



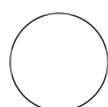
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Immunofluorescence microscopy of R1441C or VPS35 D620N MEF cells

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We use this protocol and it's working

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ABSTRACT

We present here a method to culture, fix, permeabilize, and stain R1441C or VPS35 D620N MEF cells to visualize transfected Myc-RILPL1 and endogenous TMEM55b or pRab10.

MATERIALS

Paraformaldehyde 16% (Electron Microscopy Sciences 50-980-487)
PBS (Cold Spring Harbor protocol)
TMEM55B polyclonal antibody (Proteintech 23992-1-AP)
Recombinant Anti-Rab10 (Phospho T73) antibody (Abcam ab241060)
Myc antibody (Biolegend 626802)
Donkey anti-Rabbit 568 Alexa Fluor (Thermo Fisher A10042)
Donkey anti-Mouse 488 Alexa Fluor (Thermo Fisher A-21202)
DMEM high glucose (Cytiva SH30243.01)
Fetal bovine serum (Sigma #F0926)
Microscope cover glass (Fisher 12-545-81)
FuGENE 6 transfection reagent (Promega E2691)

Culturing, plating, and treating cells

- 1 Seed R1441C MEF cells or VPS35 D620N MEF cells at 50-60% confluency in a six well plate in 2 mL of complete DMEM (DMEM containing 10% FBS and 1% penicillin-streptomycin) 24 hours before transfection.
- 2 Transfect with Myc-RILPL1 plasmid with FuGENE 6 transfection reagent (E2691) at a 3:1 FuGENE:plasmid ratio using 2µg per well according to the manufacturer's guidelines for 24 hours.
- 3 Trypsinize cells and plate onto 12 mm glass coverslips resting in a six well plate at 60% confluency and leave cells to attach to coverslips in a 37°C incubator with 5% CO₂ for 24 hours.
- 4 If needed for MLI-2 conditions, add 200nM MLI-2 and leave in the incubator for 1.5-2 hours.
- 5 For Nocodazole, add 20µM Nocodazole and leave in the incubator for 1.5-2 hours.

Fixing, permeabilizing, and mounting coverslips

- 6 Solutions needed:
 - 4% PFA in 1x PBS
 - 0.2% Saponin in 1x PBS
 - 2% BSA in 1x PBS (blocking solution)
- 7 Transfer each coverslip from the 6-well plate to a 24 well plate filled with ~400 µl 4% PFA per well and fix for 10 minutes at room temperature (all subsequent steps and solutions are at room temperature).
- 8 Wash 3x with 1x PBS.

- 9** Permeabilize cells with 0.2% Saponin in 1x PBS for 10 minutes.
- 10** Wash 3x in 1x PBS.
- 11** Block with 2% BSA in 1x PBS for 30 minutes.
- 12** Incubate primary antibody in blocking buffer for 2 hours.
 - 12.1** Dilute antibodies (see Materials) as follows:
Rabbit anti-TMEM55B PA5-61760 (1:4000)
Rabbit anti-pRab10 (1:1000)
Mouse anti-Myc (1:1000)
- 13** Wash 3x with 1x PBS.
- 14** Incubate with 2° Antibody for 1 hour in the dark in blocking buffer.
 - 14.1** Dilute antibodies (see Materials) as follows:
Donkey anti-Rabbit 568 for TMEM55b and pRab10 (1:2000) and
Donkey anti mouse 488 for Myc (1:2000)
DAPI (1:1000)
- 15** Wash 3x with 1x PBS.

- 16** Use a Kimwipe held on the edge of the coverslip to remove excess liquid; Invert the coverslip onto 3µl Mowiol on a Gold Seal glass slide (size 1x3", 1 mm thick).
- 17** Allow coverslips to dry at RT overnight.
- 18** Image