IMMUNOFLUORESCENCE ASSAY

There are two ways of fixing parasites for IF or FISH. In one method, the cells are first settled onto a coverslip and then fixed. In the second method, cells are fixed in suspension and only then settled onto coverlips. The second method is probably better to preserve the 3D-structure of the cell (especially important for co-localization studies, deconvolution with Delta-Vision, etc).

The settling of the cells onto coverslips (Fisherbrand 12mm, #15) can be performed in two ways: spinning (centrifuge) on non-sialinized coverslips or settling by gravity on sialinized coverslips. The second method is probably better to preserve the 3D-structure of the cell.

Thumb rule:
- quick and harsher method: first spin cells onto non-sialinized coverslips and then fix.
- Co-localization, "3D-quality" method: fix cells in suspension, settle by gravity onto a sialinized coverslip

Settling by spinning -> Fixation

1. Spin cells gently at RT for 3' at 2000rpm.
2. Rinse with PBS (procyclics) or TDB (bloodstream forms)
3. Spin as before and transfer cells to an eppendorf tube.
4. Resuspend in PBS/TDB in order to obtain > $1x10^7$ cells/mL.
5. Place coverslips on a Whatman paper that fits the swing bucket of the centrifuge.
6. Spot 50μL ($0.5x10^6$ cells) of cell suspension on glass coverslips
7. Spin coverslips @ 4°C for 4-5min at ≅2,000 rpm.
8. Transfer coverslips to 24-well plate.
9. Rinse once with PBS 1x, to remove unbound cells.
10. Add 100-500 μL of 2% Formaldehyde in PBS/TDB and fix for 10'.
11. Wash cells 4x with PBS 1x, 5' each wash
12. We can store coverslips at this stage for at least 2 days

Fixation in suspension -> Settling on sialinized coverslips

13. Spin cells gently at RT for 3' at 2000rpm. Discard supernatant and resuspend cells in HMI-9 in order to have > $2x10^7$ cells/mL. Transfer cells to eppendorf tube.
14. ALTERNATIVE METHOD: Rinse cells with PBS (procyclics) or TDB (bloodstream forms). Spin at RT for 3' at 2000rpm. Discard supernatant and resuspend cells in PBS in order to have > $2x10^7$ cells/mL. Transfer cells to eppendorf tube.
15. Fix cells for 10' at RT by adding an equal volume of 4% Formaldehyde in PBS/TDB. The parasite concentration at the end should be > $1x10^7$ cells/mL.

If the duration of fixation is essential, you can stop fixation by adding Glycine.

16. Spin cells as before to wash away formaldehyde (*this step is essential otherwise cells will not adhere efficiently to the sialinized coverslip*). Resuspend cells in PBS at > $1x10^7$ cells/mL
17. Spot 50-100μL ($0.5\text{-}1x10^6$ cells) of suspended cells on each sialinized coverslip
18. Allow 5-15' for cells to settle (by gravity).
19. Rinse coverslips in PBS and place them in a 24-well plate.
20. Wash coverslips 4x with PBS 1x, 5' each wash.
21. We can store coverslips at this stage for at least 2 days

Permeabilization

1. Permeabilize cells for EXACTLY 5' in 0.1% (PF) or 0.2% (BF) NP40 in PBS.

Time may have to be adjusted depending on your antigen.

2. Rinse with PBS 2-3x.

IF

1. Block twice 10' with PBG. (with shaking)
2. Incubate with primary antibody (diluted in PBG) for 90-120' at RT
3. Rinse twice quickly and wash 4x 5' with PBG (with shaking)
4. Incubate with secondary antibody (diluted in PBG) for 1-2hr at RT
5. Rinse twice quickly and wash 4x 5' with PBG (with shaking)
6. DAPI stain with DAPI stock diluted in PBS or PBG for ≅1'
7. Remove excess PBS and mount coverslips on slide in Glycerol (one slide=one experiment, with duplicate coverslips)
8. Seal with nail polish and examine under fluorescence microscope
9. Store slide at -20°C.