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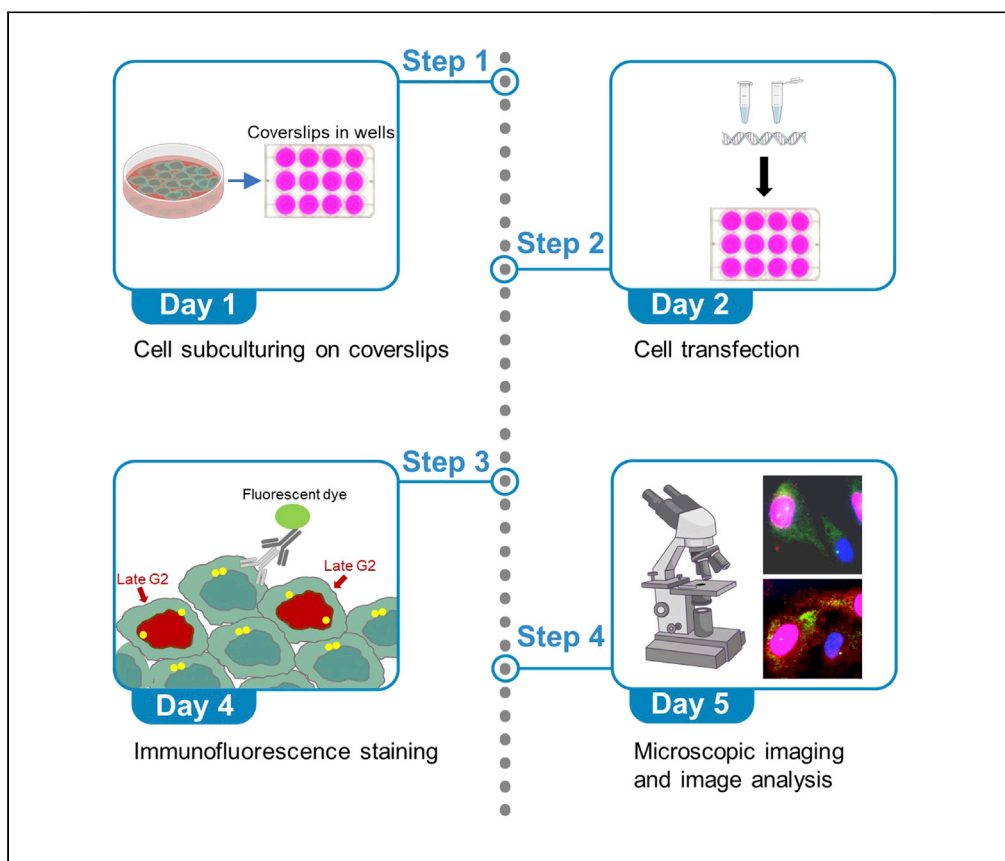**Protocol**

Protocol to measure inter-centrosome distance in adherent cells using epifluorescence microscopy

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Highlights

Protocol to measure inter-centrosome distance in adherent cells

Approach to analyze protein involvement in centrosome separation at late G2 phase

Applicable to the analysis of centrosome cohesion and separation

We present here a protocol to assay the centrosome separation events at late-G2 phase of the cell cycle by immunofluorescence microscopy. We describe the steps required for imaging and measurement of inter-centrosome distance. Here, we use GAS2L1 as an example, but the protocol can be used to test any protein for a role in centrosome separation and cohesion. The steps below are specific for hTERT RPE-1 cell lines, but other adherent cell lines (e.g., U2OS, MRC-5) are also amenable for this protocol.

Protocol

Protocol to measure inter-centrosome distance in adherent cells using epifluorescence microscopy

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SUMMARY

We present here a protocol to assay the centrosome separation events at late-G2 phase of the cell cycle by immunofluorescence microscopy. We describe the steps required for imaging and measurement of inter-centrosome distance. Here, we use GAS2L1 as an example, but the protocol can be used to test any protein for a role in centrosome separation and cohesion. The steps below are specific for hTERT RPE-1 cell lines, but other adherent cell lines (e.g., U2OS, MRC-5) are also amenable for this protocol.
For complete details on the use and execution of this protocol, please refer to Au et al. (2017) and Au et al. (2020).

