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 MgCl₂
300 mM Sucrose
Store at RT.

10x PBS
80 g NaCl
2 g KCl
14.4 g Na₂HPO₄
2.4 g KH₂PO₄
dissolve into 800 ml ddH₂O
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, heat to 60°C in fume hood. Stir until dissolved.
Add 50 ml 10x PBS and 350 ml H₂O, mix.
Store in 50 ml aliquots at -20°C.
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.

Embedding medium
Dissolve 20 mg p-phenylene diamine (Sigma P-6001) in 2 ml 10x PBS by vortexing.
Immediately add 18 ml glycerol, mix carefully without creating air bubbles. Do not vortex.
Store in 1 ml aliquots at -70°C. The solution should be colorless; discard when it turns yellow or brown. Or use ProLong Gold Antifade Reagent (Invitrogen #P36934).
Plating cells on Coverslips

1. Using ethanol-cleaned tweezers, place autoclaved cover slips onto a tissue culture plate.
2. Add media to the plate, be careful to prevent the cover slips from floating.
3. Use tweezers 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.

Fixation and Permeabilization

Formaldehyde + Permeabilization

1. Rinse cells in 1x PBS.
2. Fix cells in 2-4% formaldehyde in PBS (stored at –20°C) for 10 min at RT.
3. Wash cells 2 x with PBS for 5 min.
4. Permeabilize cells in 0.5% NP-40 in PBS for 10 min at RT. Alternatively, use 0.5% Triton-X 100 in PBS.
5. Wash cells 2 x with PBS for 5 min.
6. Proceed to blocking.

Triton X-100 Extraction (removal on nucleoplasmic proteins)

Note: be very gentle with primary cells and mitotic cells

1. Rinse cells in PBS.
2. Extract cells with Triton X-100 buffer at 4°C for 20 sec to 2 min, depending on the antibody you are using.
3. Rinse cells 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.
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 (easiest and fastest, lose some labile epitopes and membrane structure)

1. Rinse quickly in PBS (optional if MeOH fixation volume is large).
2. Plunge cells into ice cold MeOH for 5 min at -20°C.
3. Plunge cells 2 x into cold PBS for 5 min.
4. Proceed to blocking step.
Glutaraldehyde + Permeabilization (best for cytoskeleton)

1. Rinse cells in PBS.
2. Fix in 0.5% glutaraldehyde in PBS for 10 min at RT.
3. Rinse cells 3 times with 1x PBS.
4. Permeabilize cells with 3 washes in 0.5% Triton-X 100 in PBS for 5 min each.
5. Wash cells 3 x in PBS for 5 min each.
6. Proceed to blocking.

**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:
- 50 µl for round 16mm coverslips
- 100 µl for square 22mm cover slips.

For blocking and washing, use at least 5x incubation volume, more is better. 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; 2 hr @ RT or overnight @ 4°C.
3. Wash cells 3 x in PBG for 5 min.
4. Incubate with secondary antibody diluted 1:250 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. Seal with nail polish. When dry, clean cover slip with a wet tissue. The slides are now ready for microscopy.