Lazzara Lab
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Last updated by JLH February 6, 2018

Coverslip Immunofluorescence

1. Remove coverslips stored in methanol, and place in a sterile well at a slanted angle to allow the methanol to evaporate (~10-15 min). [As an alternative to methanol sterilization, 70% ethanol can be used for a minimum of 30 min.]
2. After methanol is evaporated, gently tap plate to slide coverslip flat onto the bottom of the well. Desired number of cells can now be plated into the 6/12 well plate as usual.
3. After cells have grown to desired confluence, starve and treat with drugs/growth factors, or just move onto the next step.
4. Wash 2x in PBS.
5. Fix with 4% paraformaldehyde in PBS for 20 min in fume hood. Caution: Formaldehyde is very toxic. Handle with care and make sure all work is done in proper hood.
6. You may either store overnight at 4°C and resume next day after this step, or continue. Wash 2x with PBS, and then permeabilize cells with 0.25% Triton-X 100 in PBS for 5 min.
   Note: May need to try different detergents for different amounts of times to optimize for your cells + antibodies. Don't go too long on this step however, or cells will lyse.
7. Wash 2x in PBS.
8. Prepare humidified chamber from any box that can be made airtight.
   a. Place filter paper on bottom, and add H2O to wet filter paper. Pour out excess water.
   b. Add parafilm on top of filter paper, then pat down.
9. Prepare primary antibody at desired dilution (e.g., 1:500 for Shp2, 1:250 for EGFR) in Odyssey Blocking Buffer. Abs can also be diluted in 3% BSA in PBS, but OBB tends to provide superior results.
   Note: Centrifuge antibody for 1min at max RPMs in order to settle any particulates.
10. Use tweezers/forceps to transfer coverslips to the humidified chamber, cells facing up.

a. Dot slide on a Kimwipe first to dry before putting into the chamber.

11. Add 100-150uL (125 µL for a size 18 coverslip) of antibody solution to each coverslip, just need enough to equally coat the entire surface.
12. Incubate at 37C for 3 hr. Can either use a water bath or incubator, just something that will not be disturbed. Alternatively, can incubate overnight in the cold room.
13. Transfer coverslips back to 6/12 well plate, and wash 5x in PBS-Tween for approx. 3 min on a rotator/rocker.
14. Prepare secondary antibody at desired dilution (1:750 of antibody that is already diluted 1:2 in glycerol) in OBB. Can also add DNA stain if desired (e.g., 1:2000 of Hoescht).
    Note: Make sure you use Alexa Fluor or other visible spectrum secondary antibodies, not Licor abs!
    Note: Centrifuge antibody for 10 min at max RPMs to settle any particulates.
15. Transfer coverslips to humidified chamber, dotting on a Kimwipe beforehand. Add 100-150 µL of antibody solution to each coverslip to coat the surface.
16. Incubate at 37˚C for 1 hr.
17. Wash in 0.1% PBS-Tween20 5x for approx. 3 min on a rotator/rocker.
18. Place a very small drop of mounting media (Prolong Gold Antifade) on a labeled slide, and gently put coverslip onto drop with cells facing down. Allow to dry overnight at room temp in a dark area on a level surface.
    Note: Mounting media is very viscous. Cut tip off partially on a 200 µL tip, and draw up 125 µL into cut pipet tip for a total of 6 coverslips. Avoid making bubbles.
19. After drying overnight, carefully paint a layer of clear nail polish around the edge of the coverslip to prevent mounting media from drying out quickly.