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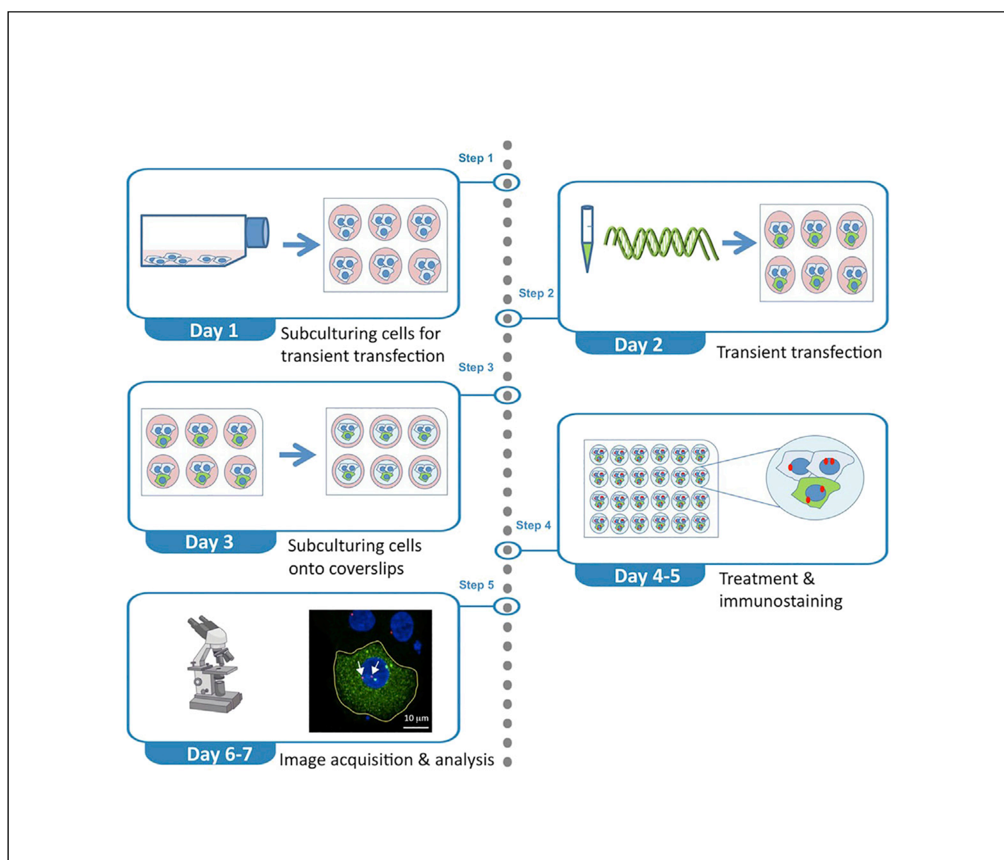
Protocol to measure centrosome cohesion deficits mediated by pathogenic LRRK2 in cultured cells using confocal microscopy



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Highlights

Protocol for robust quantification of inter-centrosomal distances in cultured cells

Step-by-step guide for cell culture, transient transfection, and immunocytochemistry

Rapid quantification of inter-centrosome distances from distinct cell types

Precise description of centrosome splitting determination mediated by pathogenic LRRK2

The present protocol allows for quantification of inter-centrosome distances in G2 phase cells by confocal fluorescence microscopy to determine centrosome cohesion deficits. We describe transfection and immunofluorescence approaches followed by image acquisition and analysis of inter-centrosome distances. This protocol is for adherent A549 cells transiently overexpressing pathogenic LRRK2 and for immortalized murine embryonic fibroblasts endogenously expressing LRRK2 but is amenable to any other cultured cell type as well.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol to measure centrosome cohesion deficits mediated by pathogenic LRRK2 in cultured cells using confocal microscopy

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SUMMARY

The present protocol allows for quantification of inter-centrosome distances in G2 phase cells by confocal fluorescence microscopy to determine centrosome cohesion deficits. We describe transfection and immunofluorescence approaches followed by image acquisition and analysis of inter-centrosome distances. This protocol is for adherent A549 cells transiently overexpressing pathogenic LRRK2 and for immortalized murine embryonic fibroblasts endogenously expressing LRRK2 but is amenable to any other cultured cell type as well.
For complete details on the use and execution of this protocol, please refer to Fdez et al.[1] and Lara Ordóñez et al.[2]

BEFORE YOU BEGIN

Timing: 2 weeks

Before experiments, cells are thawed up and recovered from a frozen stock. The recovered cells are maintained in full medium (A549 cell culture medium for A549 cells, or MEF cell culture medium for MEF cells, see materials and equipment for details). The protocol below describes the specific steps for maintaining A549 and MEF cells in culture, including recovery of cell lines from frozen stocks and subculturing steps. A549 cells have been obtained from Prof. D. Alessi, but are also commercially available (ATCC, CCL-185). R1441C-LRRK2 and wildtype littermate control MEF cells isolated from mouse embryos at day E12.5 were spontaneously immortalized by prolonged passaging,[3] and were obtained from Prof. D. Alessi (see experimental models: cell lines). Similar procedures can be used for other cell lines. Cells should never be allowed to reach 100% confluency and the passage number of cells should be annotated at all times. Do not work with cells passaged >40 times (we consider the stock of cells we originally obtained as passage 0).

