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A Cell-Free System for Real-Time Analyses of Centriole Disengagement and Centriole-to-Centrosome Conversion

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Abstract

Centriole or centrosome number in cycling cells is strictly maintained through coordinated duplication and segregation. Duplication is limited to once only per cell cycle by separating the assembly event that occurs in S/G2 phase from the two licensing events, centriole disengagement and centriole-to-centrosome conversion, both of which occurs in mitosis. In addition to duplication licensing, centriole-to-centrosome conversion also enables centrioles to associate with spindle poles and thereby to segregate equally during cell division. Centriole disengagement and centriole-to-centrosome conversion thus constitute the major regulatory module ensuring centrosome homeostasis in cycling cells. Using *Xenopus* egg extracts and purified engaged centrioles, we here describe an in vitro assay allowing us to synchronously induce the initiation of centriole disengagement and centrosome formation, pause the reaction anytime during the process, and more importantly, preserve “reaction intermediates” for further analyses.

Keywords

Centriole; Centrosome; Disengagement; Duplication licensing; Centriole-to-centrosome conversion; MTOC; PCM; *Xenopus* egg extract

¹We use HeLa cells for centriole isolation, because centrosomes can be easily detached from nuclei through the procedure described here, which is not the case for some cell types. However, after a few strokes of douncing, check the lysed cells under microscope to make sure that centrosomes are released from cells and efficiently detached from nuclei.

²Do not use a tight pestle to break cells, as it may fragment the nuclei into small pieces.

³Sucrose gradients should be freshly made. Do not disturb the gradient when loading the lysate.

⁴Engaged centrioles normally peak at fractions 9–11.

⁵The robustness of the assay critically depends on the quality of the extract and thus the quality of eggs. Energy mix is not required for this assay.

⁶The coverslip needs to be removed quickly while the squashed extract remains frozen.

⁷It is not a good idea to fix the squashed, frozen extract with PFA, as the extract would melt, and then quickly come off the slide during the fixation.

⁸Importantly, in the frozen extract, the relative position of mother and daughter centriole in each pair of “disengaging centrioles” can be instantly captured, and permanently maintained after fixation, allowing further analyses by immunostaining (Fig. 3).

⁹A PBS wash (at the end of a wash series) to remove Triton X-100 is critical, as the residual detergent on the slide would affect the surface tension of the antibody solution added later, making the immunostaining process problematic.

¹⁰Similarly, antibodies (both primary and secondary) should be diluted in PBSB free of detergent.

¹¹The clearing solution (benzyl alcohol–benzyl benzoate) is not water soluble, so the extract must be completely dehydrated with 100 % methanol and air-dried before mounting.

¹²The clearing solution possesses little or no anti-fade activity, so the subsequent imaging process needs to be done quickly with care.

1 Introduction

Animal centrioles form the core of the centrosome or microtubule-organizing center (MTOC), and have profound effects on nearly all microtubule-based processes including spindle formation and cell division. Centriole or centrosome numbers in cycling cells are carefully maintained [1]. Vertebrate cells begin the cell cycle with two centrioles, each of which duplicates in S phase, generating two pairs of mother–daughter centrioles that are tightly attached or engaged to each other. In mitosis, the two pairs of engaged centrioles segregate equally to two daughter cells through association with spindle poles, and subsequently lose their tight engagement at late mitosis, restoring the normal copy number of centrioles for the next cell cycle.

Centrioles can duplicate and segregate (through association with spindle poles) only when they have been converted to centrosomes [2]. The conversion is a process in which newborn centrioles acquire the competence to recruit the pericentriolar material (PCM) and thereby function as the MTOC or centrosome. Centriole-to-centrosome conversion starts in early mitosis depending on Plk1 and CEP295 [2, 3], and completes at the end of the cell cycle together with centriole disengagement, giving rise to one previously converted and one newly converted centrioles that are both MTOC-competent and disengaged. These disengaged, converted centrioles serve as mother centrioles that duplicate in the following S phase and carries the newborn, MTOC-non-competent daughter centriole to which it is engaged through the segregation process, ensuring centriole homeostasis. Importantly, only centrioles that are both converted and disengaged can duplicate, whereas non-converted centrioles, engaged or not, are “infertile” [2, 3]. These two requirements, centriole-to-centrosome conversion and centriole disengagement, fully exclude unlimited duplication in one cell cycle.

