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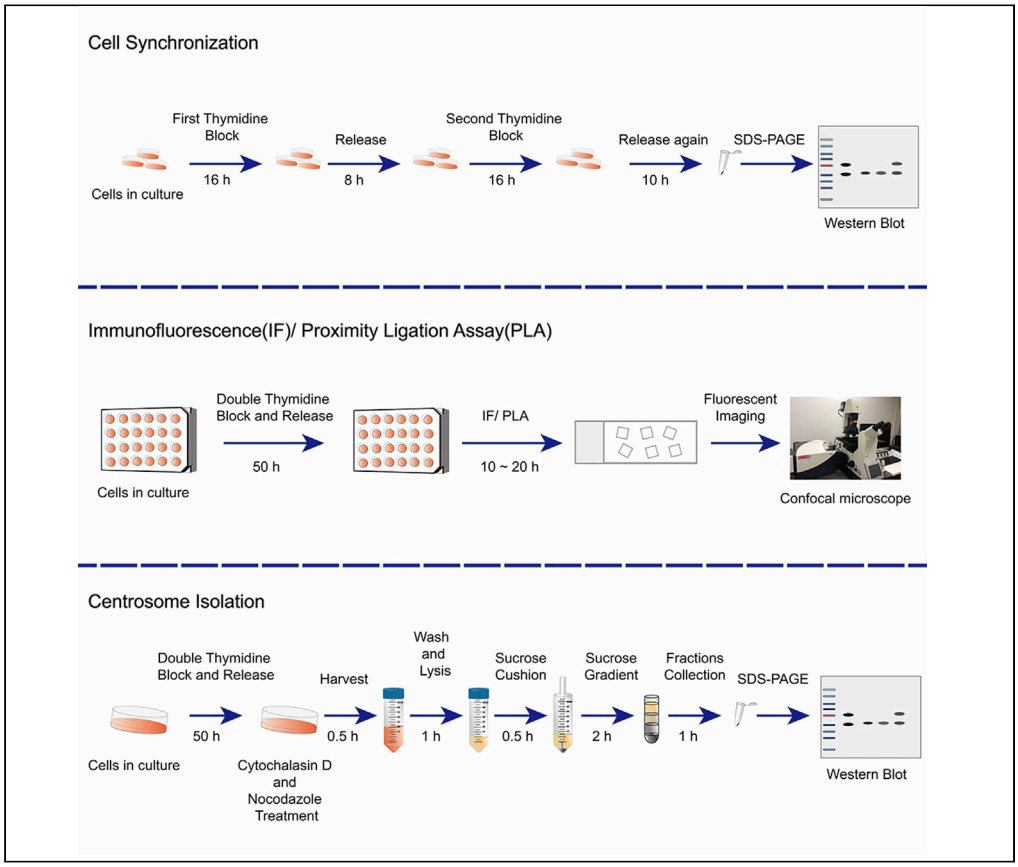
Protocol to identify centrosome-associated transcription factors during mitosis in mammalian cell lines



During eukaryotic cell mitosis, the nuclear envelope disintegrates and transcription factors are dissociated from condensed chromosomes. Here, we describe a protocol to study centrosomal translocation of nuclear receptor RXRα. We detail procedures for HeLa cell synchronization followed by immunofluorescence, in situ proximity ligation assay, and centrosome isolation. This protocol can be used to identify other transcription factors associated with the centrosome or other subcellular structures during mitotic progression.

Guobin Xie, Yuqi Zhou, Mingxuan Du, Qiqiang Wang, Jiajin Yi, Xiao-kun Zhang

xkzhang@xmu.edu.cn

Highlights

Complementary approaches to study RXRα translocation to the centrosome during mitosis

In situ PLA protocol to study centrosomal localization and action of RXRα

Applicable for studying other transcription factors during mitosis

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Protocol to identify centrosome-associated transcription factors during mitosis in mammalian cell lines

Guobin Xie,[1,3,4] Yuqi Zhou,[1,2,3,4] Mingxuan Du,[1] Qiqiang Wang,[1] Jiajin Yi,[1] and Xiao-kun Zhang[1,5,*]

[1]School of Pharmaceutical Sciences, Fujian Provincial Key Laboratory of Innovative Drug Target Research, Xiamen University, Xiamen 361102, Fujian, China

[2]NucMito Pharmaceuticals Co. Ltd., Xiamen 361101, Fujian, China

[3]These authors contributed equally

[4]Technical contact

[5]Lead contact

*Correspondence: xkzhang@xmu.edu.cn
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SUMMARY

During eukaryotic cell mitosis, the nuclear envelope disintegrates and transcription factors are dissociated from condensed chromosomes. Here, we describe a protocol to study centrosomal translocation of nuclear receptor RXRα. We detail procedures for HeLa cell synchronization followed by immunofluorescence, in situ proximity ligation assay, and centrosome isolation. This protocol can be used to identify other transcription factors associated with the centrosome or other subcellular structures during mitotic progression.

For complete details on the use and execution of this protocol, please refer to Xie et al. (2020)

BEFORE YOU BEGIN

This protocol integrates complementary cellular, molecular, and biochemical approaches, enabling a rapid and firm determination of RXRα as a centrosomal protein during mitosis. By using cell synchronization and centrosome isolation, we found that RXRα is heavily modified through phosphorylation by cyclin D kinase 1 (Cdk1) at the onset of mitosis, promoting its accumulation at the centrosome. We then demonstrated that both endogenous RXRα and exogenous mCherry-tagged RXRα reside at the centrosome during mitosis by immunofluorescence. We also use *in situ* proximity ligation assay (PLA) to confirm the centrosomal localization of RXRα and its interaction with polo-like kinase 1 (PLK1). This protocol can be easily modified to study the subcellular localization of other transcription factors during mitosis when they are dispersed from condensed chromosome.

The protocol below describes the specific experimental steps using HeLa cells. However, we also used this protocol in other cell lines, such as A549 cells and HepG2 cells.

Prior to the experiment, the drugs, the reagents and buffers should be prepared and stored under their recommended conditions. Protease and phosphatase inhibitor should be added to the lysis buffers right before they are used.

Drug preparation

Timing: 30 min

1. Thymidine stock solution (200 mM): weight 1 g thymidine powder and dissolve it in 20.64 mL double-distilled water. Then filter through 0.22 μm membrane filter under sterile condition and store in 1 mL aliquots at −20°C for up to 6 months.
2. Cytochalasin D stock solution (10 mg/mL): weight 5 mg cytochalasin D and dissolve it in 500 μL DMSO and store in 50 μL aliquots at −20°C for up to 6 months.
3. Nocodazole stock solution (10 mM): weight 2 mg nocodazole and dissolve it in 664 μL DMSO and store in 50 μL aliquots at −20°C for up to 6 months.