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

1. Quickly warm up the frozen cell vial at 37°C in a water bath.
2. Collect cells with 5 mL of pre-warmed cell culture medium and transfer to T25 flask containing 5 mL of pre-laid warm cell culture medium.

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3. Maintain cells in a humidified 37°C incubator containing 5% CO_2.
4. Change media the following day.
5. Inspect cells daily for health and confluency.

Note: For cell maintenance in general, A549 cells are subcultured at a ratio of 1:6-1:10 twice a week, and MEF cells at a ratio of 1:10 three times a week. For subculturing, we trypsinize cells at 90% confluency as detailed under "subculturing cells for transient transfection".

⚠ CRITICAL: Cells should be subcultured 3–6 times after recovery from frozen stock to assure cell health and adequate transfection efficiencies.

Institutional permissions

The cell types employed here do not require institutional permissions. However, if you use primary cells derived from animal model systems, human peripheral cells or iPSC-derived cell types, design experiments to conform to the relevant regulatory standards and make sure you obtain prior approval from IACUC (Institutional Animal Care and Use Committee) or IRB (Institutional Review Board), respectively.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-pericentrin (1:1000 dilution for staining)	Abcam	Cat#ab4448; RRID: AB_304461
Mouse monoclonal anti-γ-tubulin (1:1000 dilution for staining)	Abcam	Cat#11316; RRID: AB_297920
Mouse monoclonal anti-flag, clone M2 (1:1000 dilution for staining)	Sigma	Cat#F1804; RRID:AB_262044
IgG (H + L) Cross-Adsorbed Goat anti-Mouse, Alexa Fluor™ 488 (1:1000 dilution for staining)	Invitrogen	Cat# A11001 RRID:AB_2534069
Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 (1:1000 dilution for staining)	Invitrogen	Cat# A11012 RRID:AB_2534079
Chemicals, peptides, and recombinant proteins		
MLi2	Abcam	Cat#ab254528
DMEM, high glucose, no glutamine	Gibco	Cat# 11960044
Fetal bovine serum	Gibco	Cat# 10500064
L-Glutamine	Sigma	G4251
MEM non-essential aminoacids	Sigma	M7145
Sodium pyruvate	Gibco	Cat#11360070
Penicillin-streptomycin (10,000 U/mL)	Gibco	Cat#15140122
Trypsin-EDTA (0.25%), phenol red	Gibco	Cat# 25200072
LipoD293™ In Vitro DNA Transfection Reagent	Signagen	Cat# SL100668
Mounting medium with 1.5 μg/mL DAPI	Vectorlab	H-1200
Paraformaldehyde, 16% w/v aq. soln., methanol free (PFA)	Thermo Scientific	043368.9M
Bovine serum albumin (BSA)	Biowest	P6156
Triton X-100	Sigma	T9284
Phosphate buffered saline (PBS)	Sigma	P4417
Critical commercial assays		
PureYield Plasmid Midiprep System	Promega	Cat#A2492
Experimental models: Cell lines		
A549 wildtype cells (passage < 40, consider stock received as passage 0)	Ito et al.[4]	Prof. Dario Alessi, University of Dundee, UK

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
MEF wildtype cells (passage < 40, consider stock received as passage 0)	Steger et al.[3]	Prof. Dario Alessi, University of Dundee, UK
MEF R1441C/R1441C knockin cells (passage < 40, consider stock received as passage 0)	Steger et al.[3]	Prof Dario Alessi, University of Dundee, UK
Recombinant DNA		
pCHMWS 3xflag-hLRRK2	Greggio et al.[5]	Prof. Elisa Greggio, University of Padova, Italy
pCHMWS 3xflag-hLRRK2 Y1699C	Greggio et al.[5]	Prof. Elisa Greggio, University of Padova, Italy
Software and algorithms		
GraphPad Prism 9	GraphPad Software Inc.	https://www.graphpad.com/scientific-software/prism/
Fiji ImageJ	Schneider et al.[6]	https://imagej.net/Fiji/Downloads
Leica Applied Systems image acquisition software	Leica	LAS AF6000
Other		
Leica TCS-SP5 confocal microscope	Leica	N/A
6-well plates	Thermo Fisher	10119831
24-well plates	Thermo Fisher	10604903
T25 flasks	Thermo Fisher	12034917
Coverslips	VWR	631-1578

MATERIALS AND EQUIPMENT

A549 cell culture medium

Reagent	Final concentration	Amount
DMEM, high glucose, no glutamine	N/A	440 mL
fetal bovine serum	10%	50 mL
L-glutamine	2 mM	5 mL
Penicillin-Streptomycin	100 U/mL - 100 μg/mL	5 mL
Total	N/A	**500 mL**

Store at 4°C. Assure that the phenol red color indicator does not change color to dark pink, as this indicates pH change. Generally stable for 3–4 weeks. Discard if turbid (indicates bacterial contamination).

