**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.

**Materials**

* 24 well plastic tissue culture plates
* Collagen coated 12mm coverslips
* Cells: MEFs (WT/R1441C/VPS35 D260N), PPM1H-KO A549
* DMEM high-glucose with sodium pyruvate and glutamine (Gibco 11140050)
* Serum (Atlanta Biologicals S11150)
* Rabbit anti-P-Rab10 clone MJF-R21-22-5 (Abcam 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**

* Cells are cultured in high glucose DMEM medium with glutamine and sodium pyruvate, 10% fetal bovine serum, with additional non-essential amino acids and Penicillin/Streptomycin
* 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).
* Approximately 30,000 cells are plated on 12mm coverslips in 24 well plates submerged below 0.5ml medium (~50% confluency at plating)
	+ Coverslips can be pre-treated with rat tail collagen. This helps A549 cells grow flatter, providing better organelle visualization
* Cells may be visualized ~16hours after plating for immunofluorescence staining

**Paraformaldehyde (PFA) fixation and blocking**

* Cells are washed 1X with 0.5ml PBS
* Cells are then fixed with 0.5ml, 3% PFA in PBS for 30 min at room temperature (RT)
* Cells are washed 3X with 0.5ml PBS per wash
* For pRab10 staining, cells are permeabilized with 0.5ml 0.2% **Saponin** for 5 min at RT
	+ Permeabilization with 0.1% Triton X-100 is also possible but yields lower sensitivity
* Cells are washed 2X with PBS
* After permeabilization, cells are blocked with 0.5ml of 2% bovine serum albumin (BSA) in PBS for 30 min

**Alternative fixation method: Methanol fixation and blocking**

* Methanol fixation is needed to stain microtubule-based structures (centrioles)
* Fix cells by gently adding -20°C methanol to coverslips
* Incubate cells for 3-5 minutes in a -20˚C freezer
* Aspirate methanol, wash cells twice with ice cold PBS
* Rehydrate cells slowly in PBS for 5 minutes on ice
* Antigen block with 2% BSA for 30 min (crucial to avoid background and artifacts)
	+ No detergent permeabilization is needed as methanol solubilizes the lipids
* anti-phospho-Rab10 antibody works OK using this fixation method in conjunction with PPM1H-KO A549 cells and MEFs

 **Immunostaining**

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

* Primary antibody incubation: Rabbit anti-phosphoRab10 diluted to 0.5µg/ml in 2% BSA in PBS for 2 hr
	+ Higher dilutions (0.25µg/ml) work, but may decrease signal intensity
* After 2h, wash cells 3X with PBS
* Coverslips are incubated with a secondary goat anti-Rabbit Alexa-568 antibody (H+L, Invitrogen) diluted to 1µg/ml in 2% BSA in PBS for 45 min at RT
* DAPI (Invitrogen) can be diluted 10,000X in the secondary antibody solution to co-stain the nucleus
* Cells are washed 3X with PBS
* Coverslips are mounted upside down by placement on 4µl Mowiol