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Immunofluorescent Staining of phosphoRab10 in cultured cells

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This protocol can be used to detect the amount and localization of endogenous phospho-Rab10 by light microscopy. Cells that yield detectable, endogenous phosphorylated Rab10 without the need to express LRRK2 include: Mouse embryonic fibroblasts (MEFs; wild type and LRRK2 R1441C or G2019S, VPS35 D620N); A549 PPM1H knock-out; NIH-3T3; immunopanned primary rat astrocytes. In our hands, HeLa, hTert-RPE, A549, HEK-293T, and ShSy5y cells can be immunostained for phosphorylated Rab10 but require exogenous expression of wildtype or pathogenic mutant LRRK2 or addition of pharmacological agents. Cells should be Mycoplasma free.

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Materials

- 24 well plastic tissue culture plates
- Collagen coated 12mm coverslips
- Cells: MEFs (WT/R1441C/VPS35 D260N), PPM1H-KO A549
- MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher Scientific Catalog #11140050**
- Fetal Bovine Serum **Atlanta Biologicals Catalog #S11550**
- Recombinant Anti-RAB10 (phospho T73) antibody [MJF-R21-22-5] (ab241060) **Abcam Catalog #ab241060**
- Donkey anti-Rabbit-Alexa 568 highly cross-adsorbed H+L (Life Technologies)
- Paraformaldehyde (PFA, Sigma)
- Triton X-100 (Sigma)
- Saponin (Sigma)
- 2% BSA in PBS
- Methanol (Sigma) stored at **-20 °C**

Cell culture

1 Culture the cells in high glucose DMEM medium with glutamine and sodium pyruvate, 10% fetal bovine serum, with additional non-essential amino acids and Penicillin/Streptomycin.

2 MEFs are generally flat and occupy a relatively large surface area: cell counts per confluent dish are ~5X lower than other common cell lines (eg. HeLa).

3 Plate approximately 30,000 cells on 12mm coverslips in 24 well plates submerged below **0.5 mL** medium (~50% confluency at plating).

3.1 Coverslips can be pre-treated with rat tail collagen. This helps A549 cells grow flatter, providing better organelle visualization.

16h

4 Cells may be visualized ~ **16:00:00** after plating for immunofluorescence staining.

Paraformaldehyde (PFA) fixation and blocking

5

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Wash the cells 1X with **0.5 mL** PBS.

6 Fix the cells with **0.5 mL** , 3% PFA in PBS for **00:30:00** at **Room temperature** . 30m

7 

Wash the cells 3X with **0.5 mL** PBS per wash.

8 For pRab10 staining, permeabilize the cells with **0.5 mL** 0.2% **Saponin** for **00:05:00** at **Room temperature** . 5m

8.1 Permeabilization with 0.1% Triton X-100 is also possible but yields lower sensitivity.

9 

Wash the cells 2X with PBS.

10 After permeabilization, block the cells with **0.5 mL** of 2% bovine serum albumin (BSA) in PBS for **00:30:00** . 30m

Alternative fixation method: Methanol fixation and blocking

11 Methanol fixation is needed to stain microtubule-based structures (centrioles).

12 

Fix cells by gently adding **-20 °C** methanol to coverslips.

13  8m

Incubate cells for **00:03:00** - **00:05:00** in a **-20 °C** freezer.

14 

Aspirate methanol, wash cells twice with ice cold PBS.

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15 Rehydrate cells slowly in PBS for **00:05:00** **On ice** . 5m

16 Antigen block with 2% BSA for **00:30:00** (crucial to avoid background and artifacts). 30m

16.1 No detergent permeabilization is needed as methanol solubilizes the lipids.

17 Anti-phospho-Rab10 antibody works OK using this fixation method in conjunction with PPM1H-KO A549 cells and MEFs

Immunostaining 4h 45m

18 2h

Staining can be carried out following blocking after either PFA or Methanol fixation.

Primary antibody incubation: Rabbit anti-phosphoRab10 diluted to [M]**0.5 µg/ml** in 2% BSA in PBS for **02:00:00** .

18.1 Higher dilutions ([M]**0.25 µg/ml**) work, but may decrease signal intensity.

19  2h

After **02:00:00** , wash cells 3X with PBS.

20  45m

Incubate the coverslips with a secondary goat anti-Rabbit Alexa-568 antibody (H+L, Invitrogen) diluted to [M]**1 µg/ml** in 2% BSA in PBS for **00:45:00** at **Room temperature** .

21 DAPI (Invitrogen) can be diluted 10,000X in the secondary antibody solution to co-stain the nucleus.

22 

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Wash the cells 3X with PBS.

23 Mount the coverslips upside down by placement on **4 µL** Mowiol.

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