MEF cell culture medium

Reagent	Final concentration	Amount
DMEM, high glucose, no glutamine	N/A	430 mL
fetal bovine serum	10%	50 mL
MEM non-essential amino acids	1%	5 mL
Penicillin-Streptomycin	100 U/mL - 100 μg/mL	5 mL
Sodium Pyruvate	1 mM	5 mL
L-glutamine	2 mM	5 mL
Total	N/A	**500 mL**

Store at 4°C. Assure that the phenol red color indicator does not change color to dark pink, as this indicates pH change. Generally stable for 3–4 weeks. Discard if turbid (indicates bacterial contamination).

1xPBS		
Reagent	Final concentration	Amount
phosphate buffer	0.01 M	5 Tablets
potassium chloride	0.0027 M	
sodium chloride	0.137 M	
Total	N/A	1000 mL

Dissolve in 1L of deionized water and autoclave.
Store at 21°C–24°C for up to 3 weeks or at 4°C for up to 6 months.

PBST		
Reagent	Final concentration	Amount
Triton-X100	0.2% (v/v)	2 mL
Total	N/A	**1000 mL**

Mix with 1L of autoclaved PBS. Since Triton-X100 is very viscous, cut the front of a P1000 Eppendorf tip before slowly pipetting out from stock solution.
Store at 21°C–24°C for up to 3 weeks or at 4°C for up to 6 months.

Paraformaldehyde (PFA) fixing solution		
Reagent	Final concentration	Amount
Paraformaldehyde 16%	4% (v/v)	20 mL
Total	N/A	**80 mL**

Mix with 60 mL of autoclaved PBS. Prepare in fume hood, as paraformaldehyde stock solution is toxic.
Store in 5 mL aliquots at −20°C for up to 6 months.

Blocking solution		
Reagent	Final concentration	Amount
Bovine serum albumin (BSA)	0.5% (v/v)	0.25 *g*
Total	N/A	**50 mL**

Dissolve in 50 mL of PBST.
Store at 4°C for up to 1 week.

A laser confocal microscope with a 63x objective. We used a Leica TCS-SP5 confocal microscope with a 63 × 1.4 NA oil UV objective (HCX PLAPO CS).

lternatives: Other confocal microscopes could also be used such as Zeiss LSM710, LSM800, LSM880; Leica SP8; Nikon A1 or Olympus FV1200 etc.

GraphPad Prism 5.0, 8.0 or other versions could also be used for graph plotting.

STEP-BY-STEP METHOD DETAILS

Day 1: Subculturing cells for transient transfection

Timing: 1 h

To determine the effects of pathogenic LRRK2 on centrosome cohesion, we transiently transfect adherent A549 cells. All pathogenic LRRK2 mutants (G2019S, R1441C, R1441G, Y1699C, I2020T, N1437H) cause centrosomal cohesion deficits in a manner reverted by pharmacological LRRK2 kinase inhibition, but here we describe procedures employing flag-tagged wildtype and Y1699C mutant LRRK2.

1. Pre-warm A549 cell culture medium and Trypsin-EDTA solution in a 37°C water bath.

2. Aspirate the culture medium from the cells.
3. Add 1.5 mL of pre-warmed Trypsin-EDTA solution to T25 flask containing cells at 90% confluency and incubate at 37°C for maximally 5 min or until all cells are detached.
4. Resuspend the cells with 8.5 mL of culture medium and disperse by repeatedly pipetting the cell suspension.
5. Count cells with hemacytometer. For A549 cells, a T25 flask at 90% confluency typically amounts to around 2.5×10^6 cells.
6. Pre-lay 1 mL of A549 cell culture medium into each well of a 6-well plate.
7. Plate 1 mL of cell suspension per well of a 6-well plate containing pre-laid medium. For A549 cells, this means plating around 250′000 cells per well of a 6-well plate.

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

⚠ CRITICAL: Cells as plated here should be around 80% confluent the next day so that you obtain good transfection efficiencies.

Day 2: Transient transfection

⏲ Timing: 6 h

Here we describe procedures for transiently transfecting DNA constructs into A549 cells by using the LipoD293 transfection reagent.

