

## Isolation of Centrosomes from Cultured Cells

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The centrosome is a unique organelle that nucleates and organizes the interphase microtubule array and facilitates bipolar spindle assembly during mitosis. In the past, isolated centrosomes proved to be an ideal starting material for biochemical and structural studies as well as for functional assays to study microtubule-dependent processes. This chapter provides a practical method and the rationale of isolating centrosomes from adherent cultured cells by density gradient centrifugation. It further describes how to evaluate the centrosomes activity and function by microtubule nucleation and immunofluorescence assays and how to measure microtubule dynamics nucleated from isolated centrosomes in *Xenopus* egg extracts.

### 1. Introduction

Throughout the cell cycle the centrosome is the dominant microtubule-organizing center and thus involved in many fundamental microtubule-dependent processes including mitosis, cytokinesis, intracellular transport and regulation of cell shape and motility. Structurally, the centrosome consists of two centrioles, the mother and the daughter centriole, both embedded into a

pericentriolar matrix (PCM). It is duplicated once per cell cycle as bipolar spindle assembly requires two functional centrosomes.

The importance of the centrosome as a central cell-organizing center was already appreciated more than one century ago **(1)**. Still, there is an ongoing interest in understanding the molecular nature and function of centrosomes, especially since centrosomal abnormalities are linked to aneuploidy, chromosomal instability and thus cancer development **(2)**.

The successful isolation of centrosomes (or their functional equivalent) has been described from a number of species, including yeast **(3)**, surf clam oocytes **(4)**, mitotic sea urchin eggs **(5)**, the early *Drosophila* embryo **(6)**, cultured *Xenopus* cells **(7)** and mammalian tissues culture cells **(8-11)**. The purification of centrosomes from Chinese hamster ovary cells, as described in this chapter, was pioneered by Blackburn and colleagues **(12)**. However, the procedure described herein is largely based on the protocols by Mitchison and Kirschner **(8)** as well as Moudjou and Bornens **(10)**. Below, I will deconstruct the rationale of the protocol, facilitating potential adaptations for your cell line of choice.

The challenge when applying this method to a previously characterized or novel cell line is to obtain sufficiently pure centrosomes that still faithfully reconstitute all centrosome dependent activities. The treatment of cells with Nocodazole and Cytochalasin B depolymerizes microtubules and actin and consequently weakens the association of the centrosome with the nuclear envelope and helps to release the centrosome. Moreover, this treatment

reduces the contamination with tubulin, microtubule-associated proteins (MAPs) and actin. Before the cells are lysed in a low ionic strength buffer in the presence of detergent it is important (1) to remove all salts and metal ions (by washing with sucrose) and (2) to be fast (i.e. washes should happen in less than one minute per plate) as the cells swell and eventually burst. The first centrifugation serves as an initial clean up of the cell lysate, as cell debris and chromatin are spun down. The second centrifugation concentrates the centrosomes onto a Ficoll cushion, while the final density gradient centrifugation further concentrates the partially purified centrosomes into a sharp fraction according to the centrosomes sedimentation velocity. In some protocols a continuous sucrose gradient (20% - 62.5%) is used, whereas other protocols prefer a discontinuous gradient (40%, 50%, 70%). Here, I describe the use of a discontinuous gradient, as in my hands it proved to be faster and more robust. The high sucrose concentration in the peak fractions stabilizes centrosomal proteins and serves as a cryoprotectant when freezing. Finally, the isolated centrosomes can be analyzed by Western blot, functional (e.g. microtubule nucleation) as well as structural (localization of structural centrosome components by immunofluorescence) assays.

