FISH

There are two ways of fixing parasites for IF or FISH. Either the cells are settled onto non-silanized coverslips (Fisher 12mm, #15) and then fixed (faster but harsher), or the cells are fixed in suspension and then settled onto silanized coverlips (probably better to preserve the 3D-structure of the cell, for co-localization studies, deconvolution with Delta-Vision, etc).

Spinning followed by fixation

1. Spin cells gently at RT for 3 min at 2,000 rpm.
2. Rinse with PBS (procyclics) or TDB (bloodstream forms)
3. Spin as before and transfer cells to an Eppendorf tube.
4. Resuspend in PBS or TDB to > $2x10^7$ cells/ml.
5. Fix cells for 10 min at RT by adding an equal volume of 4% formaldehyde in PBS or TDB. The parasite concentration at the end should be > $1x10^7$ cells/ml.
6. Place coverslips on a Whatman paper that fits the swing bucket of the centrifuge.
7. Spot 50 µl ($0.5x10^6$ cells) of cell suspension onto glass coverslips.
8. Spin coverslips at 4°C for 4–5min at ~2,000 rpm.

Fixation in suspension followed by settling onto silanized coverslips

9. Spin cells gently at RT for 3 min at 2,000 rpm. Discard supernatant and resuspend cells in HMI-9 in order to have > $2x10^7$ cells/ml. Transfer cells to Eppendorf tube.
10. ALTERNATIVE METHOD: Wash cells with PBS (procyclics) or TDB (bloodstream forms). Spin at RT for 3 min at 2000 rpm. Discard supernatant and resuspend cells in PBS or TDB in order to have > $2x10^7$ cells/ml. Transfer cells to Eppendorf tube.
11. Fix cells for 10 min at RT by adding an equal volume of 4% formaldehyde in PBS or TDB. The parasite concentration at the end should be > $1x10^7$ cells/ml. *If the duration of fixation is critical, you can stop fixation by adding glycine*.
12. Spin cells as before to wash away formaldehyde (*this step is essential otherwise cells will not adhere efficiently to the silanized coverslip*). Resuspend cells in PBS at > $1x10^7$ cells/ml.
13. Spot 50–100 µl ($0.5\text{-}1x10^6$ cells) of suspended cells on each silanized coverslip
14. Allow 5–15 min for cells to settle (by gravity).
15. Wash cells 4x for 5 min with PBS.
16. We can store coverslips at this stage for at least 2 days.

Permeabilization

1. Permeabilize cells for EXACTLY 5 min in 0.1% (PF) or 0.2% (BF) NP40 in PBS. *Time may have to be adjusted depending on your antigen or DNA/RNA target.*
2. Rinse 2–3x with PBS.

Hybridization

1. Boil water; turn on one water-bath to 70°C and another to 37°C (or 50°C).
2. Prepare 20 ml of 70% Formamide in 2 x SSC and place it at 70°C.
3. Prepare 20 ml of 2x SSC and place it at 50°C
4. Prepare probe/hybridization mix on ice as follows (volumes are per coverslip):
    a. 2.4 µl Herring sperm DNA (0.2 mg/mL)
    b. 2.4 µl Yeast tRNA (0.2 mg/mL)
    c. 7.2 µl probe (1 µg/mL) (1 µL of 50-bp repeat oligo probe; 7.2 µL telomere probe)
    d. 48.0 µl Hybridization Mix (for oligo probes use Hyb. mix containing only 30% formamide).
5. For DNA probe, denature in hyb mix 100°C for 5-10 min.
6. Meanwhile, denature DNA from cells by placing coverslips in 70°C-pre-warmed 70% formamide / 2 x SSC for 5 min at 70°C (in 6-well plates; they float).
7. Aspirate formamide solution
8. Rinse briefly in pre-heated 2x SSC (50°C)
9. The probe/hyb mix should be denatured by now. Spot (50 µL) immediately onto parafilm in dark-humid chamber.
10. Remove excess liquid from coverslips and place them on probe/hyb mix spots (cells facing down!)
11. Incubate overnight at 37°C. Check temperature with thermometer!
12. Temperature depends on type of probe: TEL = 50°C; 5s-DNA, MC177, 50bp-repeats = 37°C.