⚠ CRITICAL: Purified DNA to be transfected must be of high quality and at a concentration of approximately 1μg/μL. Follow manufacturer's instructions of the commercial kit indicated in the key resources table.

(https://www.promega.com/products/nucleic-acid-extraction/plasmid-purification/pureyield-plasmid-midiprep-system/?catNum=A2492).

8. In an Eppendorf tube, mix 100 μL of DMEM high glucose, no glutamine medium with 1 μg of LRRK2 construct. Vortex.
9. In a separate Eppendorf tube, mix 100 μL of DMEM high glucose, no glutamine medium with 4 μL of LipoD293 transfection reagent. Vortex and incubate at RT for 3 min.
10. Add the LipoD293-containing solution to the DNA-containing solution. Vortex thoroughly and incubate at RT for 15 min.

⚠ CRITICAL: Make sure you thoroughly vortex the LipoD293/DNA mix for optimal transfection efficiency.

11. Add the 200 μL transfection mix to the cells in a well of a 6-well plate (add gently down the side wall of the well, and then gently swirl the plate to mix). Incubate at 37°C in 5% CO_2 for 5 h.
12. After transfection, gently remove medium and add fresh pre-warmed A549 cell culture medium. Incubate at 37°C in 5% CO_2 overnight (12–16 h).

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

Note: Steps 1–12 are omitted for MEF cells endogenously expressing wildtype or pathogenic LRRK2.

Alternatives: Dependent on cell type, other transfection reagents can be employed as well. With the large plasmid size encoding for full-length tagged LRRK2, we obtain decent transfection efficiencies also when using Turbofect (ThermoFisher, R0533), Jetprime

(Polyplus, 101000015), Fugene6 (Promega, E2691) or XtremeGene HP (Sigma, 6366244001) transfection reagents.

Note: Refer to Problem 1 of the section "troubleshooting" if low transfection efficiency is observed.

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

Day 3: Subculturing cells onto coverslips

Timing: 2 h

This section describes how cells are subcultured onto coverslips.

13. Pre-warm A549 cell culture medium and trypsin-EDTA solution in a 37°C water bath.
14. Aspirate the cell culture medium from the cells.
15. Add 0.5 mL of pre-warmed Trypsin-EDTA solution per well of a 6-well plate and incubate at 37°C for maximally 5 min or until all cells are detached.
16. Meanwhile, place a dry-heat sterilized 13-mm round glass coverslip (you can place up to three coverslips per well) into each well of a 6-well plate and pre-lay with 1 mL of A549 cell culture medium. If coverslips are floating, gently push them to the bottom of the well with a sterile pipette.
17. Add 4 mL of A549 cell culture medium to trypsinized cells and gently disperse cells by pipetting.
18. Add 1 mL of cell suspension to each well containing the coverslips and the pre-laid cell culture medium.

Note: For MEF cells endogenously expressing wildtype or pathogenic LRRK2, follow steps above but employing MEF cell culture medium instead.

Note: A549 cells and MEF cells attach to non-coated coverslips. For some other cell types, you may need to coat coverslips with e.g., poly-L-lysine to assure efficient cell attachment.

Note: Transfection efficiency is highest and transfection-induced toxicity is lowest when cells are around 80% confluent. Therefore, we do not transfect low-confluency cells already seeded onto coverslips.

Alternatives: The method presented here is based on the use of 13-mm round coverslips, but coverslips of other sizes can be used for different microscope settings.

Day 4–5: Treatment and immunostaining

Timing: 36 h

Drug treatment

The following day, to determine whether centrosomal cohesion deficits due to pathogenic LRRK2 are kinase activity-mediated, cells are treated with DMSO or the LRRK2 kinase inhibitor MLi2 (in DMSO) for 2 h before fixation and immunostaining. Use 100–200 nM MLi2 for cells transiently over-expressing LRRK2 and 10–50 nM for cells endogenously expressing LRRK2.

⚠ CRITICAL: Keep final DMSO concentration on cells to $\leq$ 0.1% (v/v), as higher concentrations of DMSO can interfere with various membrane trafficking steps which may influence centrosome cohesion.

⚠ CRITICAL: Prepare 1 mM stock solution of MLi2 in DMSO. Since MLi2 can precipitate when directly added to A549 cell culture medium, further dilute stock solution in A549 cell culture medium to make appropriate (1000x) working stocks. For example, for a 200 nM final MLi2 concentration, dilute 1 mM stock solution to 200 μM working stock solution in A549 cell culture medium. For the control DMSO condition, also dilute DMSO 1:5 in A549 cell culture medium to generate equivalent 1000x stock.

19. Treat cells with either DMSO or MLi2 in A549 cell culture medium (final DMSO concentration ≤ 0.1%) for 2 h at 37°C in 5% CO_2.

