IMMUNOCYTOCHEMISTRY OF RASMS GROWN ON GLASS COVERSLIPS

PRELIMINARY NOTE: The single most critical parameter in determining the quality of the image is the quality of the specimen preparation. A good staining procedure should yield a strong signal with an absolute black background. Perform dilution series of both primary and secondary antibodies to determine which dilutions provide the best signal without high background. Adjust blocking composition or length of wash. If samples are fixed in formaldehyde, make sure you block free aldehydes with NH_4Cl.

FIXATIVE: 4% paraformaldehyde in PBS, pH 7.4 (freshly made from powder) WEAR GLOVES! WORK IN FUME HOOD!

4 g paraformaldehyde
100 ml PBS (1X concentration made from 10X stock)
Heat in fume hood with stirring to dissolve. DO NOT BOIL! Add 1-2 drops of 1 N NaOH. Filter through Whatman #1 filter paper and let cool before using.

DISPOSE OF ALL PARAFORMALDEHYDE WASTE IN LABELED BOTTLES AND TAKE TO CHEMICAL WASTE FOR DISPOSAL.

BLOCKING BUFFER: PBS + 3% BSA

3 g BSA
100 ml PBS
Filter through Whatman #1 filter paper (to remove any undissolved precipitates). Use immediately or store in cold. Bacteria tend to grow in PBS+BSA, so don't store for long periods. Use this buffer for dilution of Abs.

EXTRACTION BUFFER: PBS + 0.2% (or 0.05%) Triton X-100 (from 10% Triton Stock)

800 µl 10% Triton Stock	200 µl 10% Triton Stock
39.2 ml PBS (for 0.2%)	39.8 ml PBS (for 0.05%)

QUENCHING SOLUTION: 50 mM NH_4Cl in PBS

0.078 g NH_4Cl
30 ml PBS

FOR ANTIBODY SOLUTIONS:

Centrifuge Ab solution for 10 min at 10,000 rpm at 4° C. Keep on ice in dark.

CELL CULTURE:

1. Use 22mm round glass coverslips, preferably #1.5 thickness. Clean with Kimwipe and dip quickly in 100% ethanol. Autoclave on filter paper to sterilize. In laminar flow hood under sterile conditions, use sterile forceps to place one coverslip into each of 6 multiwell dishes.
2. Trypsinize RASMs and count with hemocytometer. Dilute cells to final concentration of $2 X 10^4$ cells/ml. Add 4 mls to each multiwell ($2 X 10^4$ cell/ml X 4 ml= $8 X 10^4$ cells/multiwell).
3. Culture cells for at least 48 hours but not to confluency.

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1. Rinse cells quickly in cold PBS.
2. Fix cells in 4% paraformaldehyde in PBS 10 min RT (room temperature). **WEAR GLOVES!**
3. Rinse cells in PBS 3 X 5 min total. Room temperature from here out. (You can stop here—store fixed cells in PBS in cold room or 4°C).
4. For permeabilized cells (to detect intracellular antigens), extract cells in PBS + 0.05% (or 0.2%) Triton 5 min RT.
5. Rinse cells in PBS 3 X 5 min total.
6. Quench fixative in 50 mM NH_4Cl in PBS for 10 min RT.
7. Rinse cells in PBS 1 X quickly.
8. Block in PBS + 3% BSA for 1 hr RT.
9. PRIMARY ANTIBODY: Remove media from multiwell. Aspirate liquid from around coverslip. Add 80 µl/coverslip primary antibody solution in PBS/3% BSA. Incubate 1 hr RT or overnight in the cold (if overnight, make sure to provide a moist environment to prevent drying).
10. Rinse PBS 2 X 5 min each, then PBS/BSA 1 X 5 min.
11. SECONDARY ANTIBODY:Remove media as above and add 80 µl secondary Ab solution in PBS/BSA onto coverslip for 1 hr RT **IN DARK. KEEP CELLS IN DARK FOR REST OF PROCEDURE.**
12. Rinse PBS 3 X 5 min each.
13. If staining for F-actin using phalloidin, follow procedures starting with step #9.
14. Mount coverslips onto glass slides with Vectashield Mounting Medium (#H-1000 from Vector Laboratories, Burlingame, CA 94010). Aspirate any Vectashield from around coverslip. MUST BE DRY. Seal coverslips onto glass slides with Wet 'n Wild clear nail polish. **LET DRY COMPLETELY** before putting slides on microscope stage.
15. Store slides in covered slide tray (to keep in dark at 4° C).
16. When observing cells with confocal microscopy, be sure to use the same settings (laser power, iris diameter, gain and black level) for all cells (e.g., control vs. treated) in an experiment