Aberration in centriole/centrosome numbers is known to negatively impact spindle formation, chromosome segregation and cell division [4, 5], but mechanisms underlying centriole-to-centrosome conversion and centriole disengagement remain elusive. A useful approach is to develop an in vitro assay recapitulating both processes. Here, we describe a cell-free system in *Xenopus* egg extracts [6] allowing real-time monitoring and characterization of centriole disengagement and centrosome formation as they occur in late mitosis [7].

2 Materials

2.1 Isolation of Human Engaged Centrioles

1. HeLa cell expressing centrin2-GFP [7].
2. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin (all Invitrogen).
3. Aphidicolin (Sigma): 2 mg/ml in DMSO as the stock solution (1000×), stored in –20 °C.
4. Nocodazole (Sigma).

5. Hypotonic buffer (20 mM K-HEPES pH 7.8, 5 mM K-acetate, 0.5 mM MgCl_2 , 0.5 mM DTT make fresh).
6. Cell Scraper (Nunc).
7. Falcon Cell Strainers (Fisher).
8. DNase I (Stratagene): 10 U/ μl .
9. Sucrose gradient buffer (SGB): 10 mM HEPES pH 7.8, 0.1 % betamercaptoethanol, 0.1 % Triton X-100.
10. Dounce tissue grinder.
11. Discontinuous sucrose gradient. From the bottom of centrifuge tubes, sequentially load 5 ml of 70 % (W/W) sucrose, 3 ml of 50 % (W/W) sucrose, and 3 ml of 40 % (W/W) sucrose solutions. Sucrose solutions are made in sucrose gradient buffer.
12. Benchtop centrifuge (Fisher).
13. Ultracentrifuge with a SW 32 Ti or SW 28 rotor (Beckman).
14. Ultra-Clear™ centrifuge tube (Beckman; 344058).
15. Microscope (Zeiss; Axio Imager A1).

2.2 CSF Extract Preparation from *Xenopus laevis*

1. Pregnant mare serum gonadotropin (PMSG) (Sigma): 100 U/ml in sterile water.
2. Human chorionic gonadotropin (HCG) (Sigma): 100 U/ml in sterile water.
3. MMR buffer: 100 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 5 mM HEPES, pH 7.8.
4. Extract buffer (XB): 10 mM potassium HEPES pH 7.7, 1 mM MgCl_2 , 0.1 mM CaCl_2 , 100 mM KCl, 50 mM sucrose—make fresh.
5. CSF-XB: 10 mM HEPES pH 7.7, 2 mM MgCl_2 , 0.1 mM CaCl_2 , 100 mM KCl, 5 mM EGTA, 50 mM sucrose—make fresh.
6. 2 M sucrose.
7. Dejellying solution: 2 % cysteine in XB, pH 7.8—make fresh.
8. Protease inhibitors (1000 $\times$): 10 mg/ml of leupeptin, chymostatin, and pepstatin in DMSO
9. Cytochalasin D in DMSO (10 mg/ml; 1000 $\times$).
10. 18 gauge needle (BD 305195).
11. Swing bucket rotor (e.g., H-6/Sorvall or SW-55/Beckman) and centrifuge tubes (e.g., Sorvall 03124).