Reagent and buffer preparation

Timing: 10 min

4. Cell fixing buffer (−20°C methanol): pour 30 mL methanol into 50 mL centrifugation tube and place it at −20°C for 12∼16 before it is used.
5. Cell permeabilizing buffer (0.05% Triton X-100): add 20 μL Triton X-100 to 40 mL 1× PBS buffer and dissolve it by vortex. Then store it at 4°C for 1 h before it is used.

Cell culture assessment

Timing: 3 days

6. HeLa cells are maintained in MEM/EBSS Medium containing 10% fetal bovine serum (FBS) and 1% PS (penicillin streptomycin antibiotic) in an incubator 37°C with 5% CO_2. Cells need to be passaged when they are 70%–90% confluent and the subcultivation ratio is 1:2 to 1:4 with medium changed 3 to 4 times per week.
   a. For cell synchronization followed by Western Blot analysis, cells are cultured in 3.5-cm plates.
   b. For immunofluorescence and *in situ* proximity ligation assay (PLA), cells are mounted on 8 mm × 8 mm round glass coverslip in 24-well plate.
   c. For centrosome isolation, cells are cultured in 10-cm plates.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-PLK1 (208G4) (reactivity: human, mouse, monkey)	Cell Signaling Technology	4513S
Rabbit polyclonal anti-phospho-PLK1 (Thr210) (reactivity: human)	Cell Signaling Technology	5472S
Mouse monoclonal anti-PLK1 (reactivity: human, mouse, rat)	Invitrogen	37-7000
Mouse monoclonal anti-PLK1 (alternative) (reactivity: human, mouse, rat)	Abcam	ab17056
Rabbit monoclonal anti-β-actin (reactivity: human, mouse, rat, monkey, bovine, pig)	Cell Signaling Technology	4970S
Mouse monoclonal anti-β-actin (alternative) (reactivity: human, mouse, rat, monkey, bovine, pig, canine, feline, carp, chicken, sheep, *Hirudo medicinalis*)	Sigma	A5441
Rabbit monoclonal anti-Cyclin B1 (reactivity: human, rat)	Cell Signaling Technology	12231S

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit monoclonal anti-p-Histone H3 (reactivity: human, mouse, rat, monkey, zebrafish)	Cell Signaling Technology	3377S
Rabbit polyclonal anti-p-Histone H3 (alternative) (reactivity: human, mouse, rat)	ABclonal	AP0840
Rabbit polyclonal anti-RXRα (D-20) (reactivity: human, mouse, rat)	Santa Cruz Biotechnology	sc-553
Mouse monoclonal anti-Lamin B1 (reactivity: human, mouse, rat)	Santa Cruz Biotechnology	sc-56145
Mouse monoclonal anti-γ-tubulin (reactivity: human, mouse, rat, bovine, chicken, Xenopus, hamster, canine)	Sigma	T5326
Rabbit polyclonal anti-pS56-RXRα (reactivity: human, mouse)	This paper	N/A
Goat Anti-Mouse IgG F(ab')2 Secondary Antibody, HRP conjugate	Pierce	31436
Goat anti-Rabbit IgG F(ab')2 Secondary Antibody, HRP conjugate	Pierce	31461
FITC-labeled Goat Anti-Mouse IgG (H+L)	Beyotime	A0568
Cy3-labeled Goat Anti-Rabbit IgG (H+L)	Beyotime	A0516
Bovine serum albumin (BSA)	YHSM (Beijing)	N/A
Chemicals, peptides, and recombinant proteins		
Thymidine	Sigma	T1895
Nocodazole	Sigma	M1404
Ro-3306	Selleck Chemicals	S7747
BI2536	Santa Cruz Biotechnology	sc-364431
VX680	Selleck Chemicals	S1048
Cytochalasin D	Sigma	C8273
DNase I	Roche	10104159001
Protease inhibitor	Roche	11836153001
Phosphatase inhibitor	Roche	4906837001
Lipofectamine 2000	Invitrogen	11668019
Mounting medium	Vector	H-1400
Paraformaldehyde	Sigma	16005-1KG-R
Critical commercial assays		
Pierce BCA Protein Assay Kit	Thermo Scientific	23225
Duolink™ In Situ Red Starter Kit Mouse/Rabbit	Sigma	DUO92101-1KT
Experimental models: cell lines		
HeLa	ATCC	CCL2
Recombinant DNA		
pmCherry-C1-RXRα	This paper	N/A
Software and algorithms		
LAS X (Leica)	Open source	https://www.leica-microsystems.com/
Other		
0.22 μm Membrane filter unit	Millipore	SLGPR33RB
70 μm Nylon cell strainer	Falcon	352350
Confocal laser scanning microscope system	Leica	SP8
Ultra-clear centrifuge tube	Beckman Coulter	344059
Preparative Ultracentrifuge	Beckman Coulter	Optima L-100 XP
Piston Gradient Fractionator	BioComp	152

MATERIALS AND EQUIPMENT

Reagent	Final concentration and storage conditions	Volume
1×PBS	3.628 g $Na_2HPO_4 \cdot 12H_2O$, 0.24 g KH_2PO_4, 8.0 g NaCl, 0.2 g KCl in ultrapure water. Store at 4°C or 20°C~25°C for up to 1 month.	1000 mL
RIPA buffer	50 mM Tris•HCl, 150 mM NaCl, 0.5% sodium dexycholate, 0.1% SDS, 1 mM EDTA, 1× Protease inhibitor, 1× Phosphatase inhibitor, PH=7.4 (PH is titrated by HCl) . Store at −20°C for up to 1 month.	100mL
4% paraformaldehyde	40 g paraformaldehyde in 1× PBS buffer. Store at −20°C for up to 12 months.	1000 mL
0.05% Triton X-100	50 μL Triton X-100 in 1× PBS buffer. Store at 4°C for up to 1 month.	100 mL
Lysis buffer	1 mM Hepes, 0.5% NP-40, 0.5 mM $MgCl_2$, 0.1% β-mercaptoethanol, 1× Protease inhibitor (Roche, 11836153001), 1× Phosphatase inhibitor (Roche, 4906837001), PH=7.2 (PH is titrated by HCl). Store at −20°C for up to 1 month.	100 mL
Gradient buffer	10 mM Pipes, 0.1% Triton X-100, 0.1% β-mercaptoethanol, 1× Protease inhibitor (Roche, 11836153001), 1× Phosphatase inhibitor (Roche, 4906837001), pH=7.2 (PH is titrated by NaOH). Store at −20°C for up to 1 month.	100 mL
Sucrose solutions	70% sucrose in gradient buffer (w/w) (i.e., 70 g sucrose is dissolved in 30 g gradient buffer) 60% sucrose in gradient buffer (w/w) 50% sucrose in gradient buffer (w/w) 40% sucrose in gradient buffer (w/w) Store at 4°C for 1 day.	10 mL