## **2. Material, Solutions and Equipment**

### **2.1. Cell Culture**

1. Chinese hamster ovary (CHO) cells

2. MEM  $\alpha$  medium (Invitrogen #32561037), supplemented with
  - Fetal bovine serum, dialyzed (Invitrogen #26400044); final conc. 10%
  - Penicillin-Streptomycin; final conc. 100 units penicillin and 100  $\mu$ g streptomycin per 500 ml medium
  - L-Glutamine; final conc. 2 mM
3. Cytochalasin B (Sigma C6762); stock solution 10 mg/ml in DMSO
4. Nocodazole (Sigma M1404); stock solution 10 mg/ml in DMSO
5. Incubator preset to 37°C and 5% CO<sub>2</sub>
6. Tissue culture dishes (245 x 245 x 25 mm) (Nunc #166508)

## **2.2. Isolation of Centrosomes**

1. 2-Mercaptoethanol
2. EDTA
3. Ficoll®; lyophilized powder (Sigma F8016)
4. Glycerol p.a.
5. NP-40 / Tergitol® solution, 70% in H<sub>2</sub>O (Sigma NP40S)
6. PIPES
7. SigmaFAST™ Protease Inhibitor Cocktail Tablet (Sigma S8830)
8. Sucrose, UltraPure
9. TRIS

The following solutions can be prepared one day before the actual isolation of the centrosomes. Store all solutions at 4°C.

1. 1 mM Tris, pH 8.0 (before use add 8 mM 2-Mercaptoethanol)
2. 1xPBS (137 mM NaCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>)
3. 8% Sucrose in 0.1xPBS (w/w) (see **Note 1**)
4. 8% Sucrose in ddH<sub>2</sub>O (w/w)
5. 50xPE (500 mM PIPES, pH to 7.2 with KOH; 50 mM EDTA)
6. Lysis buffer (1 mM Tris, pH to 8.0 with HCl; 0.5% NP-40; before use add 8 mM 2-Mercaptoethanol and protease inhibitors)
7. Ficoll cushion (20% Ficoll (w/w) in 1xPE, 0.1% NP-40); stir at RT for several hrs to dissolve; before use add 8 mM 2-Mercaptoethanol
8. Standard solution 10% Ficoll in 1xPE, 0.1% NP-40 to calibrate the refractometer
9. Sucrose density gradient solutions
  - 40% Sucrose in 1xPE, 0.1% NP-40 (w/w)
  - 50% Sucrose in 1xPE, 0.1% NP-40 (w/w)
  - 70% Sucrose in 1xPE, 0.1% NP-40 (w/w) (see **Note 2**)
10. Centrifuges
  - BECKMAN Preparative Ultracentrifuge Optima LE-80K
  - Heraeus Megafuge 1.0R
11. COREX® II Centrifuge Tubes (30 ml)
12. Pasteur pipette
13. Refractometer
14. Rotating platform
15. Rotors

- JS-13.1, Swinging-Bucket (Beckman Coulter)
  - SW 28 Ti, Swinging Bucket (Beckman Coulter)
16. Tubes, Thinwall, Ultra-Clear™, 38.5 ml (Beckman #344058)
17. Vacuum trap

## **2.3. Centrosome Analysis**

### **2.3.1. Western Blot Analysis**

1. Mouse anti- $\gamma$ -tubulin monoclonal antibody (SIGMA T6557)
2. Standard material, solutions and equipment for SDS-PAGE and Western blotting.

### **2.3.2. Structural and Functional Analysis**

1. EGTA
2. Ethanol
3. Fetal Bovine Serum
4. Glutaraldehyde, 25% in H<sub>2</sub>O, EM grade (Sigma G5882)
5. Glycerol p.a.
6. GTP
7. Labeled tubulin (see **Note 3**)
8. MgCl<sub>2</sub>
9. Methanol
10. Mounting medium

11. Mouse anti- $\gamma$ -tubulin monoclonal antibody (Sigma T6557) or any other antibody of interest
12. NaBH<sub>4</sub>, pellets, Cobalt (Doped) (Sigma-Aldrich #247677)
13. 1xPBS
14. PIPES
15. Secondary antibody labeled with a fluorescent dye; e.g. Alexa Fluor® 594 donkey anti-mouse IgG (Sigma A21203)
16. Triton X-100
17. Tubulin (see **Note 3**)
18. 1xPE (100 mM PIPES, pH to 7.2 with KOH; 10 mM EDTA)
19. 5xBRB80 (400 mM PIPES, pH to 6.8 with KOH; 5 mM MgCl<sub>2</sub>, 5 mM EGTA)
20. Cushion (1xBRB80, 25% glycerol, 1 mM GTP)
21. Fix solution (1xBRB80, 10% glycerol, 0.1% TX-100, 0.25% glutaraldehyde, 1 mM GTP)
22. PBSTS (1xPBS, 0.1% TX-100, 10% FBS)
23. *Xenopus* egg extract (see **Note 4**)
24. Coverslips ( $\varnothing$  12 mm) (see **Note 5**)
25. Flat bottom PP-Tubes (Greiner bio-one GmbH #863504)
26. Heraeus Megafuge 1.0R
27. Microscope slides (see **Note 5**)
28. Needle (18G x 1.5 Inch)

