Step-by-step protocol for whole mount immunofluorescence of tissues/embryos.  
*Adapted from Alanentalo et al, 2007 (Nature Methods)*

Use 15ml and 50ml Falcon tubes for all steps besides clearing in BABB which should be performed in glassware. All washes and antibody incubation steps are carried out at room temperature (RT).

1. Dissect the tissue in ice-cold PBS. Remove as much membranes as possible.
2. Fix in fresh 4% paraformaldehyde (PFA) in PBS at 4°C for 2-3h.  
   **Note:** Old PFA solution tends to cause stronger endogenous fluorescence.
3. Wash for 10-30 minutes in PBS.
4. Dehydrate the tissue stepwise in methanol (33%, 66%, and 100% for ≥15 min at each step).  
   Tissue can be stored for a few months at -20°C at this point, alternatively continue with the next step.
5. Incubate the tissue in freshly prepared MeOH: DMSO: H₂O₂ (2:1:3 i.e. 15% H₂O₂) at room temperature for 12-24h to quench the autofluorescence.
6. Wash 2x30 minutes in MeOH.
7. Bring the samples to -80°C 3-5 times for at least 1h each time and back to RT to ensure that antigens in the deeper parts of the tissue are rendered accessible.
8. Rehydrate the tissue back to TBST (Rehydrate the tissue in a series of TBST (33%, 66% and 100% for 15 min at each step).
9. Block the tissue in blocking solution (10% serum in TBST (+NaAz 0.01%)) for 12-24h (use sera from the same species as in which the secondary antibody is derived).
10. Incubate the tissue with primary antibodies, diluted in blocking solution containing 5% DMSO for 48 h.
11. Wash extensively with TBST O/N.
12. The secondary antibody incubation is carried out as with the primary antibody (the secondary antibody solution should be filtered through a 0.45µm filter, Acrodisk 25mm syringe filter or equivalent).
13. Wash extensively with TBST O/N

The following steps (which are applicable for mounting of any sample for OPT scanning) should be done at SANTA OR until such time as the user is familiar with the protocol and has purchased the necessary mounts and handling equipment:

14. Embed the tissue in 1% low melting agarose (dissolved in MQ) in a tall petri dish, remove sample in agarose block slightly larger than the mount’s surface diameter.
15. Mount the block onto the mount’s surface using superglue (acrylate-based) and trim agarose around the specimen using clean slices as outlined in the users manual.
16. Magnetically attach mount to metal lid of glass ‘immersion’ container and dehydrate the embedded tissue in 100% MeOH for 12-24h, with 2 to 3 changes of MeOH.
17. Replace the final MeOH step with BABB solution (1:2 Benzyl alcohol, Benzyl benzoate) for 12-24h. Change BABB if/as necessary to ensure removal of all MeOH.

18. Scan in OPT (submersed in BABB). Depending on fluorochrome (Alexa488, Alexa594, Cy3, Qdots) the specimens can be stored in BABB (in the dark) for up to several weeks.

TBST: 50mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% TritonX-100.

Blocking solution: 10% heat inactivated serum (derived from same species as secondary antibody) in TBST (+NaAz 0.01%).

H₂O₂: generally supplied as a 30% solution, stored at 4°C, lasts 6 months.

4% PFA (100ml): Pre-heat 1xPBS at 65°C. Add 4g Paraformaldehyde powder and let it dissolve for 15min at 65°C. Also add 12.5µl 10M NaOH to the solution. Swirl the bottle gently to dissolve the PFA).

Timing:
Step 1: approximately 1h
Step 2: 2-3h
Step 3: 10-30min
Step 4: approximately 1h
Step 5: 12-24h
Step 6: approximately 1h
Steps 7-8: approximately 1day
Step 9: 12-24h
Step 10: 48h
Step 11: over night
Step 12: 48h
Step 13: over night
Step 14-15: 1h
Step 16: 1h/12-24h
Step 17: 12-24h
Total: 8-10 days depending on tissue/organ and size.