STEP-BY-STEP METHOD DETAILS

Cell synchronization

Timing: 50 h

Cell-cycle progression is orchestrated via complex networks of reversible posttranslational modifications including phosphorylation, ubiquitination and SUMOylation (Fisher et al., 2012, Kirkin and Dikic, 2007, Wan et al., 2012). Nuclear proteins are often phosphorylated during mitosis (Jesper V. Olsen et al., 2010). This part of protocol is used to study whether the transcription factor of interest undergoes phosphorylation during mitosis and identify kinase responsible for its phosphorylation. The method can also be used to study other posttranslational modifications, such as ubiquitination and SUMOylation.

Thymidine, an effective inhibitor of DNA synthesis, can arrest cells in either G1 prior to DNA replication or S phase. If thymidine is removed, cells can reenter the cell-cycle progression. When subjected to double thymidine block (TT block), cells can be synchronized at G1/S boundary (Uzbekov, 2004, Fang et al., 1998). After releasing into normal medium, cells can be collected at different time points and analyzed by Western Blot to determine whether a transcription factor undergoes modification during mitosis.

1. Cells are cultured in 3.5-cm virgin polystyrene plates (n=8) with 20% confluence for 18~24 h.
2. Replace medium with 2 mL fresh medium containing 2 mM thymidine in incubator at 37°C for 16 h.
   a. The medium with thymidine should be freshly prepared right before added to cells.
   b. The confluence of cells is about 40% now.
3. Remove medium containing thymidine completely and wash cells two times with 2 mL pre-warmed 1× PBS buffer and discard PBS.
   a. 1× PBS buffer is pre-warmed in incubator at 37°C.

⚠ CRITICAL: Ensure that thymidine is completely removed during releasing because a residual amount of thymidine can delay mitotic progression.

4. Add 2 mL fresh medium without thymidine to cells and place the cells in incubator at 37°C for 8 h.
5. Discard the medium and add 2 mL fresh medium containing 2 mM thymidine for another 16 h in incubator at 37°C.
   a. Cells are now synchronized at the G1/S boundary and its confluence is around 80%.
6. Cells are washed twice with 2 mL pre-warmed 1× PBS buffer, and released into fresh medium without thymidine again for 0, 2, 4, 6, 8, 10, 12, 14 h.
   a. 1× PBS buffer and fresh medium are pre-warmed in incubator at 37°C.
   b. Discard the medium followed by washing cells with 2 mL 1× PBS buffer carefully; store plates with cells in −80°C freezer until lysis.
   c. For kinase inhibitors treatment, discard the medium and add 2 mL pre-warmed (37°C) fresh medium containing 0.1% DMSO (vehicle control), 5 μM RO3306 (CDK1 inhibitor), 0.25 μM BI2536 (PLK1 inhibitor), or 5 μM VX680 (Aurora A inhibitor) to the mitotic cells for 15 min in incubator at 37°C.

⚠ CRITICAL: Because of decreased adherence, mitotic cells should be washed with caution to avoid their loss. If mitotic cells are already suspended in media, they can be collected by centrifuge at 850 × *g* for 3 min.

7. Lyse cells in plates with RIPA buffer and measure protein concentration using BCA Protein Assay Kit. Cell lysates are now ready for Western blot analysis.
   a. Cell lysates can be stored at −80°C for a few days before Western blot analysis.

Pause point: Cell lysates can be stored at −80°C before Western blot analysis.

Note: Both Ser10 of histone H3 (S10-H3) and threonine210 of PLK1 (PLK1-Thr210) are heavily phosphorylated during mitosis, which can be used as mitotic markers in this protocol.

Note: Collecting cells at the peak of mitosis is crucial their subsequent analysis including Western Blot, immunofluorescence, *in situ* PLA and centrosome isolation. If you use other cell lines, you should examine their morphology under light microscope every other hour after they are released from TT block, because most animal cells undergo a near spherical morphology during mitosis, known as mitotic cell rounding (Figure 1A). In our study, mitosis is peaked with 60% mitotic cells at 10 h after cells are released from TT block. So, we collect cells at 0, 2, 4, 6, 8, 10, 12, 14 h after they are released from TT block and analyze them by Western Blot using anti-pT210-PLK1 and anti-pS10-H3 antibodies. Unexpectedly, we found that RXRα undergoes phosphorylation during mitosis, implying that phosphorylated RXRα (p-RXRα) may be involved in mitotic progression. An example of such an analysis is presented in Figure 1B.

Note: Cdk1, PLK1 and Aurora A are master kinases that phosphorylate numerous substrates to regulate mitotic progression (Joukov and Nicolo, 2018, Lindqvist et al., 2009). If a transcription factor undergoes phosphorylation during mitosis, it is likely that one of these kinases is responsible, which can be easily studied by using chemical inhibitors of these kinases. We used several inhibitors to help identify Cdk1 as the kinase responsible for RXRα phosphorylation (Xie et al., 2020). There are two key points that one should pay attention when using a kinase inhibitor. First, a kinase inhibitor should be added to cells already synchronized at mitotic phase, but not asynchronous cells, in order to avoid its effect on mitotic progression. Secondly, the treatment of mitotic cells with a kinase inhibitor should be as short as possible to exclude its indirect effect on the phosphorylation of nuclear proteins.

