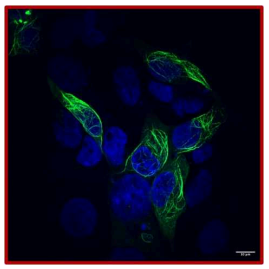
Jan 11, 2024

Visualization of LRRK2 filaments in 293T cells



The Journal of Biological Chemistry

DOI
dx.doi.org/10.17504/protocols.io.8epv5xpz4g1b/v1

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.8epv5xpz4g1b/v1

Protocol Citation: Eva Karasmanis, Kathryn S Hatch 2024. Visualization of LRRK2 filaments in 293T cells. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.8epv5xpz4g1b/v1**

Manuscript citation:
doi: https://doi.org/10.1101/2023.11.14.567123

Protocol status: Working
We use this protocol and it's working

Created: January 11, 2024

Last Modified: May 31, 2024

Protocol Integer ID: 93405

Keywords: ASAPCRN, visualization of lrrk2 filament, lrrk2 filaments in the presence, lrrk2 filament, cell, percentage of cell, lrrk2, visualization

Funders Acknowledgements:
ASAP
Grant ID: ASAP-000519

Abstract

Visualization of LRRK2 filaments in 293T cells

GOAL: Express GFP-LRRK2 with or without DARPin E11 and quantify the percentage of cells with LRRK2 filaments in the presence and absence of MLi-2 in 293T cells.

Image Attribution

Eva Karasmanis

Troubleshooting

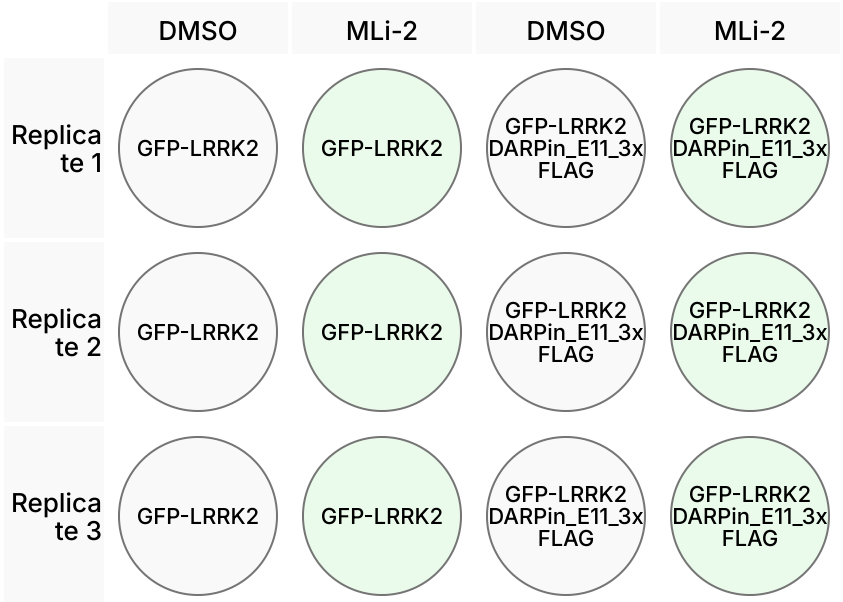
Day 1: fibronectin coating and cell plating

1 **Visualization of LRRK2 filaments in 293T cells**

GOAL: Express GFP-LRRK2 with or without DARPin E11 and quantify the percentage of cells with LRRK2 filaments in the presence and absence of MLi-2 in 293T cells.

Constructs needed:

1) CMV-8xHISDaprin C12-FLAG
2) CMV- His-daprin E11-FLAG
3) CMV-GFP-LRRK2



2 **Fibronectin Coating (Sigma cat# F0895, 0.1% solution, 1 mg/ml):**

2.1 Make [M] 0.01 µg/µL solution of fibronectin, stock @ 1 mg/mL For 6× 35 mm dishes (12 ml fibronectin working stock)- 0.12 mL fibronectin + 11.88 mL 1X PBS

2.2 Lay one 22 mm x 22 mm glass coverlsip into each 35 mm dish or 6 well.

2.3 Add 2 ml of 10 ug/mL fibronectin per 35 mm dish or 6 well.

2.4 Incubate at 37 °C 5% CO_2 for 00:45:00 45m

2.5 Wash with PBS and let dry for 00:45:00 in the tissue culture hood (no UV) 45m

3 **Plate cells onto Fibronectin coated dishes**

3.1 Dissociate cells, count and plate 6 well plate with 200K cells /well. For transfection, plate in antibiotic-free media (DMEM+10% FBS)

Day 2 Transfect cells with GFP-LRRK2 and Darpins: 2d 3h 2m

4 Transfect 800 ng LRRK2 and 400ng Darpin with 5 µL PEI /well.
- cells should be 50-60% confluent
In a sterile tube dilute 800 ng GFP-LRRK2 and 400 ng DARPin E11 plasmid DNA in Optimem (150 µL /well)

Per Well:
a) 150 µL Optimem (prewarmed)+ 5 µL PEI
b) 150 µL Optimem (prewarmed) + 800 ng GFP LRRK2 and 400 ng DARPin E11 DNA.
or
c) 150 µL Optimem (prewarmed) + 800 ng GFP LRRK2

Add PEI to diluted DNA - (Per reaction: 5 µL of [M] 1 µg/µL stock). Mix immediately.

