IF and IF-FISH

Required Solutions

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
Make up to 1000 ml
autoclave
Make a 1x working solution. The pH should be 7.4 at 25°C after 10x dilution.

Formaldehyde (2-4%)

10-20 g paraformaldehyde (Sigma P-6148) + 100 ml H_2O.
Add 200 µl 2N NaOH.
Keep in 65°C water bath, shake every 10-15 min, usually takes
20-30 min to dissolve (alternatively - CAREFULLY microwave in
5-10 sec intervals until completely dissolved)
Add 50 ml 10x PBS and 350 ml H_2O, mix.
Store in aliquots at -20°C. Only use once after thawing.
OPTIONAL: Check pH using pH paper - should be 7.2-7.3. If pH is too high the chromosomes will look fuzzy.

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 4°C.

Cytoskeleton Buffer 1

10 mM PIPES pH 6.8
100 mM NaCl
300 mM Sucrose
3 mM $MgCl_2$
1 mM EGTA
0.5% Triton X-100

Cytoskeleton Stripping Buffer 2

Prepare without $MgCl_2$ and Tween, then add those immediately before use

10 mM Tris HCl pH 7.4
10 mM NaCl
0.5% Sodium Deoxycholate
1% Tween-40
3mM $MgCl_2$

Blocking Solution

(in PBS, store at -20ºC in 10 ml aliquots)
1 mg/ml BSA
3% goat serum
0.1% Triton X-100
1 mM EDTA pH 8.0

Blocking reagent (Roche 11096176001)

Stock is 10%, dissolved in maleic acid buffer:
100 mM maliec acid
150 mM NaCl
Adjust pH to 7.5 (20°C) with NaOH. Store at 4ºC

Hybridizing solution

Make fresh. Final concentrations in dH_2O.
70% formamide (from deionized stock, Sigma F9037)
0.5% blocking reagent (from 10% stock)
10 mM Tris-HCl pH 7.2
PNA probe (1:1000-2000)

PNA probes (Purchased from PNABio)

Resuspend lyophilized probes as per the manufacturer's instructions (50 μM in formamide). Long term storage at -80ºC. Once thawed, can be stored at 4ºC in the dark.

Washing solution

70% formamide (EMD Millipore #344206)
10 mM Tris-HCl pH 7.2

3000x DAPI

Dissolve 0.5 mg/ml 4',6-diamino-2-phenylindole (Sigma D-9542) in H_2O. Stable for at least one year at 4°C.

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 ensure 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.

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.
   Note: 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.
3. OPTIONAL - Permeabilize cells in Triton-X buffer for 10 min at RT. [It is optional because the Blocking Solution has 0.1% triton-X 100]
4. Wash cells 2 x with PBS for 5 min.
5. Proceed to blocking for best results.

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) for 5 min at -20°C.
3. Plunge cells 2 x into cold PBS for 5 min.
4. Proceed to blocking step.

Pre-extraction of nucleoplasmic proteins

OPTION 1: Triton X-100 Extraction

Note: be very gentle with primary cells and mitotic cells

OPTIONAL: Use Poly-D-Lysine coated cover slips (BD: #354085), place no more than 7 coverslips /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 1-5 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. OPTIONAL - 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.

OPTION 2: Cytoskeleton stripping buffer extraction

1. Steps 1-4 on ice (you can use metal blocks in an ice bucket with your 6 cm TC dishes on top): wash twice in ice cold PBS. 3 ml appropriate for 6 cm TC dishes.
2. Incubate 5' in ice cold Cytoskeleton Buffer 1
3. Incubate 5' in ice cold Cytoskeleton Stripping Buffer 2
4. Wash thrice in ice cold PBS
5. At RT on benchtop, fix in 3% PFA, 2% Sucrose for 10'.
6. Wash twice in RT PBS, 5' each.
7. On third PBS wash, add 4 ml PBS, wrap dishes with parafilm and store at 4˚ until IF.
8. Re-permeabilize cells in cold Triton X-100 buffer for 5 min
9. Rinse cells 3x in PBS for 5 min.
10. 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:

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 Blocking Solution.
2. Incubate with primary antibody diluted in Blocking Solution, 1-2 hr @ RT or overnight @ 4ºC.
3. Wash cells 3x in PBS for 5 min.
4. Incubate with secondary antibody diluted 1:500-1:1000 in Blocking Solution; 45 min @ RT.
5. Wash cells 3x in PBS for 5 min.
   If only performing IF, add DAPI to second wash and proceed to Step 14.

FISH

6. Fix cells once again in 2-4% formaldehyde in PBS (stored at –20ºC) for 6-10 min at RT.
7. Wash 2x in PBS for 5 min.
8. Dehydrate the cells in ethanol, consecutively 70%, 95%, 100% EtOH, 5 min each. Aspirate the ethanol completely and let the cover slips dry for a couple of minutes.
9. Denaturation of DNA can be done in two ways:
   Option 1: Place 3 to 4 cover slips **(cells up)** on microscope slide, drop of hybridizing solution (100 µl) on each cover slip.

   Option 2: Use Liquid Blocker (Super PAP PEN) to draw rectangles or circles slightly bigger than cover slips on microscope slide and let dry for 1 min, drop 50-80 µl hybridizing solution inside rectangles or circles, place cover slips **(cells down)** on hybridizing solution.
10. Denature with hybridizing solution for 5-10 min (10 min for human cells with short telomeres, 5 min for mouse cells) at 80ºC by placing slides on heat block.
11. Incubate in the dark for 1-2 hours at RT or overnight at 4ºC.
12. Wash 2 x 15 min in washing solution.
13. Wash 3 x 5 min in PBS; add DAPI to the second wash.
14. Air dry at RT for 10-30 minutes.
15. 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.
16. 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 -20ºC. (IF signal should be stable for a year stored at -20ºC.)