Washings (24-well plate)

1. Defrost PBG and store it on ice. Pre-heat formamide / 2xSSC to 37°C (formamide % should be identical to the one used for O/N hybridization)
2. Transfer coverslips to new 24-well plate containing same % of formamide / 2xSSC
3. Incubate for 5 min at hybridization temperature (same oven as for hybridization)
4. Repeat wash, using vacuum pump to aspirate washing solution
5. Wash at RT as follows:
6. 2 x 5 min, 2x SSC
7. 2 x 5 min, 1x SSC
8. 2 x 5 min, 0.1x SSC
9. Wash in PBS
10. Block twice in PBG for ≥10 min.

Developing reaction (24-well plate)

Remember:
TEL PCR fragment: labeled with DIG-dUTP = develop with α-DIG (sheep), then **FITC-α-sheep**
50-bp repeat oligo: labeled with Biotin at 5´ end = develop with **ExtrAvidin-Cy3**

1. Prepare dilutions in PBG as follows (use 100–200 µl / coverslip):
    a. 1:1000 Sheep anti-DIG (Stock: antibody box; freezer in Joanna's room)
    b. 1:1000 ExtrAvidin-Cy3 (SIGMA E4142) (Stock : White Box, cold-room)
2. Incubate for 1–2 h at RT (or O/N at 4 °C) with primary Ab diluted appropriately in PBG
3. Wash 6 x 5 min with PBG
4. Incubate in secondary Ab (FITC donkey anti-sheep, diluted 1:200 in PBG) for ≥ 1 h at RT
5. Wash 4 x 5 minutes with PBG
6. Rinse with PBS
7. DAPI stain for ~ 1 min with 1000x stock diluted in PBS
8. Meanwhile, label slides.
9. Rinse coverslips with PBS.
10. Place glycerol at RT
11. Drop carefully to avoid bubbles 20 µl of glycerol containing p-phenylene diamine OR vectashield. Vectashield is not good for 3D-reconstruction, as it contains little "bubbles" that act as microlenses.
12. Remove excess PBS from coverslip and mount cover slips on glycerol (cells facing down!). 2 coverslips / slide is ideal but 3-4 is possible.
13. Seal with plain nail polish and examine.
14. Stores slides at –20° C.

Solutions

10X PBS

10x stock solution, 1 liter:
80 g NaCl
2 g KCl
11.5 g $Na_2HPO_4.7H_2O$
2 g KH_2PO_4

PBG=PBS plus

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

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 10xPBS by vortexing. Immediately add 18 ml glycerol, mix carefully without creating air bubbles: do not

vortex. Store in 1ml aliquots at -70°C. The solution should be colorless; discard when it turns yellow or brown.

Formaldehyde (2–4%)

Dilute from 37% stock

TDB

		5x concentrate	1x working solution
0.005	M KCl	1.86 g/l	0.37 g/l
0.080	NaCl	23.4	4.68
0.001	$MgSO_4.7H_2O$	1.24	0.25
0.020	Na_2HPO_4	14.2	2.84
0.002	$NaH_2PO_4.2H_2O$	1.55	0.31
0.020	glucose	18.0	3.60

Dissolve $MgSO_4.7H_2O$ separately and add slowly, with stirring, once the other components have been dissolved and made up almost to final volume. Check pH ONLY AFTER IT IS DILUTED TO 1X — it should be 7.7 and NOT need adjustment! If the pH is wrong this indicates that components were incorrect or misweighed, etc). Bottle 5x concentrate in exactly 50 ml amounts; 1x working solution in 100 ml amounts and store in freezer.

To generate a working solution from 5x concentrate, thaw concentrate and warm to around 50° C; mix and check that everything has re-dissolved. Make up to 250 ml using H_2O. Check pH. Store 4° C.

Hybridization mix

1 ml Hyb I
1 ml Hyb II
5 ml formamide
1 ml Hyb III

mix, store at –20 °C in ~ 500 µl aliquots

Hyb I 50% dextran sulfate (Sigma D-6001) in water.
(Dissolve by stirring and heating). Store at –20 °C.

Hyb II 3% BSA (Sigma A-2153)
3% ficoll (Sigma F-9378)
3% polyvinylpyrrolidone (P-2307) in water.
Store at –20 °C.

Hyb III 20X SSC (autoclaved)