Fixation

20. Remove cell culture medium.
21. Fix the cells with 2 mL PFA fixing solution (4% (v/v) PFA in PBS) for 15 min at 37°C.
22. Remove fixing solution.
23. When staining with anti-γ-tubulin antibody, fix cells additionally with ice-cold absolute MeOH for 5 min in freezer at −20°C.

Note: Specifically for MEF cells, only fix with ice-cold MeOH for 10 min in freezer at −20°C.

⚠ CRITICAL: Pre-chill absolute MeOH at −20°C for at least 24 h for optimal fixation.

Note: Refer to Problem 3 of the section "troubleshooting" if poor staining with anti-γ-tubulin antibody is observed.

Permeabilization and staining with primary antibody

24. Permeabilize the cells for 10 min by incubation with 2 mL of PBST at RT.

Note: For MEF cells, we use 0.5% Triton-X100 in PBS instead of 0.2% Triton-X100 in PBS throughout.

25. Transfer the coverslips to a 24 well plate to minimize all subsequent incubation volumes.
26. Block for 1h at RT with 500 μL of blocking solution.

Alternatives: You can block overnight (12–16 h) at 4°C.

Note: Refer to Problem 4 of the section "troubleshooting" if high background staining is observed.

27. Incubate at 4°C overnight (12–16 h) with 100 μL of blocking solution containing primary antibodies (note that mouse anti-flag, rabbit anti-pericentrin and mouse anti-γ-tubulin antibodies are all diluted at 1:1000 (v/v)).

Note: Permeabilization and blocking steps are performed on a rocker-shaker.

Note: For determination of distances between duplicated centrosomes in non-transfected cells, simultaneously stain cells with rabbit anti-pericentrin and mouse anti-γ-tubulin antibodies. For determination of centrosome splitting in transfected A549 cells, simultaneously stain cells with both rabbit anti-pericentrin and mouse anti-flag antibodies.

Note: The same protocol as outlined here is applicable to HEK293T, SH-SY5Y, primary murine astrocytes, primary human dermal fibroblasts, lymphoblastoid cell lines, iPSCs and iPSC-derived

neural precursor cells (NPCs).[1,7–9] Optimal fixation and permeabilization conditions for other cell types need to be independently determined.

Staining with secondary antibody

⏲ **Timing: 3 h (for steps 28 to 35)**

The following morning, coverslips are stained with secondary antibodies and mounted onto slides as follows:

28. Wash coverslips 3 times with 1 mL of PBST (0.2% Triton-X100/PBS) for 10 min at RT.

    Alternatives: You can wash coverslips with blocking solution instead.

29. Incubate for 1 h at RT with 100 µL of blocking solution containing fluorescently-conjugated secondary antibodies (goat anti-mouse AlexaFluor488 and goat anti-rabbit AlexaFluor 594 are diluted at 1:1000 (v/v)).
30. Wash 3 times with 1 mL of PBST for 10 min at RT.

    Note: Incubation of coverslips with secondary antibodies and subsequent washing steps should be performed in 24-well plates protected from light.

31. Carefully pick up the coverslips with forceps.
    a. Dip into small beaker containing deionized water and then into small beaker containing 70% EtOH.
    b. Place coverslips onto a Kimwipe with cells facing up and let coverslips air-dry.
32. Put a drop (around 4 µL) of mounting medium with 1.5 µg/mL DAPI onto a slide.

    ⚠ CRITICAL: Ensure that no air bubbles are present in the drop of the mounting medium.

33. Place dried coverslip onto drop of mounting medium (cells facing down).
34. Remove excess mounting medium from the edges of the coverslip using a Kimwipe.
35. Have a member in your lab code the individual coverslips so that you will perform image acquisition and analysis blind to conditions.

    Note: Washing steps are performed on a rocker-shaker.

    Pause Point: Slides can be stored at 4°C for 1–3 months in the dark.

Image acquisition and analysis

⏲ **Timing: 2 h/coverslip**

Here we describe image acquisition and analysis for MEF cells endogenously expressing wildtype or R1441C-mutant LRRK2, but the same procedures are used for other cell types endogenously expressing or transiently over-expressing LRRK2.

36. Image acquisition
    a. Acquire images on a Leica TCS-SP5 confocal microscope using a 63 × 1.4 NA oil UV objective (HCX PLAPO CS).
    b. Collect images using single excitation for each wavelength separately and dependent on secondary antibodies.
        i. A 488 nm Argon laser line and a 510–540 nm emission band pass (for Alexa Fluor 488).