BEFORE YOU BEGIN

Cell culture

⏱ **Timing: 1 week**

Before the experiment, hTERT RPE-1 (RPE-1) cells are thawed and recovered from a frozen stock, and the recovered cells are maintained in DMEM/F-12 containing 10% fetal bovine serum and 100 U/mL penicillin-streptomycin mix. The doubling time of healthy RPE-1 cells should be ~15–20 h.

The protocol below describes the specific steps for maintaining RPE-1 cells in culture, including the recovery of the cell line from a vial of frozen stock and the subsequent subculturing steps. Similar procedures can be used for other cell lines. Cells should not be allowed to reach 100% confluence before subculture, and the passage number of cells should be monitored; we generally discard cells that have been passaged >40 times.

Note: Perform the following steps in a sterile class II biosafety cabinet.

1. Prewarm culture media in a 37°C water bath to minimize thermal stimulation of cells.
2. Place the cryogenic vial of the frozen cell stock in a 37°C water bath to rapidly thaw the cells. Spray the vial with 70% ethanol and wipe carefully after thawing. Transfer the thawed RPE-1 cell suspension into a 15-mL conical tube and dilute with 5 mL of prewarmed culture medium (DMEM/F-12 supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin mix).
3. Pellet cells by centrifugation at 300×g for 5 min. Resuspend the pellet with 10 mL of culture medium, plate the cells on a 100-mm culture dish, and maintain the dish of cells in a humidified 37°C incubator containing 5% CO_2.

Cell subculturing and cell seeding on coverslips

○ Timing: 1–2 days

Note: Perform the following steps in a sterile class II biosafety cabinet. RPE-1 cells are passaged when they are 80%–90% confluent, with a subculture ratio of 1:5 to 1:10. Two to three rounds of subculture aid cell recovery from thawing.

4. Prewarm culture media to 37°C as described in step 1.
5. Aspirate the culture medium in the 100-mm culture dish of RPE-1 cells (∼80%–90% confluent) and rinse the cell monolayer briefly with 5 mL of prewarmed PBS.
6. Add 1 mL of trypsin-EDTA solution to cells and incubate at 37°C for 2–3 min until all cells are detached from the dish.
7. Resuspend cells with 9 mL of culture medium and disperse the cells by repeatedly pipetting the cell suspension.
8. Plate 1–2 mL of the cell suspension in a new 100-mm culture dish containing 9 mL of fresh culture medium for subculture and maintenance of the cell line.
9. Place a sterilized 18-mm round glass coverslip into each well of 12-well plates, add 1 mL of fresh medium to each well, and then seed a volume of the cell suspension containing ∼0.1–0.3 × 10^5 RPE-1 cells onto the coverslips for subsequent experiments. Cells are counted using a hemacytometer.
10. Allow cells to adhere to the culture dish or coverslips for 12–24 h in a humidified 37°C incubator containing 5% CO_2.

Alternatives: The method presented here is based on the use of 18-mm round coverslips. Coverslips of other sizes can be used for different microscope settings.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-γ-tubulin antibody (1:500 v/v dilution)	Sigma-Aldrich	Cat#T5326; RRID: AB_532292
Rabbit polyclonal anti-CENP-F antibody (1:200 v/v dilution)	Santa Cruz	Cat#sc-22791; RRID:AB_2078927
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:1,000 v/v dilution)	Thermo Fisher Scientific	Cat#A21202; RRID:AB_141607
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (1:1,000 v/v dilution)	Thermo Fisher Scientific	Cat#A21207; RRID:AB_141637
Chemicals, peptides, and recombinant proteins		
Hoechst 33258	Sigma-Aldrich	861405; CAS: 23491-45-4
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12	Thermo Fisher Scientific	Cat#11320033
OPTI-MEM Reduced Serum Medium	Thermo Fisher Scientific	Cat#22600050
Fetal bovine serum	Thermo Fisher Scientific	Cat#10099141
Penicillin/streptomycin	Thermo Fisher Scientific	Cat#15070063
Trypsin-EDTA (0.25%)	Thermo Fisher Scientific	Cat#25200056
Lipofectamine RNAiMax	Thermo Fisher Scientific	Cat#13778150
Bovine serum albumin	Sigma-Aldrich	A9418; CAS: 9048-46-8
Mowiol 4-88	Sigma-Aldrich	81381; CAS: 9002-89-5
Experimental model: Cell lines		
hTERT RPE-1 cells	ATCC	CRL-4000
Software and algorithms		
Zen 3.0 (blue edition)	Carl Zeiss	https://www.zeiss.com/microscopy/int/products/microscope-software/zen-lite.html
Other		
Axio Observer ZI microscope	Carl Zeiss	N/A