2.3 Analyses of Centriole Disengagement and Centriole-to-Centrosome Conversion in Egg Extracts

1. Glass slides and 12 mm round coverslips (Fisher Scientific).
2. Liquid nitrogen.
3. Razor blade (Fisher Scientific; heavy duty; S95932).
4. Slide jar (Wheaton).
5. 100 % methanol (−20 °C).
6. PBS: phosphate buffered saline.
7. PBST: 0.1 % Triton X-100 in PBS.
8. PBSB: 3 % bovine serum albumin (BSA) in PBS.
9. Antibodies: anti-GFP (BioLegend), anti-human C-Nap1 [7], anti-human SAS-6 (Santa Cruz), anti- γ -tubulin (Santa Cruz), anti-pericentrin (Abcam), and appropriate secondary antibodies (Invitrogen), all diluted in PBSB.
10. Clearing solution [8]: mix 1 volume of benzyl alcohol to 2 volumes of benzyl benzoate (1:2; both from Sigma).
11. Nail polish.
12. Fluorescence microscope (e.g., Axio Imager A1, Zeiss).

3 Methods

3.1 Isolation of Engaged Centrioles Labeled with Centrin-GFP

1. Our centrosome isolation is based on previously published protocols [9–11], with some critical modifications.
2. Grow 7–10 large plates (150 mm) of HeLa cells expressing Centrin-GFP to ~70 % confluency.
3. Add 2 μ g/ml aphidicolin and incubate for 18 h to arrest cells at S phase.
4. After 18 h, add nocodazole to 5 μ g/ml, and incubate for 1 h to depolymerize microtubules, which facilitates the detachment of centrioles from nuclei.
5. Remove the medium and rinse cells twice with ice-cold hypotonic buffer (HB).
6. Add 20 ml of HB to each 150 mm plate and incubate at 4 °C for 10 min to swell cells.
7. Remove HB and scrape cells off the plate using cell scrapers.

8. Break the swollen cells in a Dounce tissue grinder using a loose pestle, and transfer the lysate to a Falcon tube. 5–10 strokes are sufficient to release centrosomes.
9. Centrifuge the lysate at $2000 \times g$ in a benchtop centrifuge with a swing bucket rotor at 4 °C for 5 min to pellet nuclei, and collect the supernatant.
10. Wash the pellet with 10 ml of HB to release trapped centrosomes and spin again at $2000 \times g$ at 4 °C for 5 min. Collect the supernatant.
11. Combine supernatants, add Triton X-100 to 0.5 %, and spin again at $2000 \times g$ at 4 °C for 5 min. Collect the supernatant.
12. Filter the supernatant through 70 μ m cell strainers.
13. Incubate the supernatant with 1 μ g/ml (2 U/ml) of DNase I on ice for 30 min.
14. Load the final supernatant onto discontinuous sucrose gradients in ultraclear centrifuge tubes (25 $\times$ 89 mm; Beckman) and spin at $122,000 \times g$ with SW 28 or SW 32 Ti rotor at 4 °C for 1 h.
15. Puncture the bottom of the ultracentrifuge tube with 18 gauge needles and collect 0.5 ml fractions in 1.5 ml tubes on ice. Centrosomes are normally trapped at the interphase of 70 and 50 % sucrose gradients.
16. Spot 1 μ l of each fraction on a slide and check the yield of centrioles (GFP labeled) with fluorescence microscopy. An example of engaged centrioles in a peak fraction is shown in Fig. 2a.
17. Aliquot and freeze centrosome-containing fractions in liquid nitrogen, and store at –80 °C. The concentration of centrosomes in peak fractions is around $2.5 \times 10^4/\mu$ l.

3.2 CSF Extract Preparation from *Xenopus laevis*

1. Our preparation of CSF extracts is similar to what is described by Murray [6].
2. Day 1: Inject female frogs (*Xenopus laevis*) with 50 U pregnant mare serum gonadotropin (PMSG) to prime ovulation.
3. Day 5: Inject the same female frogs with 250 U human chorionic gonadotropin (HCG) to induce final ovulation. Each injected frog should be placed individually in a static tank containing 1.5 l of $1\times$ MMR buffer at 18–20 °C for 16 h during which ovulation occurs.
4. Eggs are collected at day 6 and washed with fresh MMR buffer at 18 °C.
5. Remove MMR, treat eggs with 2 % cysteine in extract buffer (dejelling solution) to remove the jelly coat.
6. Gently wash dejellied eggs three times with extract buffer (XB), three times with CSF-XB, and two times with CSF-XB supplemented with

proteinase inhibitors (leupeptin, chymostatin, and pepstatin at 10 µg/ml), and cytochalasin D (10 µg/ml).