Immunofluorescence

Timing: 10 h or 18∼20 h

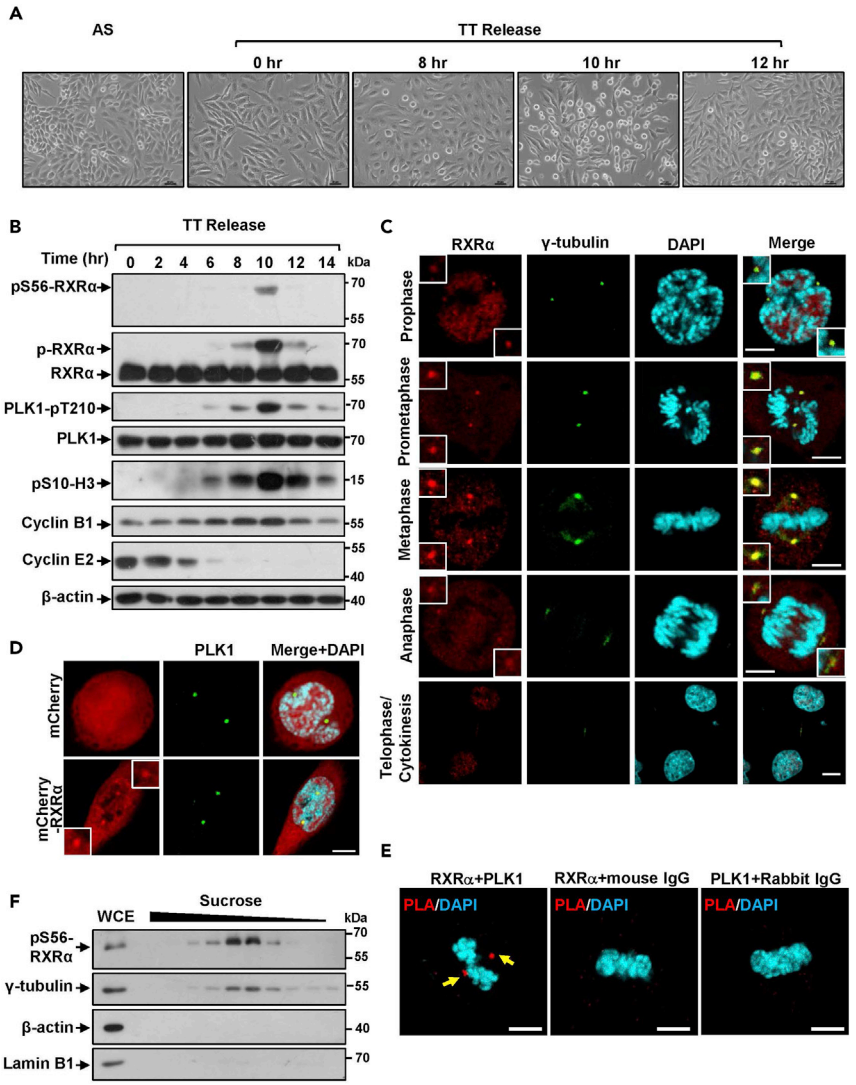

Figure 1. Identification of transcription factor RXRα as a centrosomal protein

(A) Live image analysis of HeLa cells released from TT treatment for the indicated time. AS, asynchronous cells. Scale bar, 50 μm.

(B) Modification of RXRα in HeLa cells released from TT block for the indicated time was analyzed by WB.

(C) Centrosomal localization of endogenous RXRα in HeLa cells revealed by immunostaining RXRα (red) and γ-tubulin (green). DAPI (blue). Scale bar, 10 μm.

(D) Centrosomal localization of transfected mCherrry-tagged RXRα (red) in HeLa cells. PLK1 (green) was revealed by immunostaining. DAPI (blue). Scale bar, 10 μm.

(E) Analysis of RXRα interaction and colocalization with PLK1 at the centrosome of HeLa cells by PLA assay. Scale bar, 10 μm.

(F) Centrosomal localization of endogenous pS56-RXRα was revealed by WB analysis of centrosomal fractions collected after sucrose density centrifugation of lysates prepared from mitotic HeLa cells. WCE, whole cell extract.

Mitosis is characterized by dramatic changes in chromatin organization and gene expression in eukaryotic cells. Transcription factors are dissociated from mitotic chromatins when they are condensed during mitosis (Martínez-Balbás et al., 1995, Egli et al., 2008, Palozola et al., 2017). Their localization and function during mitosis remains an enigma in the field. There are numerous proteins found to be associated with different subcellular organelles during mitosis, including centrosome, kinetochore, central spindle and midbody, hence playing a role in regulating the mitotic progression

(Zitouni et al., 2014). This part of the protocol aims to study the localization of a transcription factor from interphase to mitotic phase when it is dissociated from mitotic chromatin. Although the protocol is used to examine the accumulation of the nuclear protein at the centrosome upon phosphorylation, it can also be used to determine whether it resides at other subcellular organelles, such as kinetochore, central spindle and midbody.

8. Cells are mounted on 8 mm× 8 mm round glass coverslip in 24-well plate with 20% confluence for 18∼24 h, followed by TT block (steps 2∼5)
   a. Round glass coverslip should be clean and sterile, which is placed in the well before culture medium containing cells is added to the well.
   b. Transfect cells with plasmids containing mCherry-tagged or EGFP-tagged transcription factor of interest during the second thymidine arrest if you want to confirm the localization of the transcription factor (Figure 1D).
9. Cells are released into fresh medium without thymidine for 10 h after washed twice with 1 mL pre-warmed (37°C) 1× PBS buffer.
   a. For kinase inhibitor treatment, discard the medium and add 500 μL pre-warmed (37°C) fresh medium containing 0.1% DMSO, 10 μM RO3306 (CDK1 inhibitor), or other inhibitors, such as PLK1 inhibitor BI2536 or Aurora A inhibitor XV680, to mitotic cells for one more hour.
10. Wash one time with 500 μL 1× PBS buffer per well and discard PBS.
    a. The following steps (10∼12) are also performed in wells.

⚠ CRITICAL: Because the adherence of mitotic cells on coverslips is weak, you should gently perform the experiments below to avoid the loss of mitotic cells.

11. Cells are fixed with 500 μL −20°C methanol for 10 min at 4°C or 4% paraformaldehyde prepared in 1× PBS buffer for 10 min at 20°C∼25°C.
    a. Methanol should be pre-cooled at −20°C.
12. After discarding the fixing buffer, cells are washed twice by 500 μL 1× PBS buffer, then permeabilized with 500 μL 0.05% Triton X-100 in 1× PBS buffer for 8 min at 4°C.
    a. 0.05% Triton X-100 should be pre-cooled at 4°C.
13. Cells are then washed twice with 1×PBS again and blocked with 500 μL 1% Bovine Serum Albumin (BSA) in 1× PBS for 30 min at 20°C∼25°C.
    a. Blocking buffer (BSA in 1× PBS buffer) should be prepared right before use.