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MATERIALS AND EQUIPMENT

1× PBS

Reagent	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.24 g
Dissolve in ultrapure water to 1,000 mL.		
Store at 21°C–24°C for up to 3 months.		

PBST

Reagent	Final concentration	Amount
Tween-20	0.05% (v/v)	50 µL
Mix with 100 mL of 1× PBS.		
Store at 21°C–24°C for up to 1 week.		

Blocking buffer

Reagent	Final concentration	Amount
Bovine serum albumin	2% (w/v)	0.2 g
Dissolve in 10 mL of PBST.		
Freshly prepare on the day of experiment.		

Mounting medium

Reagent	Final concentration	Amount
200 mM Tris-HCl, pH 8.5	100 mM	12 mL
Glycerol	25% (w/v)	6 g
Mowiol 4-88	10% (w/v)	2.4 g
Incubate and stir at 50°C until Mowiol 4-88 is dissolved. Top to 24 mL with ultrapure water.		
Store at 4°C for up to 6 months.		

STEP-BY-STEP METHOD DETAILS

siRNA transfection

⏱ Timing: 2–3 days

To test GAS2L1 function in centrosome separation, we have used siRNA-mediated knockdown of GAS2L1 expression in RPE-1 cells. Here, we describe the procedures for transfecting siRNAs into cells by using the Lipofectamine RNAiMax transfection reagent.

⚠ CRITICAL: Cells should be ~20% confluent at the beginning of transfection to ensure efficient siRNA transfection.

1. Replace the medium in each well of 12-well plates (containing RPE-1 cells on coverslips; see steps 9 and 10 from the section "before you begin") with 0.9 mL of prewarmed fresh culture medium.
2. Transfect RPE-1 cells with 20 nM siRNAs (control and *gas2l1*-targeting siRNAs) by using Lipofectamine RNAiMax according to the manufacturer's protocol. Specifically, separately dilute 2 µL of Lipofectamine RNAiMax reagent and appropriate amounts of siRNAs in 50 µL of Opti-MEM

medium. After incubation for 5 min, add the Opti-MEM–Lipofectamine mix into the Opti-MEM–siRNA mix and incubate further for 15 min before applying to the cells.

3. Maintain the cells in a humidified 37°C incubator for 36–48 h until the cells are 80% confluent (similar to the high density shown in this website: https://www.atcc.org/products/crl-4000).

   Alternatives: Lipofectamine 2000 or Lipofectamine 3000 can be used instead of Lipofectamine RNAiMax.

   Note: Knockdown efficiency should be tested in parallel by immunoblotting with an antibody against GAS2L1 (Au et al., 2017). Refer to Problem 1 of the section "troubleshooting" if low knockdown efficiency of target proteins is observed.

   Note: Refer to Problem 2 of the section "troubleshooting" if massive cell death occurs after transfection.

Immunofluorescent staining

🕐 Timing: 3 h

To study centrosome separation at late G2, cells are immunostained with an antibody against γ-tubulin to label centrosomes and an antibody against CENP-F to identify late-G2 cells. Inter-centrosome distance is measured in late-G2 cells as an indicator of the centrosome-separating activity.