### 2.3.3. Measuring Microtubule Dynamics

1. Catalase (10 mg/ml in 1xBRB80, 50% glycerol; store at -20°C)
2. Glucose (1M in ddH<sub>2</sub>O; store at -20°C)
3. Glucose oxidase (10 mg/ml in BRB80, 50% glycerol; store at -20°C)
4. Hemoglobin
5. Labeled tubulin (see **Note 3**)
6. Antifading mix (5 µl of catalase solution, 5 µl of glucose oxidase solution, 5 µl of 1M glucose)
7. Saturated hemoglobin solution (30-40 mg/ml in ddH<sub>2</sub>O; spin and pipette off the supernatant. Prepare freshly!)
8. *Xenopus* egg extract (see **Note 4**) or *in vitro* (see **Note 6**)
9. Coverslips (22 x 22 mm) (see **Note 5**)
10. Imaging analysis software
11. Inverted fluorescence microscope (e.g. Axiovert 200; Carl Zeiss, Inc.)  
equipped with a
  - 100x Plan Apochromat NA 1.4 oil immersion objective lens
  - CCD camera (e.g. CoolSNAP; Roper Scientific)
  - Long-pass rhodamine filter
12. Microscope slides (see **Note 5**).

## 3. Methods

### 3.1. Cell Culture

Expand the CHO cells to 15 24.5x24.5 cm plates, which should be near to confluency the day of use. On the day of the centrosome isolation, exchange CHO growth medium with medium containing Nocodazole and Cytochalasin B (final concentrations of 10 µg/ml and 5 µg/ml, respectively). Return the plates to the 37°C incubator for 90 min.

### **3.2. Isolation of Centrosomes**

All solutions and equipment should be at 4°C and all work should be done at 4°C.

1. Before starting remember to add 2-Mercaptoethanol to the 1 mM Tris washing solution and 2-Mercaptoethanol and protease inhibitors to the lysis buffer. Just before pouring the gradient add 28 µl 2-Mercaptoethanol per 50 g sucrose solution.
2. Prepare the discontinuous sucrose gradient by progressively layering less dense sucrose solutions upon one another; therefore pipette 5 ml 70 %, 3 ml 50% and 3 ml of 40% sucrose solution in a thinwall, Ultra-Clear Beckman tube (max Volume 38.5 ml).
3. Bring one cell culture dish at a time to the cold room, aspirate the medium and successively wash the cells as fast as possible with approximately 60 ml per plate of
  - 1xPBS
  - 8% Sucrose in 0.1xPBS
  - 8% Sucrose in ddH<sub>2</sub>O

- 1 mM Tris, pH 8.0; 8 mM  $\beta$ -ME
4. Finally add 30 ml lysis buffer and shake each plate on a rotating platform for at least 20 min.
  5. Combine the cell lysates from all plates, add 1/50 Volume of 50xPE and spin in 30 ml COREX tubes in a JS-13.1 rotor at 3 000 rpm (1 500 x g) for 3 min at 4°C.
  6. Prepare fresh COREX tubes with a 2 ml Ficoll cushion. Collect the supernatants after the first spin and carefully apply upon the Ficoll cushions. Alternatively, the supernatants can be underlayered with the Ficoll cushion using a syringe.
  7. Spin at 12 700 rpm in a JS-13.1 rotor (25 000 x g) for 15 min at 4°C, no brake.
  8. Aspirate the supernatant until you reach approximately 2 ml above the Ficoll cushion. Carefully collect the final 2 ml above the Ficoll cushion with a Pasteur pipette. The centrosomes should be concentrated in this layer. Pool all interfaces.
  9. To get a reasonable density of centrosomes, it is important to layer all centrosomes onto a single sucrose gradient, therefore make sure to not exceed the total volume of the Ultra-Clear Beckman tube (max Volume 38.5 ml, cushion 11 ml).
  10. Before applying the centrosomes upon the sucrose density gradient make sure the Ficoll concentration is less than 10% by using a refractometer. If the Ficoll concentration is >10%, dilute with lysis buffer with 1xPE. This is

essential to make sure that the centrosomes will layer upon the sucrose gradient.

