Immunofluorescence Detection and F-actin Staining of MTLn3 Cells

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[Abstract] Epidermal growth factor (EGF)-stimulated MTLn3 cells protrusion play an important role in cell migration. Phalloidin which binds F-actin in cells is an imaging tool used with light microscopy to investigate the distribution of actin. The protocol described here can be useful for observing signaling in the EGF pathway.

Materials and Reagents

1. MTLn3 cells
2. Type I rat tail collagen (BD Biosciences, catalog number: 354236)
3. Leibovitz's L15 media w/o phenol red (Life Technologies, Invitrogen™, catalog number: 21083-027)
4. Leibovitz's L15 media w/ phenol red (Life Technologies, Invitrogen™, catalog number: 11415-064)
5. Rhodamin phalloidin red Cy3 (Life Technologies, Invitrogen™, catalog number: R415)
6. Phosphate buffered saline (PBS)
7. Trypsin-EDTA
8. HCl
9. 95% ethanol
10. Acetic acid
11. FBS-alpha-MEM
12. Paraformaldehyde
13. Triton X-100
14. Glycine
15. BSA
16. N-propyl gallate
17. Glycerol
18. Methanol
19. Nail polish
20. Collagen solution
21. EGF (EMD Millipore, catalog number: 01-102) (see Recipes)

22. Rhodamine phalloidin (see Recipes)

Equipment

1. Centrifuges
2. Coverslips
3. Tissue culture flask
4. Parafilm
5. Culture dish
6. Humidified chamber
7. Sharp forceps
8. Water bath
9. Culture hood

Procedure

A. Collagen coated coverslip preparation:

1. Wash coverslips twice with 95% ethanol.
2. Wash and incubate the coverslips in IN HCl for 1 h at room temperature (RT).
3. Wash twice with sterilized PBS (coverslips can be stored in methanol in bulk).
4. If taken straight from methanol, wash twice with sterilized PBS to rehydrate the coverslips. From this point on every procedure needs to be conducted under sterile conditions.
5. Monolayer the coverslips on 100 mm culture dish.
6. Coat the coverslips in acetic acid-collagen solution:
   256 µl of 3.33 m ml^{-1} type I collagen into 20 ml of filter sterilize 0.01 M acetic acid. Add 20 ml of acetic acid collagen solution to the monolayer coverslips in 100 mm dish. Incubate in the culture hood at RT for 2 h.
7. After collagen coating, rinse once gently with sterilized PBS. At this point the coverslips can be stored in PBS at 4 °C for 1 week.

B. Splitting MTLn3 cells on collagen coated coverslips and starvation:

8. For 80 mm tissue culture flask, MTLn3 cells should be kept 50-70% confluent at all times. Over confluent cells do not respond to EGF stimulation well.
9. Rinse once with 1 ml trypsin-EDTA, aspirate and then trypsinize with 1 ml trypsin-EDTA at 37 °C incubator for 3-5 min until cells come off the flask.
10. Add 9 ml 5% FBS-alpha-MEM into the flask. Pipet the cells up and down a few times to break the cell clumps.

11. From a 70% confluent 80 mm flask, take 3-5 ml cells to one 100 mm culture dish of the collagen coated coverslips. Usually it takes > 24 h for MTLn3 cells to be well spread on the coverslips.
12. Wash once with warm starvation media, 0.35% BSA-L15 media, and starve the cells in this media for 3 h at 37 °C without CO_2.

C. EGF stimulation and cell fixation:

13. Place culture plate into 37 °C water bath. Add appropriate volume of EGF stimulation buffer to the plate and stimulated for 3 min.
14. Fix cell with warm 4% Paraformaldehyde in 37 °C water bath for 10 min.
15. After fixation, rinse 1x with PBS and the coverslips can be stored in PBS at 4 °C overnight.

D. Immunofluorescence/ F-actin staining

16. Permeablize the fixed cells with 0.5% triton X-100-PBS for 10 min at RT.
17. *Optional:* rinse once with 0.1 M glycine-PBS and incubate in 0.1 M glycine-PBS for 10 min at RT to reduce background.
18. Block with 1% FBS- 1% BSA- PBS at RT 4x 5 min, then transfer coverslips onto parafilm.
19. Incubate 1-2 h with primary antibody (50 µl per coverslips) usually start with 1:200 dilution in 1% BSA-PBS buffer in a humidified chamber.
20. Wash with PBS or 1% BSA-PBS 5 min 3-5 times to wash out non-specific primary antibody.
21. Incubate with 1: 200 secondary antibody in 1% BSA-PBS for 1 h at RT.
22. Aspirate the secondary antibody, and incubate 20 min with Rhodamine phalloidin (1:15 dilution in 1% BSA-PBS)
23. Wash the coverslips with PBS 5 times for 5 min each.

E. Mounting coverslips:

24. Making mounting buffer (0.11 g n-propyl gallate + 2.5 ml 2x PBS + 2.5 ml glycerol), vortex until all of it has dissolved (usually takes 40 min). Store in dark for 2 weeks.
25. Add 3 µl mounting solution on the slide. Use sharp forceps to pick up the coverslip, absorb excess buffer on the edge of the coverslips, carefully drop the coverslip on mounting solution.
26. Apply nail polish on four corners of the coverslip and seal the edge with nail polish all round. Store the slides either in -20 °C or 4 °C in slide boxes.

Recipes

1. To make EGF aliquots
   Spin down the EGF powder, add 333 µl PBS and then aliquot into either 5 µl or 10 µl aliquots. Store in -80 °C.
2. To make Rhodamine phalloidin stock
   Spin down the power and add 1.5 ml methonol to dissolve. Store in -20 °C in the dark.

References

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2. Yip, S. C., El-Sibai, M., Coniglio, S. J., Mouneimne, G., Eddy, R. J., Drees, B. E., Neilsen, P. O., Goswami, S., Symons, M., Condeelis, J. S. and Backer, J. M. (2007). The distinct roles of Ras and Rac in PI 3-kinase-dependent protrusion during EGF-stimulated cell migration. *J Cell Sci* 120(Pt 17): 3138-3146.