⚠ CRITICAL: Pre-chill methanol at −20°C for optimal fixation.

4. Fixation
   a. Aspirate culture medium from the transfected cells.
   b. Add ∼1 mL of pre-chilled absolute methanol to cells by using a Pasteur pipette and incubate in a −20°C freezer for 5 min.
   c. Aspirate the methanol and rehydrate the cells by incubating with ∼1 mL of PBS for 10 min at 21°C–24°C.

   ⏸ Pause point: Cells can be stored in PBS at 4°C for 2–3 days.

   d. Briefly rinse the coverslips twice with ∼1 mL of PBST.
   e. Incubate the coverslips with 0.5 mL of blocking buffer for 30 min at 21°C–24°C.

5. Staining with primary antibodies
   a. Dilute the rabbit polyclonal anti-CENP-F antibody (1:200 v/v) and mouse monoclonal anti-γ-tubulin antibody (1:500 v/v) in blocking buffer.
   b. Load a 30-μL drop of the diluted antibodies on a piece of parafilm.
   c. Place each coverslip on a drop of the antibody mixture by using forceps; cells are placed face down and therefore are in contact with the antibody mix.
   d. Incubate at 21°C–24°C for 1 h.

   ⚠ CRITICAL: Ensure that no air bubbles are present in the drop of antibody mix.

   Alternatives: Antibodies against pericentrin can be used instead of anti-γ-tubulin to label centrosomes.

6. Transfer the coverslips to the wells of 12-well plates by using forceps; cells are placed face up.
7. Rinse the coverslips 5 times with ∼1 mL of PBST.

8. Secondary antibody staining is performed similarly as the primary antibody staining (step 5), except that donkey anti-mouse IgG (Alexa Fluor 488-conjugated) and donkey anti-rabbit IgG (Alexa Fluor 594-conjugated) are diluted in PBST (1:1000) for the staining. Cells are incubated with antibodies at 21°C–24°C for 1 h in a dark box.
9. Transfer the coverslips back to 12-well plates; cells are placed face up.
10. Rinse the coverslips thrice with 1 mL of PBST and twice with 1 mL of PBS.
11. Incubate the coverslips with Hoechst 33258 (1 mL, diluted in PBS to 1 μM) at 21°C–24°C for 2 min to label DNA.
12. Rinse the coverslips once with ∼1 mL of PBS.
13. Mount the coverslips on microscopic slides as follows:
    a. Load a drop of mounting medium (∼10 μL) on a microscope slide.
    b. Transfer coverslips onto a Kimwipe by using forceps with cells facing up.
    c. Air-dry the coverslips for ∼1 min on the Kimwipe and then place each coverslip slowly on a drop of the mounting medium by using forceps (with the cells facing down).
    d. Remove excess mounting medium from the coverslip edge by using Kimwipes.
    e. Allow the slides to dry at 37°C for 1 h or at 21°C–24°C for 12–24 h.

> ⚠ CRITICAL: Ensure that no air bubbles are present in the drop of mounting medium at step 13c.

> ⏸ Pause point: Slides can be stored at 4°C for one month or at −20°C for >6 months in the dark.

Image acquisition and analysis

> ⏱ Timing: 1 h

Cells are imaged using an epifluorescence microscope (Axio Observer ZI) equipped with a Hamamatsu ORCA-Flash4.0 Digital CMOS camera [2048 (H) × 2048(V) effective number of pixels] and an X-Cite series 120Q lamp (Lumen Dynamics); the microscope is operated using ZEN 3.0 software (blue edition), and cell images are acquired using a Plan Apo 100× NA 1.4 oil objective. An epifluorescence microscope with a 60× or 100× objective and a conventional setup is sufficient for imaging and image acquisition. Acquired images are processed using ZEN 3.0 to determine inter-centrosome distances. The analysis can also be performed using other software, such as ImageJ (Schneider et al., 2012).