Pause point: Blocking can be run for 12∼16 h at 4°C.

14. Transfer the coverslips from 24-well plate to a covered dish to prevent fluorochrome fading.
15. Discard blocking buffer and incubate cells with 20 μL primary antibody diluted with blocking buffer per coverslip at 20°C∼25°C for 3 h.
    a. You should use 20 μL pipette tips to discard blocking buffer carefully and ensure that no liquid is left on coverslips. This prevents dilution of primary antibody.
    b. The dilution of primary antibody is often 1:100∼1:200 depending on the valence of antibody. In our case, the dilution of anti-RXRα antibody (rabbit polyclonal antibody), anit-PLK1 antibody (mouse monoclonal antibody) and anti-γ-tubulin antibody (mouse monoclonal antibody) is 1:100, 1:200 and 1:1,000, respectively.

Pause point: Incubation of primary antibody can be run for 12∼16 h at 4°C.

16. Remove primary antibody and wash with 200 μL 1× PBS (3× 5 min).
    a. For last wash, ensure that there is no much liquid left on coverslips to prevent the dilution of the secondary antibody.

17. Incubate cells with 20 μL FITC-labeled Goat anti-Mouse IgG (1:200) diluted with blocking buffer to detect PLK1 or γ-tubulin and Cy3-labeled Goat anti-Rabbit IgG (1:200) diluted with blocking buffer to visualize RXRα at 20°C∼25°C for 1 h.
    a. Dish should be covered during incubation to avoid fluorochrome fading.
18. Second antibody is removed and cells are stained with 20 μL 4′,6-diamidino-2-phenylindole (DAPI) (1:10,000 dilution) in 1× PBS at 20°C∼25°C for 5 min to visualize nuclei.
19. Discard DAPI and wash cells in 200 μL 1× PBS (4× 5 min).
20. Tap off excess PBS from coverslips and wash them in double distilled water for 3 sec by using tweezers. Then remove the residual water from coverslips and mount them on the glass slides using 3 μL mounting medium.
    a. For removing residual water, you can use paper towel to carefully touch the edge of coverslips so that water is absorbed.
    b. Mounting medium containing DAPI can be used instead of a separate DAPI staining step.

⚠ CRITICAL: Because 1×PBS buffer can form white crystal and impact the imaging if it dries, washing the coverslips using double distilled water before mounting is necessary.

21. Place slides in dark at 20°C∼25°C for 2 h to dry them before analyzing.
22. Images are taken under a LEICA TCS SP8 confocal laser scanning microscope system (Leica).
    a. Conventional confocal microscopy should be good for imaging, and the 40× or 63× oil objective is recommended.
    b. Interphase or mitotic cells undergoing different stages of mitotic phase can be easily identified according to the morphology of nucleus and chromosome revealed by DAPI staining. In our case, we examined the subcellular localization of endogenous RXRα during mitosis (Figure 1C), and found that RXRα is colocalized with γ-tubulin, a known centrosomal marker, during prophase of mitosis, although it mainly resides in the nucleus at this stage of mitosis. RXRα association with the centrosome became very prominent during prometaphase and metaphase. After cells exit from mitosis, RXRα returned back to the nucleus. Furthermore, transfected mCherry-tagged RXRα also colocalized with PLK1 at the centrosome in mitotic cells (Figure 1D). These results allowed us to conclude that RXRα translocates from the nucleus to the centrosome during mitosis.
    c. For the identification of kinase responsible for RXRα phosphorylation, we found that RO3306 can inhibit centrosomal RXRα staining, which help us concluding that Cdk1-dependent phosphorylation of RXRα is required for its centrosomal translocation (Xie et al., 2020).
    d. After imaging, slides can be stored at −20°C in dark for up to 6 months.

Note: In order to have enough time for transfected plasmids to express protein and to avoid interference with S-phase completion (Arroyo et al., 2017), we suggest that you perform the transfection experiment once cells are released from first thymidine arrest.

Note: Different fixing reagents are required for the exposure of different antigens. So, the choice of fixing reagents depends on the primary antibody used. In our case, methanol is suitable for anti-γ-tubulin antibody. But both of methanol and paraformaldehyde can be used for anti-RXRα and anti-PLK1 antibodies. You can find further information at: https://blog.cellsignal.com/successful-immunofluorescence-fixation-and-permeabilization, or check the datasheet of antibody of interest or related publications for optimal fixation conditions.

In situ proximity ligation assay (PLA)

Timing: 8∼10 h

Cdk1 phosphorylation of their substrates provides them with docking sites for recruiting PLK1, which may occur at distinct subcellular localizations, such as centrosome, kinetochore and central spindle

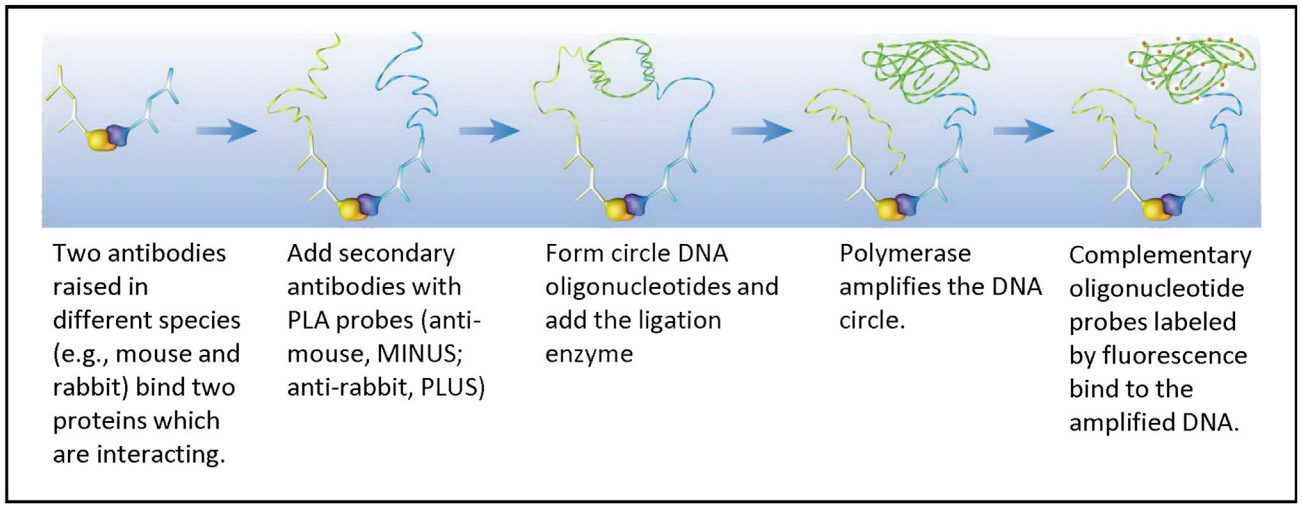

Figure 2. The principle of In situ proximity ligation assay (PLA)
This figure is re-edited from the original figures distributed by Sigma company.