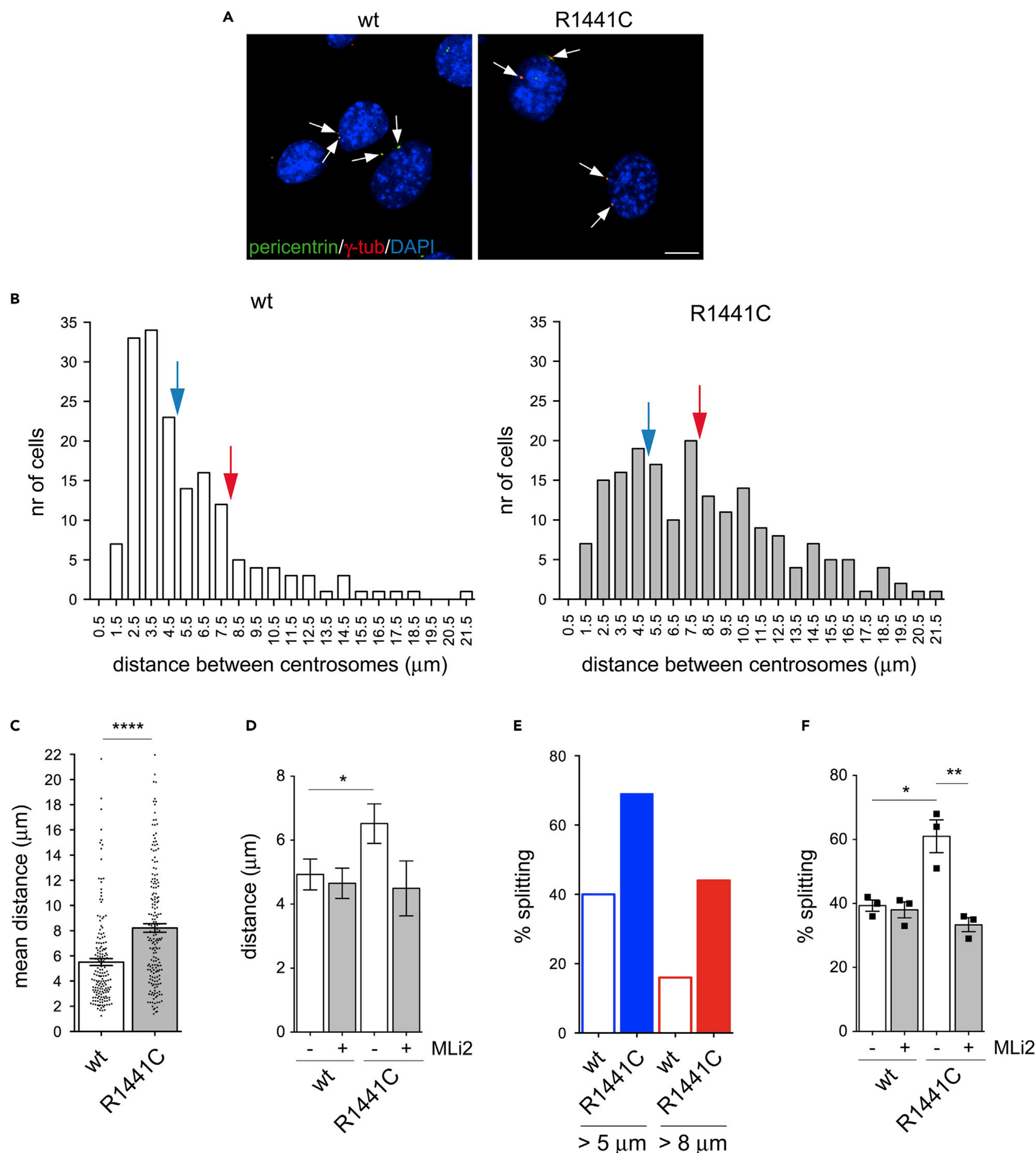

Figure 1. Centrosomal cohesion deficits in R1441C-LRRK2 knockin MEF cells

(A) Representative images of wildtype (wt) or R1441C-LRRK2 knockin MEFs stained with antibodies against two centrosomal markers (pericentrin and γ-tubulin) and with DAPI. Arrows point to duplicated centrosomes as labeled by both centrosomal markers. Scale bar, 10 μm.

(B) Frequency histogram distribution of the number of wildtype (left) or R1441C-LRRK2 (right) MEFs displaying duplicated centrosomes within binned distances as indicated.

(C) Distances between duplicated centrosomes from wildtype or R1441C-LRRK2 MEFs. This allows for determination of the mean distance from one given experiment (wildtype: 5.5 μm, 167 cells with duplicated centrosomes; R1441C-LRRK2: 8.2 μm, 189 cells with duplicated centrosomes). Bars represent mean ± SEM (****$p < 0.001$).

Figure 1. *Continued*

(D) Quantification of the mean distances from wildtype or R1441C-LRRK2 MEFs with or without MLi2 treatment from three independent experiments. Bars represent mean ± SEM (*p < 0.05).