14. Image acquisition

Acquire epifluorescence images by using DAPI (ultraviolet excitation filter), Alexa Fluor 488 (blue excitation filter), and Alexa Fluor 594 (green excitation filter) channels for imaging the signals of nuclear DNA, γ-tubulin, and CENP-F, respectively.

> Note: The two separated or unseparated centrosomes in a majority of cells are well-focused in the same focal plane. Cells containing centrosomes at different focal planes are excluded from the image acquisition and analysis. Both CENP-F–positive and –negative cells are included in the image acquisition.

15. Image analysis and measurement of inter-centrosome distance
    a. Late-G2 cells are identified by the positive staining of CENP-F at nuclei.
        i. We determine the nuclear intensity of CENP-F by measuring the mean intensity of CENP-F within the nuclear region (drawn manually using the Contour graphic tool of ZEN software), and we derive the cytosolic intensity as the mean intensity of three 10-μm-diameter circles (created using the Circle graphic tool of ZEN software) randomly positioned in the
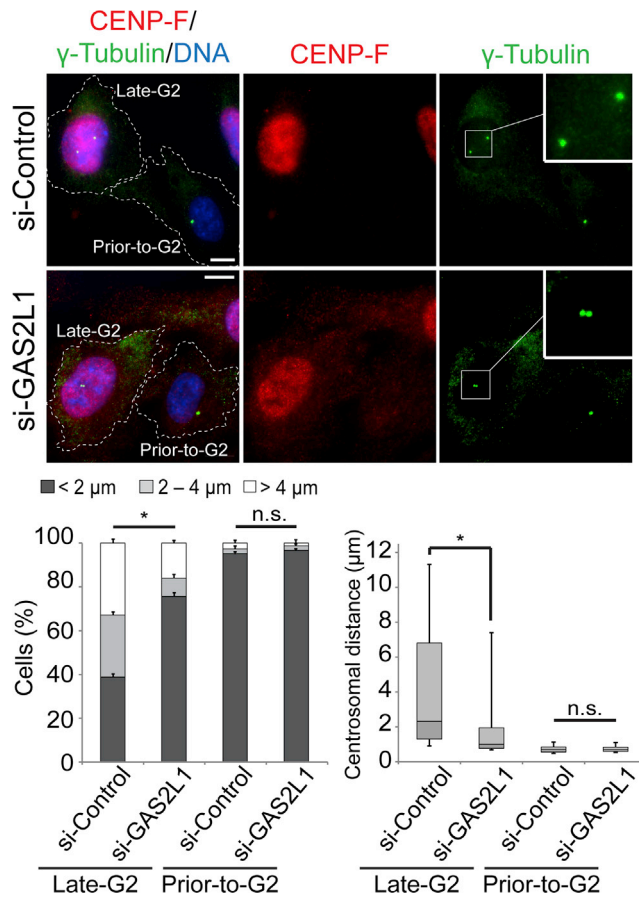
Figure 1. RPE-1 cells were transfected with a control siRNA or a *gas2l1*-targeting siRNA (si-GAS2L1)
Inter-centrosome distances were measured from late-G2 cells (n = 152 and 194, si-Control and si-GAS2L1, respectively) and prior-to-G2 cells (n = 188 and 186, si-Control and si-GAS2L1) in three independent experiments. White dashed lines indicate the cell boundary. Scale bars, 10 μm. Error bars denote SD of three independent experiments. Box-and-whisker plots: lower and upper edges of the boxes represent lower and upper quartiles (25%–75%), respectively; lines within the boxes are the medians; lower and upper whiskers extend from the lower quartile down to 10% and from the upper quartile up to 90%, respectively. *, p < 0.01; n.s., not significant (p > 0.5); unpaired Student's t test. [Reproduced from (Au et al., 2017)].

       cytosolic region. The fluorescence intensity of a non-cell area in the micrograph is recorded for background subtraction. Other methods can be used to obtain the same information.

    ii. Cells featuring a nuclear-to-cytosolic fluorescence-intensity ratio of >2.5 are scored as late-G2 cells. We measure inter-centrosome distances in both cells in late G2 and cells in stages preceding late G2 (Figure 1).

  b. Measurement of inter-centrosome distance.

    i. Manually position a circle surrounding centrosomes as defined by γ-tubulin staining by using the Circle graphic tool of ZEN software. The software tool automatically marks the circle centers, which are the centrosome centers.

    ii. Use the LINE tool of ZEN software to measure the distance between the centers of two centrosomes, which provides the inter-centrosome distance.