(Zitouni et al., 2014). *In situ* PLA is a powerful technique that visualizes protein interaction *in situ* (Figure 2). We used this method to detect the interaction of RXRα and PLK1 at the centrosome. The protocol is performed using a Doulink assay kit (Sigma/Aldrich) based on the manufacturer's instructions (https://www.sigmaaldrich.com/technical-documents/protocols/biology/duolink-fluorescence-user-manual.html) with modifications. The protocol can also be modified to study whether the interaction occurs at a distinct subcellular structure during mitosis.

23. Mount cells with 15%∼20% confluence on 8 mm× 8 mm round glass coverslip in 24-well plate for 18∼24 h, followed by TT block (steps 2∼5).
    a. In manufacturer's instruction, cells should be deposited on glass slides. But we have modified it so that cells are mounted on glass coverslip.
24. Cells are released into fresh medium without thymidine for 10 h after washed twice with 1 mL pre-warmed (37°C) 1× PBS buffer.
    a. The optimal confluence of cells is 50%∼70%.
25. Wash cells twice with 500 μL 1× PBS buffer per well and discard PBS.
    a. The following steps (26∼28) are also performed in 24-well plate.
    b. All wash steps below should be performed at 20°C∼25°C.
26. Fix cells with 500 μL cold methanol (−20°C) for 5 min at 4°C.

Pause point: After fixation, cells can be left in 1× PBS at 4°C for several days.

27. After discard fixing buffer, cells are washed twice by 500 μL 1× PBS buffer, then permeabilized with 500 μL 0.05% Triton X-100 in 1× PBS buffer for 8 min at 4°C.
28. Wash cells two times by 1× PBS and transfer the coverslips from 24-well plate to a covered dish.
    a. Place some wet cotton balls around the inner edge of the covered dish to prevent coverslips from drying.
    b. We use a covered dish with wet cotton balls instead of a humidity chamber suggested by manufacturer's instruction.
29. Block cells with a drop of Blocking Solution per coverslip for 60 min at 37°C incubator.
    a. Blocking Solution and the following reagents except primary antibodies are supplied by the company.
    b. Be sure to cover the entire sample with Blocking Solution.

Pause point: Blocking can be run for 12∼16 h at 4°C.

30. Discard Blocking Solution and incubate cells with 25 μL primary antibody diluted in Antibody Diluent per coverslip for 60 min at 37°C incubator.
    a. For 8 mm× 8 mm round coverslip, 25 μL of solution is enough for coverage.
    b. Use 20 μL pipette tips to tap off Blocking Solution as much as possible to prevent dilution of the primary antibody.
    c. Dilute your primary antibodies to a suitable concentration. In our case, the dilution of anti-RXRα antibody and anit-PLK1 antibody is 1:100 and 1:300, respectively.

Pause point: Incubation of primary antibody can be run for 12∼16 h at 4°C.

31. Discard primary antibody solution and wash coverslips 4 × 5 min with 1× Wash Buffer A.
    a. In order to reduce the background signal, we increase the times of washing in this step and the following steps, which are more than that suggested by the manufacturer's instruction.
    b. Tap off excess wash buffer to avoid dilution of the PLA probe solution.
32. Incubate cells with PLA labeled secondary antibodies (anti-mouse, MINUS; anti-rabbit, PLUS) for 60 min in a 37°C incubator.
    a. For a 25 μL reaction, take 5 μL PLA probe MINUS stock, 5 μL PLA probe PLUS stock and 15 μL Antibody Diluent. If multiple coverslips are used, you can scale up volume accordingly.
33. Discard the PLA probe solution and wash the coverslips 4× 5 min again with 1× Wash Buffer A.
34. Add 25 μL of Ligation solution including ligase to each coverslip for 30 min at 37°C incubator.
    a. For a 25 μL reaction, first add 5 μL the 5× Ligation buffer to 20 μL ultrapure water to obtain 25 μL 1× Ligation buffer, then add 0.625 μL of Ligase to 24.375 μL 1× Ligation buffer. If multiple coverslips are used, scale up volumes accordingly.
35. Discard ligation solution and wash coverslips 4× 5 min with 1× Wash Buffer A.
36. Incubate cells with 25 μL Amplification buffer including polymerase per coverslips for 100 min at 37°C incubator.
    a. For 25 μL reaction, first add 5 μL 5× Amplification buffer to 20 μL ultrapure water to obtain 25 μL 1× Amplification buffer, then add 0.3125 μL Polymerase to 24.6875 μL 1× Amplification buffer. If multiple coverslips are used, scale up volumes accordingly.
37. Discard the amplification solution and wash the coverslips 3× 5 min with 1× Wash Buffer B and one time in 0.01× Wash Buffer B for 1 min.
    a. Tap off excess wash buffer from coverslips at the last step.
38. Mount coverslips by using a minimal volume of mounting medium with DAPI.
    a. Use clear nail polish to seal the edge of coverslips to the glass slides because the mounting medium is aqueous and does not solidify.
    b. Transferring mounting medium to glass slide by 20 μL pipette carefully and slowly to avoid air bubbles under the coverslip.
39. Place glass slide in dark at 20°C∼25°C for 15 min before analyzing in a LEICA TCS SP8 confocal laser scanning microscope system (Leica).
    a. Conventional confocal microscopy combined with 40× or 63× oil objective is good for detecting the interaction.
    b. The PLA signal is recognized as red fluorescent spots in cells. In our case, PLA fluorescent spots at the centrosome during mitosis can only be observed when both antibodies are present, demonstrating the high specificity of the interaction. The PLA result can be used to illustrate the interaction between RXRα and PLK1 and the location that the interaction occurs during mitosis (Figure 1E).
    c. After imaging, store slides in the dark at 4°C for up to 4 days or at −20°C for up to 6 months.