(E) Centrosomes were defined as split when either > 5 μm (blue) or > 8 μm (red) apart, and the percentage of cells with duplicated centrosomes bigger than the respective cut-off plotted from data shown in (B). A splitting phenotype in the R1441-MEFs as compared to wildtype MEFs is observed in both cases.

(F) Quantification of split centrosome phenotype from wildtype or R1441C-LRRK2 MEFs with or without MLi2 treatment from three independent experiments. Bars represent mean ± SEM (**p < 0.01; *p < 0.05). (Figure modified from.[1])

ii. A 561 nm HeNe laser line and a 596–628 nm emission band pass (for Alexa Fluor 594).
iii. A 405 nm UV diode and a 420–470 nm emission band pass (for DAPI).

Note: Use the same laser intensity settings and exposure times for image acquisition of individual experiments.

c. Acquire 15–20 image sections with a z-step size of 0.5 μm.

Note: If imaging centrosome cohesion from cells transiently overexpressing LRRK2, select areas based on the presence of transfected cells, and only image healthy-looking transfected cells.

Note: If imaging cells endogenously expressing LRRK2, image random areas across the coverslip.

Note: Refer to Problem 5 of the section "troubleshooting" if you encounter blue dots in the DAPI channel.

Note: Refer to Problem 6 of the section "troubleshooting" if you encounter very few cells with duplicated centrosomes.

37. Image analysis

Note: The average distance between duplicated centrosomes is cell type-dependent.[1,10] Therefore, for any given cell type, you first need to determine the mean distance between duplicated centrosomes from control wildtype cells so as to define centrosome splitting as described below for that given cell type.

a. Generate maximal intensity projections of all acquired images.
b. Process and analyze images using Leica Applied Systems (LAS AF6000) image acquisition software.

Alternative: Image analysis can be performed in ImageJ.[6]

Note: In all cases and based on DAPI staining, exclude mitotic cells from further analysis.

i. Non-mitotic cells display either one centrosome (G1 phase) or two centrosomes (late G2 phase). From 100 random non-transfected interphase cells, quantify the percentage of cells displaying two centrosomes.

Note: The percentage of cells displaying duplicated centrosomes is an indicator of the percentage of cells in G2 phase of the cell cycle, and this number should be similar to the percentage of cells in G2/M phase as independently determined by FACS analysis using propidium iodide.[1]

ii. Make a zoom of an area of interest. Manually measure the distance (center to center) between duplicated centrosomes from 150–200 independent wildtype cells (Figure 1A).

iii. Plot a frequency histogram of the distances between duplicated centrosomes from control wildtype cells using GraphPad Prism 9 software (Figure 1B). Perform the same analysis with cells endogenously expressing pathogenic LRRK2 (Figure 1B).
iv. Plot the distances between duplicated centrosomes in wildtype versus R1441C-LRRK2 cells (Figure 1C). As evident from the data, you can see a shift toward larger inter-centrosome distances in the frequency histogram of the R1441C-LRRK2 as compared to the wildtype MEFs, which is reflected by an increase in the mean distance between duplicated centrosomes (Figure 1C).
v. Repeat the experiment twice more to obtain the average mean distances between duplicated centrosomes from wildtype versus R1441C-LRRK2 MEFs and in either the absence or presence of MLi2 (Figure 1D).
vi. The definition of "centrosome splitting" is arbitrarily defined as the percentage of cells with a distance between duplicated centrosomes greater than a given value.[10] In the example provided, 40% of cells with duplicated centrosomes are considered to display a splitting phenotype when the distance between the duplicated centrosomes is > 5 μm (blue, Figures 1B and 1E). In contrast, 15% of cells are considered to display a splitting phenotype when the distance between the duplicated centrosomes is > 8 μm (red, Figures 1B and 1E). In both cases, the R1441C-LRRK2 MEFs display an increased centrosome splitting phenotype as compared to the wildtype cells (Figure 1E).
vii. Repeat the experiment twice more to obtain the average splitting phenotype (here defined as > 5 μm) between duplicated centrosomes from wildtype versus R1441C-LRRK2 MEFs in either the absence or presence of MLi2 (Figure 1F).

Note: We determine the maximal diameter of the nucleus from around 20 cells as identified by DAPI staining to obtain a mean nuclear diameter value. We exclude the occasional cells where the distances between their duplicated centrosomes are larger than the mean nuclear diameter. This is to assure that we do not quantify centrosome distances from cells in early phases of mitosis (specifically prophase), where DAPI staining does not accurately identify such cells in all cases. In the case of MEF cells, we exclude cells with inter-centrosome distances > 20 μm (1 cell, Figure 1B). In the case of A549 cells, we exclude cells with inter-centrosome distances > 5 μm.

⚠ CRITICAL: Only analyze cells where the centrosomes are co-stained by both centrosomal marker antibodies (pericentrin and γ-tubulin).

