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Immunofluorescent Staining of phosphoRab10 in cultured cells

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We use this protocol and it's working

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Abstract

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.

Attachments

DOCX

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20KB

Materials

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.

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

Paraformaldehyde (PFA) fixation and blocking

5 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 Incubate cells for 00:03:00 - 00:05:00 in a -20 °C freezer. 8m

14 Aspirate methanol, wash cells twice with ice cold PBS.

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

> Note
>
> 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 After 02:00:00 , wash cells 3X with PBS. 2h

20 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 . 45m

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

22 Wash the cells 3X with PBS.

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