⚠ CRITICAL: For PLA experiment, the identification of optimal conditions for primary antibodies, including sample processing (fixation and permeabilization), antibody concentration, blocking solution and antibody diluent, is crucial. We suggest to use IF staining to

identify conditions for primary antibody, which can then be used for PLA experiment. However, because PLA provides greater sensitivity and signal amplification as compared to IF, the concentration of primary antibodies used for PLA may be less than that used for IF.

⚠ CRITICAL: Make sure that reagents and buffers are prepared and stored under their recommended conditions and that some reagents are completely thawed (e.g., ligation buffer and amplification buffer) and resolved prior to usage.

Note: Perform all steps at the appropriate incubation times for best results, in particular the primary antibody incubation step and enzymatic steps (ligation and amplification).

Note: The use of negative controls (e.g., isotype IgG) is highly recommended when performing PLA experiment.

Note: It is important that images should be taken with the same acquisition parameters between experimental and control samples. Settings can be optimized using positive negative controls.

Centrosome isolation

Timing: 6 h

The presence of a nuclear protein at the centrosome can also be detected by Western blot analysis of purified centrosomes So, this part of the protocol is used to prepare centrosomes from mammalian cultured cells, which is adapted from (Wigley et al., 1999, Gogendeau et al., 2015) with modifications, followed by separation of cellular components through a discontinuous sucrose gradient (Figure 3).

40. Cells cultured in 10-cm plates (n=20) are released from TT block for 10 h, and treated with 1 μg/mL cytochalasin D and 0.2 μM nocodazole for 1 h at 37°C to disrupt the actin cytoskeleton and microtubules.
    a. Cytochalasin D and nocodazole are added to the medium.
    b. After the treatment, all subsequent steps are carried out at 4°C.
41. Collect the cells in 50 mL centrifugation tube by centrifugation at 350 × *g* for 8 min.
    a. The adherence of cells at this stage is weak, so they can be detached from plates by pipetting up and down a few times (3-5 times) before harvesting.
42. Wash the resulting cell pellet in 20 mL 1× PBS buffer and centrifuge cells at 350 × *g* for 8 min, followed by resuspending with 20 mL of 0.1× PBS/8% sucrose.
    a. Cells should be handled carefully afterward because they swell due to the hypotonic media.
43. Cells are recentrifuged at 350 × *g* for 8 min and resuspended in 2 mL 0.1× TBS/8% sucrose followed immediately by addition of 8 mL lysis buffer. The cell suspension is gently shaken and passed five times through a 10-mL narrow-mouth serological pipette to lyse cells. Place cell lysate on ice for 5 min.
    a. Take an aliquot of 50 μL as the whole cell extract (WCE) and store it in −80°C refrigerator.
44. Cell lysate is centrifuged once more at 2,500 × *g* for 10 min to remove swollen nuclei, chromatin aggregates, and unlysed cells.
45. The resulting supernatant is filtered through a 70 μm Nylon Cell Strainer into a 50 mL centrifugation tube.
46. Add 1 M Hepes to the supernatant to obtain a final concentration of 10 mM Hepes.
47. Add DNase I to the supernatant to a final concentration of 1 μg/mL. Mix well and incubate the supernatant on ice for 30 min.