7. Using fire polished pasteur pipettes, transfer the eggs drop by drop into centrifuge tubes (e.g., Sorvall #03124; 13 × 100 mm).
8. Remove excess CSF-XB on top of the eggs.
9. Pack the eggs by centrifuging at $200 \times g$ for 1 min and then $500 \times g$ for 1 min, and aspirate the excess buffer.
10. Crush the eggs by centrifugation at 10,000 rpm ($16,400 \times g$) for 10 min at 16 °C in a swinging bucket rotor (e.g., H-6/Sorvall with proper adaptors).
11. Collect the cytoplasmic fraction, which is the middle layer in straw color, with an 18-gauge needle by puncturing the side of the centrifuge tube. Immediately place the extract in cold eppendorf tubes on ice.
12. Add 1/1000 volume of protease inhibitors and cytochalasin D, and 1/40 volume of 2 M sucrose to the extract. Mix thoroughly with blunted pipette tips.
13. Fresh CSF extracts should be used within 1 or 2 h.

3.3 Setup of Centriole Disengagement and Centriole-to-Centrosome Conversion Reaction in CSF-Released Extracts

1. This assay is based on Tsou and Stearns [7].
2. Combine 40 µl of fresh CSF extract with 1 µl of isolated engaged centrioles (approximately 2.5×10^4) in a 0.5 ml eppendorf tube and incubate at RT (~23 °C) for 10 min. Make sure centrioles are mixed thoroughly in the extract (with blunted pipette tips).
3. Add 0.4 µl of 40 mM CaCl_2 to trigger centriole disengagement and centriole-to-centrosome conversion, both of which occur robustly in the following 30–40 min during which the CSF extract exits mitosis (CSF-released extract).
4. Reactions can be stop and preserved at different time points for future analyses by adding 1 µl of 0.5 M EDTA followed by instant freezing in liquid nitrogen. Alternatively, the behavior of centrioles or centrosomes can be examined in real time as the reaction proceeds (see Subheading 3.4 below).

3.4 Examining the Centriole Disengagement and Centrosome Assembly Reaction in Real Time

At different time points after calcium addition, aliquot 1 µl of the reaction mix onto a glass slide (Fig. 1a), and squash the extract into a thin layer with a coverslip (12 mm round) (Fig. 1b, c). The behavior of GFP-labeled centrioles in a live reaction can then be directly observed under fluorescence microscopy, as revealed in Fig. 2b for centriole disengagement.

1. Alternatively, the squashed extract can be instantly frozen by immersing the whole slide into liquid N₂ for 1 min (Fig. 1d), capturing the reaction intermediates for further analyses.
2. To immunostain centrioles in the extract, take out the slide from liquid N₂ and quickly remove or pry off the coverslip with a single-edge razor blade (Fig. 1e), leaving a thin layer of frozen extract on the slide.
3. Immediately fix the frozen extract by immersing the slide in chilled 100 % methanol (−20 °C) in a jar for 5 min (Fig. 1f). After fixation, a thin, turbid layer of the extract can be seen stably attached to the slide (Fig. 1g).
4. Rehydrate the fixed extract with PBST for 5 min, and then with PBS for another 5 min in separate jars to wash out the detergent (Triton X-100) before staining.
5. Without drying or touching the rehydrated extract, carefully dry the rest of the slide by wiping off the PBS around the squashed sample using tissue paper, leaving the extract in a small square of hydrated area for the following immunostaining.
6. Cover the rehydrated extract with 30–40 µl blocking solution (PBSB) and keep the slide in a wet chamber for 1 h at RT (23 °C)
7. To examine centriole disengagement or centriole-to-centrosome conversion, incubate the extract with 30–40 µl of selected primary antibodies (e.g., anti-GFP, γ -tubulin, C-Nap1, or pericentrin), and keep the slide in a wet chamber for 1 h at RT, or overnight at 4 °C.
8. Wash the slide in jars twice with PBST for 5 min, and then with PBS for another 5 min.
9. Wipe off the excess PBS buffer around the sample, apply 30–40 µl of secondary antibodies onto the sample, and let them incubate for 1 h at RT in a wet chamber.
10. Wash the slide three times with PBST, and dehydrate the extract by immersing the slide in 100 % chilled methanol twice in separate jars, each for 5 min. Use freshly made 100 % methanol to ensure complete dehydration of the extract.
11. Take out the slide from methanol, and let methanol evaporate at RT for 1–2 min.
12. After the dehydrated extract becomes air-dry and appears turbid, apply 10 µl of clearing solution (benzyl alcohol–benzyl benzoate in 1:2 ratio) on top of the dry extract [8].
13. As the extract instantly becomes transparent, mount a 22 × 22 mm coverslip onto the clearing solution, remove the excess solution, and seal the coverslip with nail polish. Avoid trapping air bubbles in between the slide and coverslip.

