Generic Immunofluorescence (IF) Protocol

Required Solutions

Triton X-100 buffer

0.5% Triton X-100
20 mM Hepes-KOH (pH 7.9)
50 mM NaCl
3 mM $MgCl_2$
300 mM Sucrose
Store at RT. (Keep one at 4°C for extraction.)

10x PBS

80 g NaCl
2 g KCl
14.4 g Na_2HPO_4
2.4 g KH_2PO_4
dissolve into 800 ml ddH_2O
Adjust pH to 7.4
qs to 1000 ml
autoclave
Make a 1x working solution. The pH should be 7.4 at 25°C after 10x dilution.

PBG

0.2% (w/v) cold water fish gelatin (Sigma G-7765)
0.5% (w/v) BSA (Sigma A-2153)
in PBS
Store in 50 ml aliquots at -20°C.

Formaldehyde (2-4%)

10-20 g paraformaldehyde (Sigma P-6148) + 100 ml H2O.
Add 200 µl 2 N NaOH.
Keep in 65°C water bath, shake every 10-15 min, usually takes 20-30 min to dissolve.
Add 50 ml 10x PBS and 350 ml H2O, mix.
Store in 50 ml aliquots at -20°C. Only use once after thawing.
pH should be 7.2-7.3. If pH is too high the chromosomes will look fuzzy.

DAPI

Lab stock: dissolve at 5 mg/ml 4',6-diamino-2-phenylindole (Sigma D-9542) in ddH2O. Store at -20°C.
Individual stocks: Dilute from lab stock to 0.5 mg/ml. Stable at 4°C.

NaN_3

Stock (10%): Dissolve 1 g sodium azide (Sigma S8032) in 10 ml ddH2O. Store at 4°C.
Use 1:500 dilution (0.02% final) for antibacterial purpose. Note: NaN_3 is poisonous.

Embedding medium

ProLong Gold Antifade Reagent (Invitrogen #P36934).

Alternative: VECTASHIELD (VECTOR LABORATORIES, #H-1000 w/o DAPI, #H-1200 w/ DAPI) Note: VECTASHIELD fades more than ProLong Gold Antifade.

Plating cells on Coverslips

1. Using ethanol-cleaned tweezers, place autoclaved cover slips onto a tissue culture plate. Or use Pasteur glass tube with yellow tip on vacuum to place cover slips.
2. Add media to the plate, be careful to prevent the cover slips from floating.
3. Use pipette tip to insure that all the cover slips are firmly in place.
4. Evenly distribute the cells and rock gently to mix; allow the cells to attach 12-24 hours before fixation.

   Note: Cell density for plating: 5 - 8 x 10^5/6 cm dish, place 6-7 cover slips / 6 cm dish

Fixation and Permeabilization

Formaldehyde + Permeabilization

1. Fix cells in 2-4% formaldehyde in PBS (stored at –20ºC) for 10 min at RT.
2. Wash cells 2 x with PBS for 5 min.
3. Permeabilize cells in 0.5% NP-40 in PBS for 10 min at RT. Alternatively, use 0.5% Triton-X 100 in PBS.
4. Wash cells 2 x with PBS for 5 min.
5. Proceed to blocking for best results.
   Cells can also be stored at 4ºC at this point, add 500x stock NaN_3 (10% stock in H_2O). Use caution, NaN_3 is poisonous. In general, IF works better with fresh samples stored within 3 days.

Triton X-100 Extraction (removal on nucleoplasmic proteins)

Note: be very gentle with primary cells and mitotic cells

Use Poly-D-Lysine coated cover slips (BD: #354085), place no more than 7 cover slips /6 cm dish

1. Rinse cells in cold PBS with Ca^{2+} and Mg^{2+}.
2. Extract cells with Triton X-100 buffer at 4ºC for 20 sec to 2 min, depending on the antibody you are using, let solution flow slowly to side of dish.
3. Rinse cells gently 2 x in PBS at RT.
4. Fix cells in 3% paraformaldehyde/2% sucrose in 1x PBS for 10 min at RT.
5. Wash cells 2 x in PBS 5 for min.

   Note: at this point the cells can be stored at 4ºC for no more than 3 days for best results.
6. Re-permeabilize cells in Triton X-100 buffer for 10 min at RT.
7. Rinse cells 2 x in PBS for 5 min.
8. Proceed to blocking.

Alternative Fixation Techniques

Methanol (e.g. PARP1)

1. Rinse quickly in PBS (optional if MeOH fixation volume is large).
2. Plunge cells into ice cold MeOH (Sigma 154903 500ml) for 10 min at -20°C.
3. Plunge cells 2 x into cold PBS for 5 min.
4. Proceed to blocking step.

Blocking and Antibody Incubation

Complete these steps in a flat plastic container wrapped with foil. Line the container with wet paper towels and put parafilm on the bottom. Place the cover slips on top of the parafilm.

For the antibody incubation steps use the following:
80 µl -100 µl for round 16mm cover slips
100 µl for square 22mm cover slips.

For blocking and washing, use at least 5x incubation volume, more is better (e.g. 1 ml). Do not let the coverslips dry.

1. Block non-specific sites by incubating 30 min or longer in PBG.
2. Incubate with primary antibody diluted in PBG, 1-2 hr @ RT or overnight @ 4°C.
3. Wash cells 3 x in PBG for 5 min.
4. Incubate with secondary antibody diluted 1:500-1:1000 (for FITC-antiRabbit IgG, use 1:1000 for lower background) in PBG; 45 min @ RT.
5. Wash cells 2 x in PBG for 5 min.
6. Wash cells in PBG+DAPI @ 100 ng/ml.
7. Wash cells 2 x in PBS for 5 min each.
8. Using fine tweezers, take the cover slip and drain the excess fluid. Place the cover slip (cells down) on a 20-30 µl drop of embedding medium on a microscope slide. Try to avoid trapping air bubbles. Embedding medium is viscous so just drop a ~30 µl drop on the slide without worrying about exact pipetting.
9. Drain excess embedding medium with tissue. Air dry at RT for 30 min. Seal with nail polish. When dry, clean cover slip with a wet tissue. The slides are now ready for microscopy or store at -20°C. (IF signal should be stable for a year stored at -20°C.)