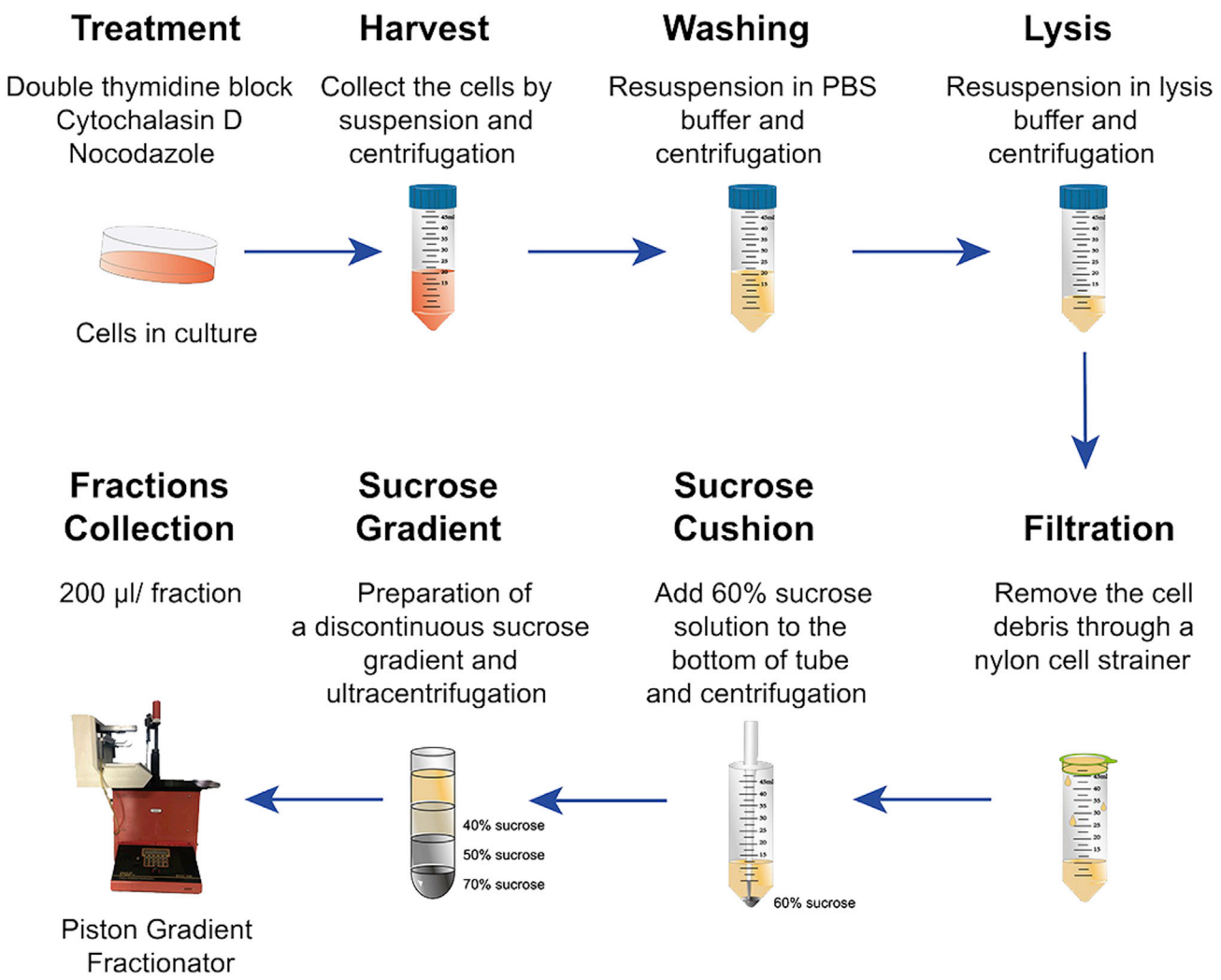

Figure 3. The procedure of centrosome isolation

48. The mixture is gently underlaid with 1 mL of 60% sucrose solution and spun at 10,000 × *g* for 30 min to sediment centrosomes onto the cushion.
49. Remove the upper 8 mL of the supernatant carefully and the remainder, including the cushion, containing the concentrated centrosomes is gently vortexed.
50. Prepare a discontinuous sucrose gradient in a 14× 89 mm Ultra-Clear Centrifuge Tube (Beckman) by adding the tube with 3 mL 70% sucrose, 3 mL 50% sucrose, and 3 mL 40% sucrose solution orderly.
    a. 70% sucrose, 50% sucrose, and 40% sucrose solutions should be prepared separately prior to use.
    b. Use the Pasteur pipet to add the sucrose solution along the inner wall of centrifuge tube slowly.
51. The reminder is then loaded onto the discontinuous sucrose gradient from the bottom and spun at 25,600 × *g* for 1 h in a Beckman Preparative Ultracentrifuge with an SW28 rotor.

52. Fractions is collected by Piston Gradient Fractionator (BIOCOMP) and subjected to Western blot analysis.
    a. The volume of discontinuous sucrose gradient is 9 mL, and fractions are collected from the top by 200 µL/fraction. Usually centrosomes are enriched in fractions nearby the boundary

between 50% and 70% sucrose. Fractions can be stored in −80°C refrigerator for a few weeks before further analysis.

Pause point: Fractions can be stored in −80°C refrigerator before further analysis.

b. For Western blot analysis, 8% SDS-PAGE gel made by ourselves is appropriate. Fractions are boiled in SDS sample loading buffer with 20 μL/fraction.

c. In our study, pS56-RXRα coaccumulates with the centrosome marker γ-tubulin. β-actin (a housekeeping protein) and Lamin B1 (a nuclear protein) are used as negative controls. This result indicates that pS56-RXRα localizes at centrosome (Figure 1F).

Note: After cytochalasin D and nocodazole treatment, the adherence of mitotic cell on plates is very weak, so you can suspend mitotic cells into culture media by either using pipette or horizontal shaker.

Note: The $MgCl_2$ concentration during the lysis step is very important, because it is crucial for dissociation of centrosome from nucleus. The concentrations may range from 0.5 mM to 1 mM depending on cell types (Gogendeau et al., 2015). For HeLa cells, the optimal concentration of $MgCl_2$ is 0.5 mM (Wigley et al., 1999).

EXPECTED OUTCOMES

Cell synchronization by the double thymidine block combined with the analysis of the effect of kinase inhibitors will illustrate if the transcription factor of interest undergoes special phosphorylation during mitosis and suggest a kinase responsible for the phosphorylation. Immunofluorescence and the analysis of purified centrosomes will determine whether the transcription factor translocates from the nucleus to the centrosome during mitosis. *In Situ* PLA will demonstrate whether the transcription factor interacts with other protein of interest at the centrosome. By using this protocol, you can further investigate key amino acid residue or domains in the transcription factor for its centrosomal localization by transfection of mutant transcription factor. Together, the protocol will identify a transcription factor as a centrosomal protein during mitotic progression. This protocol can be modified to determine whether a transcription factor can target other subcellular structures during mitosis.

LIMITATIONS

Not all cell lines are sensitive to thymidine, such as MDA-MB-231 and T47D breast cancer cells. Thus, it is important to make sure that the cell line you plan to use is sensitive to thymidine before conducting this protocol. One simple way to check is to see whether cells become round upon double thymidine block under light microscope.

Not all protein phosphorylation can result in mobility shift on SDS-PAGE. Immunoprecipitation using anti-transcription factor antibody in mitotic cells followed by Western blot using phospho-amino acid-specific antibodies may be used to confirm its phosphorylation. *In vitro* kinase assays, including radioactive assay, remain the 'gold standard' to assess protein phosphorylation.

TROUBLESHOOTING

Problem 1

Cells are not sensitive to thymidine.

Potential solution

You can choose nocodazole, a microtubule depolymerizing agent known to arrest cells at the G_2/M phase, to determine whether transcription factor undergoes modification during mitosis. To study its subcellular localization during mitosis, you can remove nocodazole, so that cells can enter mitosis again.

Problem 2
Choice of fixing buffer (steps 11 and 26)

Potential solution
The choice of fixing buffer may vary depending on primary antibodies and protein targets. −20°C methanol and 4% paraformaldehyde are two most common fixing buffers. In our experience, the methanol is suitable for anti-γ-tubulin, anti-cep192, and anti-centrin antibodies, while both methanol and paraformaldehyde can be used for anti-RXRα and anti-PLK1 antibodies. For other proteins of interest, you can try these two fixing buffers first or use published conditions.

Problem 3
Hard to find mitotic cells expressing transfected transcription factor in immunofluorescence assay.

Potential solution
Improve transfection efficiency, harvest cells at peak of mitosis, or use cells stably expressing the exogenous transcription factor of interest.

Problem 4
The background signal is too high in PLA experiment.

Potential solution
This may be due to excess amount or nonspecific binding of primary antibody, insufficient blocking, insufficient washing of reaction, drying of sample, and precipitates in buffers. You can dilute or use other primary antibody; prolong blocking time; adjust washing conditions, such as the number and duration of washing; make sure that coverslips are not dried; ensure that buffers are completely mixed prior to use. You can also check the manufacturer's instructions for more information.

Problem 5
Insufficient enrichment of centrosome.

Potential solution
Harvest cells when they are at 80% confluence, and use optimal $MgCl_2$ concentration to adequately dissociate centrosomes from nuclei. You should also avoid rocking during the preparation of the discontinuous sucrose gradient.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiao-kun Zhang (xkzhang@xmu.edu.cn)

Materials availability

This study did not generate new unique reagents.

Data and code availability

The study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, X.-k.Z.; investigation, G.X., Y.Z., M.D., and Q.W.; writing – original draft, G.X., Y.Z., and J.Y.; writing – review & editing, X.-k.Z., G.X., and Y.Z.; funding acquisition X.-k.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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