Indirect Immunofluorescent Staining of MCF-10A Acini Cultured In Matrigel (Detailed)

1) Fixation: Aspirate the medium from each well of the chamber slide and immediately fix acini with 2% formalin (Sigma) or 3-4% paraformaldehyde for 20 minutes at room temperature. Acini can also be fixed using a 1:1 mix of methanol:acetone at –20°Cfor 10-12 minutes. For 8-well chamber slides, 300-400 µl volumes are appropriate for the fixation, permeabilization, and all subsequent washing steps in this protocol. Once fixed, slides can be stored at 4°C for up to two weeks. Rinse wells once in PBS and store in 500 µl/well PBS. Wrap wells in Saran wrap to avoid drying out.
2) Permeabilization: If formalin or paraformaldehyde is used for fixation, permeabilize with PBS containing 0.5% Triton X-100 for 10 minutes at 4°C. Depending on the antibody utilized for immunostaining, the detergent concentration or duration of permeabilization may require modification.
3) Glycine Rinse: Rinse 3 times with PBS/Glycine (130 mM NaCl; 7 mM Na_2HPO_4; 3.5 mM NaH_2PO_4; 100 mM glycine), 10-15 minutes per wash at room temperature.
4) Primary Block: Incubate with 200µl/well of IF Buffer (130 mM NaCl; 7 mM Na_2HPO_4; 3.5 mM NaH_2PO_4; 7.7 mM NaN_3; 0.1% BSA; 0.2% Triton X-100; 0.05% Tween-20) +10% goat serum for 45-60 minutes at room temperature.
5) **OPTIONAL** Secondary Block: Aspirate the primary block and incubate with 100 µl/well of secondary block (IF Buffer + 10% goat serum + 20 µg/ml of goat anti-mouse $F(ab')_2$ fragment (Jackson ImmunoResearch#115-006-006) for 30-40 minutes.
6) Primary antibody: Incubate with primary antibody in block solution overnight (15-18 hours) at 4°C. Although optimal antibody concentrations should be determined empirically on a case-by-case basis, a 1:100 to 1:200 dilution of the primary antibody is a good starting point. (See Table 2 for antibodies known to work in this application).
   Occasionally, overnight incubation at 4°C elicits liquefication of the basement membrane and extensive lifting of the acini during subsequent washing steps. This varies with each lot of Matrigel™ used for the morphogenesis assay, and unfortunately, cannot easily be predicted. If this problem arises, it is advisable to perform the primary antibody incubations overnight at room temperature rather than 4°C.
7) Rinse 3 times (20 minutes each) with IF Buffer at room temperature with gentle rocking.
8) Secondary Antibody: Incubate with fluorescent conjugated secondary antibody in IF Buffer + 10% goat serum for 40-50 min at room temperature. We recommend Alexa™ conjugated, highly cross-absorbed secondary antibodies from Molecular Probes used at 1:200 dilution; in our experience, these secondary reagents exhibit low levels of background, and minimal cross-reactivity between species, making them useful for double immunostaining procedures.
9) Rinse 1 time (20 minutes) with IF Buffer at room temperature with gentle rocking.
10) Rinse 2-3 times with PBS (10 minutes).
11) In order to counterstain nuclei, incubate with PBS containing 5 µM TOPRO-3 (Molecular Probes) and/or 0.5 ng /ml 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 15 minutes at room temperature.

12) Rinse 1 time with PBS for 5 minutes at room temperature.
13) Mount with freshly prepared Prolong Antifade Reagent (Molecular Probes) and allow it dry O/N at RT. Once dry, slides can be stored at 4^{o}C for up to one week or at –20^{o}C for up to two months.