⚠ CRITICAL: Centrosome amplification can be observed in certain immortalized cell types.[11] Therefore, exclude all cells with more than two centrosomes from further analysis.

EXPECTED OUTCOMES

In the majority of G2 cells, duplicated centrosomes are physically held close together by a set of proteins. Pathogenic LRRK2 phosphorylates Rab8 and Rab10. When phosphorylated, these proteins gain the ability to bind to RILPL1, a protein localized to the subdistal appendage of the mother centriole. The phospho-Rab/RILPL1 complex displaces a protein critical for centrosome cohesion and thereby causes centrosome splitting, which is reverted upon pharmacological LRRK2 kinase inhibition.

Such kinase-mediated centrosomal cohesion deficits are observed in a wide range of cell types either overexpressing or endogenously expressing pathogenic LRRK2, indicating that they are a sensitive and dynamic cellular readout for pathogenic LRRK2 kinase activity. By contrast, inhibiting

wildtype LRRK2 does not affect inter-centrosome distances, which suggests that LRRK2 is not required for centrosome cohesion per se.

LIMITATIONS

The protocol here describes a solid and reproducible method for quantifying inter-centrosome distances from confocal images of any adherent cell type. Centrosomal distances are quantified from 3D z-stacks collapsed into 2D maximal intensity projections. This allows for quantification of duplicated centrosomes in different focal planes. However, duplicated centrosomes present at identical positions but across different z-stacks will be omitted by this type of analysis. Even though highly time-intense, these scenarios can be included by manual inspection of all individual z-stacks.

Our protocol allows us to determine centrosomal cohesion phenotypes in cultured cells *in vitro*. However, further experimental evidence is required for comprehensively analyzing centrosomal cohesion deficits *in vivo*, such as in neural stem cells in the intact adult mouse brain of pathogenic LRRK2 knockin mice.

TROUBLESHOOTING

Problem 1

Low transfection efficiency when transiently overexpressing LRRK2 constructs (steps 1–12 from the section "step-by-step method details").

Potential solution

Several measures can be taken to improve transfection efficiency. First, assure good quality and concentration of purified plasmid DNA (use endotoxin-free PureYield Plasmid Maxiprep Kit as indicated in key resources table). Second, assure that cells have been subcultured 3–6 times after recovery from frozen stock, as transfection efficiencies in recently thawed up cells is low. Third, assure that cells are at around 80% confluency at the day of transfection, as transfection efficiency drastically decreases if cells are too confluent, and do not use cells beyond passage 40. Fourth, make sure you thoroughly vortex the DNA and LipoD293 mix.

Problem 2

Massive cell death after transient transfection (steps 1–12 from the section "step-by-step method details").

Potential solution

Cell death after lipofection can be due to the toxicity of the lipofection reagent. In our hands, the toxicity of LipoD293 is generally low, but can vary by batch. The amount of LipoD293 can be lowered, but this may compromise transfection efficiency. Alternatively, other lipofection reagents can be employed. In addition, assure that cells are at around 80% confluency, as transient transfection of cells at lower confluency becomes increasingly toxic.

Problem 3

Poor staining with anti-γ-tubulin antibody (steps 20–23 from the section "step-by-step method details").

Potential solution

MeOH fixation is critical for efficient staining of many centrosome-resident proteins including γ-tubulin. If poor staining and high cytosolic background is observed, assure that MeOH solution has been kept in freezer at −20°C for at least 24 h. Increase fixation time with ice-cold MeOH for enhanced staining of centrosomes.

Problem 4

High cytosolic background staining (steps 24–27 from the section "step-by-step method details").

Potential solution

Instead of 1 h at RT, perform blocking step at 4°C overnight (12–16 h). Alternatively, increase the percentage of BSA in the blocking solution from final 0.5% (v/v) to final 1% (v/v). You can also try blocking with final 10% (v/v) goat serum (Vector Laboratories, S-1000) instead of BSA.

Problem 5

Blue dots in DAPI staining (steps 8–12 from the section "step-by-step method details").

Potential solution

Liposome-based transfection reagents including LipoD293 can give rise to autofluorescence of internalized liposomes which appear as blue dots in transfected cells. Aliquot transfection reagents into 50 μL aliquots so as to minimize oxidation of reagents upon repetitive use, and use new aliquot each time you perform transient transfections.

Problem 6

Very few cells displaying duplicated centrosomes (steps 36–37 from the section "step-by-step method details").

Potential solution

If signal is saturated, closely apposed duplicated centrosomes can appear as one big centrosome. Change acquisition settings so that you do not get signal saturation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sabine Hilfiker (sabine.hilfiker@rutgers.edu).

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.

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AUTHOR CONTRIBUTIONS

E.F, R.F., A.J.L.O., B.F., and Y.N. performed the experiments. E.F. and S.H. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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