For Staining and fixing:

Stain on Coverslips

Adapted from Kari's old protocols + https://www.future-science.com/doi/10.2144/000114610 to adjust for suspension cells

Materials

12mm circle coverslips
Cell matrix coating that works with your cell type (common are PDL, fibronectin)
24well culture plate

PBS
Blocking Buffer (2%FBS, 0.1%Triton X in PBS)
4% PFA in PBS

Before protocol

1. Coat coverslips
2. Place coated coverslips in 24 well dish

Protocol

1. Pellet cells (250 × *g* for 5 min)
2. Resuspend cells in PBS at ~1×10^6 cells/mL.
3. Pipet 300uL into coated coverslip in 24 well dish
4. Let sit for 30 min for gravity sedimentation
5. Gently remove PBS then fix cells with warm (37 °C) 4% PFA for 10 min*.
    a. TIP: You can hand aspirate, but often this gives inconsistent results. I recommend using a reduced flow aspirator. To reduce the flow on your aspirator stack unfiltered pipet tips on the aspirator, it works best if you do this in descending order. I.e. If you are using a glass pasture pipet aspirator add a p1000 tip, then a p200 tip, then a p20 tip, you can add more tips to slow it down further based on your suction.
    b. * You may need to change fixation based on your antibodies or your cells
    c. We have been finding the 2.5% PFA for 20min is better for many suspension cell types to stop blebbing because it help keep the cell pressurized while fixing!
6. Wash 2 x 5 min with PBS
7. Permeabilize and block for 0.5-1hr (2%FBS, 0.1%Triton X in PBS-this is what I used, if you use BSA it should be fine as well)
    a. NOTE: if just looking at outer membranes, you may want to withhold from permeobilization!

8. Primary antibody staining (in general times can vary per antibody):
    a. Add antibody and incubate at 37 °C) for 1 hr
    b. Remove antibody and wash 3x5min with PBS
    c. Repeat with other primaries
    d. NOTE: All antibodies should be used to fully cover the coverslip, in a 24 well dish this is generally about 200-250uL. If you want to save on antibody, you can always place 50uL on parafilm and gently turn the coverslip upside down on top of it. Remember to and create a humid chamber around it (wet kim wipes and a plastic container) so it does not dry out.
9. Secondary staining
    a. Using Alexa fluor antibodies at 1:500 in PBS (if multiple are being used, can mix in same tube)
    b. Incubate at RT for 1hr
10. Add DAPI (use 1:1,000 of 1mg/mL DAPI in PBS) and incubate for 5min
11. Wash 3x with PBS and mount on slides
12. Slide mounting tips:
    a. 12mm coverslips only require 3uL of mounting media
    b. Leave PBS in the wells so the coverslips do not vacuum seal to the bottom of the well
    c. To retrieve the coverslip from the well, it is recommended that you create a coverslip picking-up tool from a syringe needle (form a hook on the end of a syringe needle with some pliers) to help pick up/tilt up the coverslip with one hand so you can easily grab it with tweezers in the other.

Notes: users have had fairly good luck with the Plasma membrane marker:

Plasma membrane	Postasim ATPase abcam	Rat (ab283345)	Goat anti Rat