4.1 In a sterile tube dilute 800 ng GFP-LRRK2 and 400 ng DARPin E11 plasmid DNA in Optimem (150uL/well)

Per Well:
a) 150 µL Optimem (prewarmed)+ 5 µL PEI
b) 150 µL Optimem (prewarmed) + 800 ng GFP LRRK2 and 400 ng DARPin E11 DNA.
or

c) 150 µL Optimem (prewarmed) + 800 ng GFP LRRK2

Add PEI to diluted DNA - (Per reaction: 5 µL of [M] 1 µg/µL stock). Mix immediately.

4.2 Incubate at Room temperature for 00:15:00 15m

4.3 Add DNA/PEI mixture to cells dropwise.

4.4 Swirl the plate to distribute Incubate at 37 °C 5% CO_2 for 48:00:00 2d

Day 4 MLi2 or DMSO treatment 2d 3h 2m

5 Treat Cells with MLi2 or DMSO (5uL per well of a 1 mM stock) for 02:00:00, 37 °C 5% CO_2 2h

Fixing and Staining of Cells 2d 3h 2m

6 **Fixing:** Prewarm freshly made 3% PFA, 4% sucrose in 1X in PBS 1X. You will need 1 mL per well.·

6.1 Aspirate media

6.2 Immediately add prewarmed fixation buffer (3% sucrose, [M] 4 % (v/v) PFA in PBS 1X)·

7 Incubate 00:12:00 Room temperature 12m

8 2X rinse with PBS 1x

9 2X Wash with PBS1x for 00:05:00 at Room temperature 5m

10 2X Wash with PBS 1x for 00:05:00 at Room temperature . 5m

11 **Quenching:** 2X rinse and 2x 00:10:00 of 0.4% NH4Cl [M] 75 millimolar (mM) in PBS 1X 10m

12 **Blocking/Permeabilizing:** incubate cells blocking/permeabilizing buffer (2% BSA + 0.1 % triton X-100 in PBS 1X) for 00:20:00 Room temperature 30m

During blocking make and spin the antibody (ab) mix. Each coverslip needs ~40-50 uL uL of ab mix. Antibodies are diluted in Blocking buffer (2% BSA in PBS 1X) and spun at 4 °C 00:10:00 m 50000 x g, 4°C to clear aggregates. Remove spun ab and place in new tube. Mix to get even concentration. HERE: we used 1:200 rabbit polyclonal anti-FLAG DYKDDDDK tag – (ptg labs Cat no : 20543-1-AP)

13 2X Rinse with Wash 2X (00:05:00 , Room temperature) with blocking buffer 5m

14 Add primary antibody mix : 3h 5m
Add 40uL on parafilm and flip coverslip on the antibody. Incubate 03:00:00 Room temperature or 4 °C Overnight . If incubating overnight, make a humidity chamber before placing in fridge.

HERE: we used 1:200 rabbit polyclonal anti-FLAG DYKDDDDK tag – (ptg labs Cat no : 20543-1-AP)

15 Rinse 2X with blocking buffer

16 Wash 2X 00:05:00 Room temperature with blocking buffer 5m

17 Add secondary mix in PBS (1:200 Goat a-rabbit Alexa568; SIGMA A-11011 +1:5000 DAPI**) 00:45:00 Room temperature 45m
** DAPI can alternatively be added as a separate step at 1:1000 dilution for 15 min

18 5X Rinse with PBS 1X

19 Mount in Fluorsave hard media (Millipore 345789)

20 Let dry for at least an hour. Store in Fridge 4oC if not imaging immediately. Check coverslip is set with tweezers before imaging.

Imaging and analysis

21 Blind your mounted slides before imaging to prevent bias during aquisition.

22 Find areas with transfected cells. Acquire Z stacks by determining top and bottom with a 0.3 um step size. (about 20-25 z stacks) ·

23 **Analysis in Fiji**: Go through each image, make max projections, and mark each transfected cells with a ROI (region of interest).

24 In an excel sheet keep track of each cell you mark and score as 0 if no LRRK2 filaments are present or 1 if some are. ·

25 Include at least 50 cells/ sample. ·

26 Calculate % cells with filaments (number of transfected cells with filaments /total number of transfected cells *100) ·

27 Unblind

28 Transfer values to prism to generate graph and statistics.