Note: Acquired cell images can be imported into ImageJ to identify late-G2 cells and to quantify inter-centrosome distance by using similar methods as described above.

EXPECTED OUTCOMES

In the majority (∼61%) of late-G2 control cells (CENP-F-positive cells), centrosomes are separated by ≥2 μm; however, after siRNA-mediated depletion of GAS2L1, the proportion of cells with an inter-centrosome distance of ≥2 μm is reduced to ∼24% (Figure 1). This indicates that GAS2L1 is necessary for the centrosome separation that occurs in G2. By contrast, GAS2L1 knockdown does not affect the inter-centrosome distance in cells that are in stages preceding G2 (Figure 1; CENP-F-negative cells), which suggests that GAS2L1 is not required for centrosome cohesion.

Overall, either depletion or overexpression of a protein of interest might cause defects in centrosome separation in late G2, which would be reflected by a statistically significant reduction in the inter-centrosome distance. Conversely, an increase in the inter-centrosome distance in cells before G2 would suggest a defect in centrosome cohesion.

LIMITATIONS

This protocol describes a readily applicable method for quantifying inter-centrosome distance from 2D epifluorescence images of adherent cells that are mostly flat, such as RPE-1, U2OS, and MRC-5 cells. In late G2, the majority of such cells contain two separated centrosomes that are present in the same focal plane and are thus highly suitable for the analysis. However, cells that are cultured in 3D systems frequently harbor centrosomes in distinct focal planes, and this requires 3D imaging and analysis by confocal microscopy.

Centrosome separation is controlled by various mechanisms, including the Nek2-dependent phosphorylation of several centrosomal proteins that mediates centrosome disjunction and the subsequent microtubule motor Eg5-dependent separation of the centrosomes. Nek2-mediated phosphorylation occurs at late G2 before mitotic entry and induces the phosphorylation-dependent disassembly of the centrosome linker and activation of GAS2L1. Eg5 is fully activated after mitotic entry for centrosome splitting, but low activity of Eg5 has been detected in late G2 and the involvement of Eg5 in pre-mitotic centrosome separation has also been reported (Smith et al., 2011). Our protocol allows us to quantify the contribution of proteins in late-G2 centrosome separation before mitotic entry. However, further experimental evidence is required for comprehensively elucidating the precise mechanism by which candidate proteins mediate centrosome separation.

TROUBLESHOOTING

Problem 1

Low knockdown efficiency of target proteins (steps 1–3 from the section "step-by-step method details").

Potential solution

Several measures can be implemented to overcome this problem. First, cells should be seeded at an appropriate level of confluence (∼20% at the beginning of transfection). Second, siRNAs should be stored properly. After dissolving in nuclease-free water, siRNAs are stored in aliquots at −80°C. Third, multiple siRNA sequences for a candidate gene should be tested for knockdown efficiency. A mixture of multiple siRNAs targeting a single gene can be considered for transfection if the knockdown efficiency of single siRNAs is low.

Problem 2

Massive cell death after transfection (steps 1–3 from the section "step-by-step method details").

Potential solution

Cell death can be attributed to the toxicity of Lipofectamine RNAiMax. In our experience, the toxicity of Lipofectamine RNAiMax varies from batch to batch. The amount of Lipofectamine RNAiMax used for transfection can be lowered, although this might compromise the knockdown

efficiency. Seeding more cells (e.g., at ∼30% confluence) for transfection is another potential solution for this problem.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Robert Z. Qi (qirz@ust.hk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze unique codes or datasets, respectively.

AUTHOR CONTRIBUTIONS

F.K.C.A. performed the experiments. F.K.C.A., E.S.W.L., and R.Z.Q. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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