14. The slide is now ready for viewing. An example of the immunostaining result that reveals centriole disengagement and centriole-to-centrosome conversion is shown in Fig. 3 (*see also* Note ¹³ below).

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¹³Figure 3 shows the coincidence of centriole disengagement (Fig. 3a–c) and centriole-to-centrosome conversion (Fig. 3b, c) in CSF-released extracts. GFP-centrin marks all centrioles, whereas C-Nap1 highlights mother centrioles, and SAS-6 labels only the daughter. At the 20 min time point after calcium addition, daughter centrioles start to recruit γ -tubulin and pericentrin as they are disengaging from the mother (Fig. 3b, c), marking the conversion of centrioles to centrosomes. Note that as the egg extract naturally lacks APC^{cdh1}-mediated protein degradation, SAS-6 is stably maintained in disengaged daughter centrioles, serving nicely as a daughter marker.

Fig. 1.

Preparation of a thin layer of frozen extract for immunostaining. **(a)** Aliquot 1 μ l of the reaction mix onto a glass slide. **(b, c)** Squash the extract into a thin layer using a 12 mm rounded coverslip. **(d)** Instantly freeze the squashed extract by immersing the whole slide into liquid N₂ for 1 min. **(e)** Take out the slide from liquid N₂ and quickly remove or pry off the coverslip with a single-edge razor blade while the extract is still frozen. **(f)** Immediately fix the frozen extract by immersing the slide into chilled 100 % methanol (–20 °C) in a slide jar for 5 min. **(g)** After fixation, a thin, turbid layer of the extract is left on the slide.

Fig. 2.

Centriole disengagement in real-time reactions. **(a)** GFP-labeled, engaged centrioles purified from HeLa cells. GFP signals are visualized by fluorescence microscopy. **(b)** Monitoring of centriole disengagement. Purified engaged centrioles were incubated with CSF extracts, and calcium was added to release the arrest. Centrioles at indicated time points after calcium addition were visualized directly by fluorescence microscopy. Note the separation of daughter centrioles (weak/small GFP foci) from mothers

Fig. 3.

Analyses of centriole disengagement and centriole to centrosome conversion in frozen extracts by immunostaining. (**a–c**) Centrioles and the relative position of the mother and daughter in the extract were fixed and examined with indicated antibodies at indicated time points after calcium addition. GFP-centrin marks all centrioles, C-Nap1 highlights mother centrioles (**a**), and SAS-6 labels only the daughter. Note that daughter centrioles start to recruit γ -tubulin (**b**) and pericentrin (**c**) as they are being disengaged from the mother (20 min time points). SAS-6 is stably maintained in disengaged daughter centrioles, as the egg extract naturally lacks the activity of APC^{cdh1} mediated protein degradation. At the 40 min time point, centrioles were fully disengaged, showing only the mother centriole