Volumes of the spindle isolation buffer. Disrupt cells by gentle pipetting and/or vortexing. 4 Flasks-- (resuspend in **300-400ul** buffer)

14. Check the spindle isolation by phase-contrast microscopy. Under these hypotonic conditions, chromosomes become dissociated and the spindle should be free from any cell debris. I incubated for ~15-20min **-Mullins said---immediately spin down**

15. Sediment spindles/midbodies at 2000rpm for 20min. at RT in a clinical centrifuge.

At this point you can take a sample and antibody stain for actin and tubulin to check for proper isolation of structures. Just drop 1-5ul on a slide and fix.

Midbody extraction (to get rid of MTs):
You need to do this for better peptide determination by mass spec (actin and tubulin are considered contaminants in mass spec due to their abundance)
16. Chill midbody pellets on **ICE** and wash with 50mM MES, pH 6.3 (1000g 2 min).
-use 1ml washes (transfer to ufuge tube) Bring pellet up in 100ul of MES

17.Put 100ul solution over a 40% glycerol cushion

18. Spin 5min at full speed

19. Wash in MES

20. Bring up in 100ul of MES

21. Protein were concentrated by precipitation

- 100ul protein soln + 400ul MeOH
- Vortex
- 100ul Chloroform
- Vortex
- 400ul ddH2O
- Vortex
- Spin at full speed 2min
- Remove upper layer
- + 300ul MeOH
- Vortex
- Spin for 5min
- Air dry
- Resuspend in 20-40ul of 2X sample buffer
- Run 5ul to check

Electrophoresis:
Pellets of isolated midbodies or spindles are resuspended in SDS-sample buffer, boil for 10min, and run on 7.5% acrylamide gels and stained with Coomassie Blue or Silver Stain.

Midbody isolation (Mullins and McIntosh, 1982)

For what it's worth here is the synchronization procedure we used:

(1) Block in 2.5 mM thymidine (in standard F-12 medium plus 5% FBS)for 16 h

(2) Remove the thymidine by pouring off the medium from the culture flask/bottle and then washing the monolayer 2x with sterile PBS (Ca++ and Mg++ free) in generous amounts

(3) Pour off last PBS and add culture medium with 0.12 ug/ml nocodazole

(4) allow cells to enter mitosis and accumulate as arrested mitotic cells in the nocodazole medium

(5) Flasks were shaken / roller bottles swirled to dislodge adherent mitotic cells (CHO's become nicely rounded in mitosis and it usually doesn't take much force to dislodge them from the substratum)

(6) Cells were pelleted (1K rpm, 1 min in a Sorvall GLC1 table-top centrifuge) and pellets were resuspended in fresh, warm (37 C) medium, and pooled into 15 ml Corex, round bottom centrifuge tubes

(7) a small magnetic stir bar was placed in the bottom of each Corex tube, and the tube was placed into 37 C water (water was in a plastic basin and temperature was maintained with a circulating heater; the basin was positioned on top of a magnetic stirrer; gentle stirring with the stir bar kept cells circulating in the tubes so that they did not settle during this incubation. The tubes were capped with Parafilm to prevent water from the bath splashing in)

(8) I monitored small aliquots of the cell suspension, using phase contrast microscopy to determine when most of the cells had furrowed - usually this took 20-30 minutes. The rest of the procedure is provided in decent detail in the Methods section of the paper.

(Mullins and McIntosh, 1982)

1. As soon as the vast majority of cells had completed furrowing (~25-35min)
Pellet cells at 200g for 3min. Gently resuspend in 25 volumes of

Hypotonic swelling solution:

1M hexylene glycol (2-methyl-2, 4-pentandiol)
20uM MgCl2
2mM PIPES, pH 7.2 at RT

2. Immediately pellet cells at 200g for 3min and vigorously resuspend in 50 volumes of

Lysing Solution:

1M hexylene glycol
1mM EGTA
1% Nonidet P-40
2mM PIPES, pH 7.2

Warmed at 37C

3. Vigorously vortex for 30sec in Lysing Solution.
4. Stabilize midbodies by chilling on ice
5. Add 0.3 volumes of COLD

pH lowering solution:

1M hexylene glycol
50mM MES, pH 6.3
6. Centrifuge at 250 g for 10min to remove large debris.
7. Take supernatant and layer it over a cushion of 40% glycerol (w/v) in 50mM MES, pH 6.3
8. Centrifuge at 2800 g for 20min to pellet midbodies
9. Resuspend pellet in 50mM MES, pH 6.3
10. Centrifuge again at 2800g for 20min
11. Wash 2 X with 50mM MES buffer
12. Resuspend in 50mM MES to appropriate concentration.

Extraction…

13. Add Sarkosyl NL-30 (1% wt/vol) in 50mM MES, pH 6.3 and mildly vortex to a final conc of 0.2-0.6%.
14. Extraction was allowed to proceed on ICE and monitored by phase-constrast microscopy. When extraction was considered complete, the preps were centrifuged at 3700 g for 15min, the supernatant removed, and the pellet washed in 50mM MES,
15. Proteins in the supernant were concentrated by precipitation with
10 Volumes of COLD acetone & centrifuged at 3000g for 15 min.

Run proteins on 7.5% acrylamide gels
Stain with Coomassie Blue

50mM MES, pH 6.3

500mls	**100mls**
4.8g MES	0.96g MES
450mls ddH2O	90mls ddH2O
pH	pH
bring up to 500mls	bring up to 100mls

50mM MES, pH 6.3; 0.35% Sarcosyl

100mls

98mls MES, pH 6.3
1.2mls 30% Sarcosyl

1M PIPES, pH 6.9

100mls

33.5g PIPES
80mls ddH2O
pH
bring up to 100mls

Tissue Culture Procedures

Hood In Use
UV OFF, FAN ON, POWER ON
Bring pipets, tips, pasteur pipets, racks, marker, EtOH into hood

Prewarm Media, trypsin, PBS (Ca2+, Mg2+Free)

Write on NEW flask:
Name
Cell types
Dilution
Date
passage #

Add 7mls of media to new flask (150cm^2)

How to Trypsinize
Suck of media by aspiration
Wash w/PBS (3mls) & rock around to coat bottom & aspirate out
Add trypsin ~1ml (enough to coat the bottom
Check on scope @2-3minute to see if cells have detached
Suck off excess (not all)
Bang and detach cells on table top
Add 4mls media to resuspend, make sure to suck up back and forth to detach cells 2X
Suck up and put in 15ml conical
Add dilution ul amounts to flasks that you need to passage

Any left over cells should be aspirated into liquid waste
Turn off Light & Fan
Turn on UV