Characterizing localization differences between WT and KO fibroblasts

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Abstract

Question: Determine if there are differences in localization of MRTF and SRF between wildtype and pirin KO fibroblasts.

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Protocol

Wash and lift cells

Step 1.

1. Aspirate old media from flask
2. Add 5 ml PBS to wash, and then aspirate
3. Add 3 ml of Trypsin to flask
4. Incubate for 5 minutes to lift cells
5. Add 3 ml of EMEM media to stop trypsinization
6. Pipette up and down to clear all cells from plate
7. Transfer all media and cells to new labeled tubes
8. Centrifuge (at 4000 rpm for 5 minutes) to create a cell pellet
9. Aspirate off supernatant
10. Add 1 ml of EMEM media
11. Pipete up and down to resuspend cells

Stain cells with Trypan Blue

Step 2.

(Trypan blue stains dead cells)

1. Label eppendorf tubes
2. Pipette 10 microliters of Trypan Blue into each tube
3. Add 10 microliters of designated cell solution into tubes with Trypan Blue
4. Pipette up/down for good suspension
5. Pipette 10 microliters of stained cell solution into a labeled Countess slide
6. Use Countess Machine to count alive vs. dead cells. ** Use ZOOM function to adjust microscope EVERY TIME for an accurate count.

Plate cells

Step 3.

1. For 8 well plate, we used 10,000 cells/ well.

(20,000 cells/ ml) x (0.5 ml/well) = 10,000 cells/ well

2. Use M1V1=M2V2 equation to determine master mix requirements. OR you can use the Countess Count function.
3. Knowing the cell dilution, make master mixes with determined amounts.
4. Add 500 microliters of master mix cell solutions to each well.
5. Label and incubate for 24 hours.

Fix cells

Step 4.

1. With existing media (which is 500 microliters), add 50 microliters of 37% formaldehyde to each well to give a 1:10 dilution.
2. Let stand for 15 minutes.
3. Aspirate off media
4. At this point, you can save slide in refridgerator or countinue with treatment.

Blocking buffer

Step 5.

1. Make blocking buffer (0.5 g BSA powder + 30 microliters of Triton X-100 + 10 milliliters of PBS)
2. Wash wells with 500 microliters of PBS.
3. Aspirate
4. Add 300 microliters of blocking buffers to each well for 1 hour.
5. Set plate on room temperature rotating tray for 1 hour.

Primary antibody treatment

Step 6.

1. Make antibody dilution (0.1g BSA powder + 30 microliters of Triton X-100 + 10 ml of PBS)
2. Make 1:200 dilution of primary antibodies (1ml of antibody dilution + 5 microliters of primary antibody)
2. Remove plate from rotating tray
3. Aspirate off blocking buffer
4. Add 400 microliters of dilution to control wells and 400 microliters of dilution WITH antibodies to other wells.
5. Incubate on rocker overnight in the cold room.

Secondary antibody treatment

Step 7.

1. Make secondary antibody dilution, if needed. Can use extra stock from previous antibody dilution (01. g BSA + 30 microliters Triton X-100 + 10 ml PBS)
2. Make a 1:500 secondary antibody dilution. (1 ml of antibody dilution + 2 microliters of secondary antibody).
3. Keep secondary antibody dilution covered in foil. Keep Dark!
4. Get plate from cold room.
5. Aspirate off primary antibody mix
6. Wash wells with 500 uL PBS three times.

7. Add 400 uL of secondary antibody dilution ot every well.
8. Cover plate with tinfoil to keep dark.
9. Incubate in room temperature on rocking tray for 1 hour.
10. Wash 3x with 500 uL of PBS.

Mount

Step 8.

1. Using tool, pry off well walls.
2. Add 1 small drop of DAPI to every well floor
3. GENTLY place coverslip on top of DAPI drops. DO NOT PRESS DOWN.
4. Cover plate with foil/ and do not disturb for 30+ minutes. The coverslip will adhese to the plate.

View with EVOS

Step 9.

View slides with EVOS using the manufacturers manual.