CELL FIXATION & PERMEABILIZATION USING PARAFORMALDEHYE

Reagents

- 4% (w/v) Paraformaldehye in PBS [usually prepare a 20% stock]
    - Preparation of stock solution is performed in the chemical fume hood. If you have to make a stock solution yourself for the first time then you should have prewarmed PBS in a flask (inside the fume hood) and add the powdered paraformaldehyde to it. Heat the solution to hot but not boiling level on a heater/stir plate until the solution becomes clear. Check with a pH paper that the pH is around (7.5). Cover the solution and let it cool down inside the fume hood and aliquot that into 15 ml universal tubes and freeze them.
    - Upon use, you can warm (thaw) an aliquot in the 37^{o}C water bath. Store what is left at 4^{o}C and use within a week, otherwise you can refreeze it.
- 1XPBS containing final concentration of 1mM $MgCl_2$ and 1mM $CaCl_2$
    - Recently I have been preparing all solutions in just autoclaved 1X PBS without $MgCl_2$ and $CaCl_2$ and it worked even better.
- Blocking solution: make up 3% (w/v) BSA in PBS/1mM $MgCl_2$/1mM $CaCl_2$.
- Washing solution: it is 1:10 dilution of blocking solution, dilute in PBS/1mM $MgCl_2$/1mM $CaCl_2$.
- Permeabilization solution: 0.05% (v/v) TritonX-100 in PBS. In a couple of times I used 0.1% (v/v) TritonX-100 and it worked fine. It did not affect the labelling with BXP-21.

Procedure

1. Your cells should be grown on sterile cover slips to the desired confluence level. Prior to seeding, cover slips could be incubated, under tissue culture sterile conditions, in serum containing medium overnight or at least 5 hours before seeding the cells. This would enhance cells adhesion and spreading. If you need to transfect your cells, then transfection can be performed as you would normally do it, but this time cells would be on cover slips ☺
2. **Fixation:**
    a. Remove the medium from cells, rinse them 1x with PBS, remove PBS and immediately add 1ml of 4% paraformaldehyde (DO NOT LET CELLS DRY). Leave the fixing agent on cells for about 5 minutes at room temperature without shaking [this step should be done in the tissue culture hood].

b. Use vacuum line to remove paraformaldehyde from cells then slowly rinse them 3x with PBS by adding it to the side of the well (or dish containing your cells) in order not to disturb the attached cells.
    i. If at this stage you cannot continue with the procedure, you can store cells in PBS in the cold room for overnight.

3. **Permeabilization: [**This step is done only when you are using BXP-21 against ABCG2**]**
    a. Cells are washed once with PBS, and then 1 ml of permeabilization solution is added to the cells and incubated for 5 minutes at room temperature.
    b. Rinse cells 3x with PBS, gently as mentioned earlier then remove it.
    c. Add 1 ml (or any volume that would cover the cells) of blocking solution and incubate the cells with it for at least and hour at room temperature. Cells could be gently shaked on a rocker.
4. **Primary antibody addition:**
    a. Wash cells once with PBS after removing the blocking solution.
    b. Prepare your primary antibody in the washing buffer at the proper dilution you usually use and incubate the cells with it for about an hour at room temperature. You can incubate the cells with the primary antibody at 4^{o}C overnight.
5. **Secondary (staining) antibody addition:**
    a. Was cells from the primary antibody solution using washing buffer. Do the wash gently, 3x, with gentle shaking during the wash then vacuuming the solution. Performa a last wash with PBS.
    b. Prepare the secondary antibody at the proper dilution in washing buffer (in our case we use AlexaFlour 488 and prepare it at a 1:1000 dilution). 0.5ml would be enough to cover the cells, so make up the dilution in way that saves the antibody you are using!
    c. Add 0.5ml of the secondary antibody solution to the cells and incubate them for an hour at room temperature.
6. **Final step, mounting cover slips:**
    a. Do the same washing step as you did for the primary antibody.
    b. Keep the cells wet in PBS while you prepare for mounting the cover slips
    c. Life cover slips carefully from the well, using sharp ended foresceps and needle if you wish, holding the coverslip from the corner and tap the corner on a paper towel to dry it a bit. Rest the cover slip with the cells' side facing up, add a tiny drop of VECTASHIELD mounting medium containing DAPI on this side. Lay a microscopic slide on top of the cover slip and press gently so that you get rid of any air bubbles

and have the mounting medium dispersed evenly between the cover slip and the slide. Finally, you can lift up the slide with coverslip attached to it and seal the edges of the coverslip with bit of transparent nail polish for better attaching the coverslips to the slide for a longer time.

d. Slides need to be stored in dark cold place and they could be stable for couple of months or even longer.