11. Spin in a SW 28 Ti rotor at 27 000 rpm (130 000 x g) for 90 min at 4°C, no brake.
12. Fractionate the gradient manually from either the bottom (by puncturing the bottom of the tube) or the top or by using an automated fraction collector. Collect 0.5 ml fractions and determine the sucrose density by refractometry. Check the fractions between 40% and 60% sucrose for centrosomes by Western blot analysis (as described in 3.3.1.).
13. Pool the centrosome-containing fractions (see **Note 7**) and snap-freeze 20 µl aliquots in liquid nitrogen. Store long-term at -80°C.

### **3.3. Centrosome Analysis**

#### **3.3.1. Western Blot Analysis**

To determine in which fraction the centrosomes peak, take a 10 µl sample of each fraction in the 40-60% sucrose range and process it for SDS polyacrylamide gel electrophoresis and subsequent Western blot analysis.

In a NUPAGE 4-12% Bis-Tris gel (Invitrogen)  $\gamma$ -tubulin travels at 51 kDa (HiMark™ Pre-stained Protein Standard, Invitrogen). If you are interested in the enrichment throughout the isolation process, samples at different steps of the purification procedure can be taken and probed against  $\gamma$ -tubulin (see **Figure 1**).

### 3.3.2. Structural and Functional Analysis

The centrosomes can be assayed functionally by (1) an *in vitro* polymerization reaction or (2) incubating them in *Xenopus* egg extracts. In either case, the samples can be further processed for immunofluorescence to visualize a protein of interest.

Set up one of the following reactions:

1. *In vitro* polymerization reaction
  - Tubulin (3 mg/ml)
  - Cy3-labeled tubulin (0.25 mg/ml at a 1.5 labeling ratio)
  - GTP (1 mM)
  - Centrosomes (1  $\mu$ l of the peak fraction)
  - 1xBRB80 (ad 20  $\mu$ l)
  - Incubate at 37°C for 20 min.
2. *Xenopus* egg extract reaction
  - Cy3-labeled tubulin (0.25 mg/ml at a 1.5 labeling ratio)
  - Centrosomes (1  $\mu$ l of the peak fraction)
  - *Xenopus* egg extract (ad 20  $\mu$ l)
  - Incubate at 18°C for 20 min.
3. Place a coverslip into a flat bottom tube and overlay with 3 ml cushion (1xBRB80, 25% glycerol, 1 mM GTP).
0. Stop the *in vitro* / *Xenopus* egg extract reaction by adding 1 ml fix solution / 20  $\mu$ l reaction.

1. Spin 1 ml over the glycerol cushion onto a coverslip at 3 000 rpm, 20°C for 15 min in a clinical centrifuge.
2. Aspirate the solution and most of the cushion, leave about 0.5 ml.
3. Pick up the coverslip by pushing with a needle from the bottom of the plastic tube.
4. Fix in cold methanol at -20°C for 5 min.
5. Quench 2 x with 0.1% NaBH<sub>4</sub> in PBS for 10 min.
6. Wash 3 x with 1xPBS.
7. Process for immunofluorescence:
  - Block in PBSTS for 60 min at RT or overnight at 4°C.
  - Incubate with 1<sup>st</sup> antibody (e.g. anti-γ-tubulin diluted 1:1 000 in PBSTS) for 4h at RT or overnight at 4°C.
  - Wash 3 x with 1xPBS.
  - Incubate with 2<sup>nd</sup> antibody (e.g. Alexa Fluor® 594 donkey anti-mouse IgG diluted 1:500 in PBSTS) for 60 min at RT, in the dark.
  - Wash 3 x with 1xPBS.
  - Embed with mounting medium onto a microscope slide.

### **3.3.3. Measuring Microtubule Dynamics**

#### ***Time-Lapse Microscopy***

Ideally the microscope room or if available an incubation chamber at the microscope is set to 20°C.

