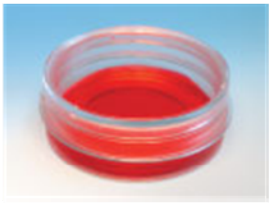
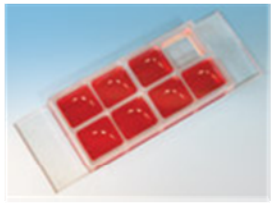
Immunofluorescence protocol for Adherent HeLa cell Actin and Nuclei labeling-IGB Core 010410

For cells grown in Ibidi micro cell culture wells:

1. Plate 1000-2000 cells per well (if you are using the 8 well IBIDI plate) in 10% DMEM, no phenol red (Cells should be grown in plastic bottles (up to 90% confluence) in phenol red free medium washed in PR red free medium twice and trypsinized in PR free medium and transferred to wells). I feel the 4000 cell count is bit too much for that well size, so make it smaller but let it grow longer so that you will have new/fresh cells not just the cells trypsinized).
2. Let cells grow for 72 hours (or until the confluence is reached 50-60%), then immunostain cells.

3. One slide per antibody. You can repeat this with any other primary antibody made in Mouse. If the Primary antibody made in Rabbit, then change the Goat anti-Mouse to Goat Anti-Rabbit and follow all the protocol steps as usual. If you double label a mouse and rabbit primaries with two different targets, incubate the cells with both primaries mixed at the same time and sequentially incubate the secondaries for ex (Goat anti-Mouse conj Alexa 488 and Goat Anti Rabbit conj Alexa 546 each for 2 hours) and follow all the steps together with either DAPI or TOPRO3 counter staining of Nuclei. Call Shiv 3-1214 with your questions.
    a. **8/Single chamber slide with coverglass #1.5 equivalent (plastic) bottom setup:**





http://www.ibidi.de/products/disposables/S_8082X_Slide8well.html

+ Actin Primary + Goat anti-mouse secondary	+ Actin Primary + Goat anti-mouse secondary +TOPRO3	+Goat anti-mouse Secondary only and No primary	+Signal FX only (Blocking buffer)
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Quick overview of steps involved:
PFA → rinse → Block → rinse → primary → rinse → secondary → rinse → TOPRO3 → rinse → Prolong Gold → store at 4 degree C → Imaging

4. Wash cells with fresh medium. Begin Fixation Procedure.

Fixation: (Most steps at Room Temperature except the primary and secondary antibody incubation done at 37degree C);

- DO NOT LET SAMPLE DRY OUT!!!
- PROCESS ONE WELL AT A TIME!!!
    - Remove medium and replace it immediately to that well and proceed

1. Wash cells in PBS (RT) 3x quickly with fresh PBS each time (to remove debris and serum)
2. Fix all cells in Paraformaldehyde (From EMS) (4%) in PBS (1x) for 30 minutes.
    a. Make 4% PFA with 1mL of 16% PFA and 3 mL of 1xPBS
    b. Kept 16% PFA aliquots (one in desk, four others at 4 degreeC).
        i. Undiluted PFA should be used within a maximum of two weeks after break open the vial.
3. Wash wells 3x (5 min each) in PBS.
4. Incubate slides in 0.5% Triton X 100 in PBS for 15 min
    a. Wash once in PBS
    b. Keep HPLC Grade Methanol around 10 mL in -20 deg C in a closed glass vial.
    c. Incubate every well with this ultracold Methanol for 15 min by keeping the whole dish in -20 deg C with methanol
    d. Take out and remove methanol one by one well and replace with PBS.
    e. Incubate in PBS at RT for 30 min.
5. Blocking:
    a. Add ITsignal FX to wells (be generous to cover the entire surface-4-5 drops) and incubate for 30 minutes
6. Rinse wells 1x in PBS.
7. Dilute PRIMARY Ab in PBS/ and one drop of IT Signal FX
8. Centrifuge it for 5 minutes at 12,000 RPM
    a. Add supernatant to wells
9. Incubate with primary antibody (actin at 1-100 dilution with one drop of ITSignal FX and PBS) or at appropriate concentrations for 2h up to over night at 37 degree C (apply sufficient volume and keep the lids closed in the incubator).
    a. KEEP wells that will receive secondary antibody only and IT Fx only in blocking buffer until it is time to incubate with secondary antibodies or wash step after second antibody incubations.
10. Wash wells 3x (5 minutes each) with 1x PBS.

(All steps from here on, keep the cell dish covered with a small box on the desk so that they are in the dark)

11. Dilute secondary antibody Goat Anti-Mouse Alexa 488Ab in PBS with two drops of IT FX for the total volume of 2 mL (sufficient for 8 well ibidi) and centrifuge it for 5 minutes at 12,000 RPM
    a. Keep other wells with blocking buffer where secondary antibody will not be used.
    b. Incubate with secondary for 2-4 h
12. Wash all wells 3x (5 minutes each) with 1x PBS.
13. Incubate all plates in TOPRO3 nucleic acid stain (50 uM working concentration made from 1mM stock for 30 min) or DAPI (10-ug/mL in 1x PBS) for 15 minutes. (need 4uL of DAPI stock (in drawer) + 1,996uL of 1x PBS for 2mL = enough for ONE 8-chamber slide.
14. Wash all plates briefly (couple of seconds) in 1x PBS 3x.
    a. Make Prolong gold 1mL in to 20 aliquots of 500 microliter each. Store 9 of them in -20 and leave 1 in the refrigerator.
15. Bring Prolong Gold (in the refrigerator) to room temperature first 2-4 h at RT after taking it from Refrigerator. Then mount in Prolong Gold. Before that make sure you drain the final PBS as much as possible. One may use a pointed kimwipe around the well area, and then add PG without trapping bubbles. May use one tip per well. Add approximately 120ul to cover all cells as solution in wells will form a depression in the center of wells. (Made 500uL aliquots of Prolong Gold, kept at -20C).
16. Keep the dish in dark with lid loosely on top at room temp for 24hrs for the prolong gold to cure.

17. Store in dark at 4C with lids closed or sealed with parafilm until analysis.
18. Call Shiv 333-1214 if you have any questions.