1. Set up the following reaction:
  - 10  $\mu$ l CSF-extract
  - 1  $\mu$ l purified human centrosomes
  - 0.25 mg/ml Cy3-labeled tubulin (1.5 labeling ratio)
  - 0.5  $\mu$ l of saturated hemoglobin solution
  - 0.33  $\mu$ l of antifading mix
2. Gently mix the reaction and keep at 20°C.
3. Squash 2.7  $\mu$ l of the mixture under a 22 x 22 mm coverslip.
4. Locate a single centrosome (as shown in **Figure 2**)
5. Determine the minimum exposure time.
6. Start recording time lapses, ideally in 2-s intervals for at least 1 min, (it might be necessary to refocus while imaging) and compile the images into stacks.

### ***Image Analysis***

Use an appropriate imaging analysis software, e.g. ImageJ or MetaMorph, to track the microtubules. Track the position of the centrosome in the first frame of the stack and subsequently the (+) end of a given microtubule at least over 20 frames (i.e. 40 s). The centrosome is set as the origin of the microtubule, while in each following frame the (+) end's pixel location is noted. The distance between the two points measured, i.e. the centrosome and the microtubule (+) end, thus simply represents the microtubule length. Pixel location over time consequently represents microtubule growth, shrinkage or

pausing. Finally, for each microtubule tracked you obtain a file with two columns containing (1) time in seconds and (2) length of microtubules in pixels (ideally converted into  $\mu\text{m}$ ). These coordinates can be read by a Matlab macro (see **Note 8**), which will calculate microtubule dynamics, which are characterized by four parameters: (1) rate of growth ( $\mu\text{m}/\text{min}$ ), (2) rate of shrinkage ( $\mu\text{m}/\text{min}$ ), (3) catastrophes defined as the frequency of transition from growth to shrinkage ( $\text{min}^{-1}$ ), and (4) rescues defined as the frequency of transition from shrinkage to growth ( $\text{min}^{-1}$ ). In addition, the pausing time is given. In order to get reasonable statistics, I would recommend analyzing at least 60 microtubules for a minimum of 30 s each.

#### 4. Notes

- (1) Some of the solutions in this protocol are w/w. This means for a 20% sucrose solution you weigh out 20 g sucrose and then add buffer until the weight is 100 g.
- (2) 70% sucrose does not go into solution completely until it is heated.
- (3) Porcine tubulin can be ordered (Sigma T6954) or prepared as described in **(13)**. Labeled tubulin can be ordered (Cytoskeleton, Inc. TL331M) or prepared as described in **(14)**.
- (4) CSF-arrested *Xenopus* egg extract is prepared as described in **(15)**. Extract prepared by this protocol is arrested in M-phase. If you are interested in interphase processes, an interphase egg extract can be prepared as describe in **(16)**.

- (5) Coverslips and microscope slides should be washed in ethanol, rinsed in ddH<sub>2</sub>O, dried on a Whatman paper and kept dust-free in a tissue culture dish.
- (6) Microtubule assembly from centrosomes can also be observed in BRB80 buffer in the presence of tubulin as described in (17).
- (7) To determine the centrosome concentration, spin the centrosomes onto coverslips (as described in 3.3.2.), assume a quantitative sedimentation and count the number of centrosomes in a chosen area. A good yield is  $1 \times 10^8$  centrosomes/0.5 ml.
- (8) The Matlab macro was developed by Francois Nedelec (EMBL, Heidelberg) and is available online (<http://www.cytosim.org/>).

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### Figures Captions

**Figure 1: Centrosomal Isolation Profile** Throughout the centrosome isolation procedure samples were taken at the indicated steps and processed for Western blot analysis, 5  $\mu$ l of each sample were separated on a 4-12% Bis-Tris gel and probed against  $\gamma$ -tubulin.

**Figure 2: Dynamic Measurements of Centrosomally Nucleated Microtubules in Metaphase.** CSF-arrested egg extracts was supplemented with isolated centrosomes and Cy3-labeled tubulin. Still image of fluorescence recording from time-lapse video, note that the fluorescent image is shown as an inverted image making the (+) end tracking of microtubules more convenient. Scale bar: 10  $\mu$ m.