Immunofluorescence
Paraffin Sections
(w/o Trilogy)

Note: This IF method is for use on tissues prone to falling off slides (e.g. adult skulls), which cannot withstand the high-pressure, 15-minute antigen retrieval step. See IF for Paraffin w/ Trilogy for samples with better slide-adhesion. Trilogy shortens the dehydration and antigen retrieval steps, so it is faster than this classical method, and also is reportedly a more effective antigen retrieval (e.g. more positive cells based on cleaved Caspase staining)

Tissue Processing Procedure

1. Fix tissues in formalin (or 4%PFA) immediately after collection 1hr/mm of tissue (perfused tissue is best)
2. Wash in 1X PBS for 3x10 min
3. Put in 70% EtOH, process through EtOH steps, Histoclear & Wax (see Manual Processing Protocol)
4. Section embedded tissues to make 6um thick sections

Day 1 (~2.5 hours)

5. Deparaffinize slides 60min:

   Done using 250 ml of solution in staining buckets with slide rack

   Note – never let your slides dry out!!!!!! Throughout this whole procedure

   Deparaffinize
   - 1.) Histoclear x 10min
   - 2.) Histoclear x10min

   Rehydrate
   - 3.) 100% EtOH (or Flex 100) x 3-5 min
   - 4.) 100% EtOH (or Flex 100) x 3-5min
   - 5.) 95% EtOH (or Flex 95) x 3-5min
   - 6.) 95% EtOH (or Flex 95) x 3-5min
   - 7.) 70% EtOH x 3-5min
   - 8.) MQ H20 x 5 min

   Note all of the above steps do not have to be exact… you can leave for longer

6. Antigen retrieval (Heat Induced Epitope Retrieval) 30min:
   a. In **Plastic** Coplin jar(s), place slides in **Dako Antigen Retrieval Buffer** (To prep: in fridge, make excess of 1:10 dilution with distilled water, pH to 6.0-6.2, optimal is pH 6.1, using HCl if necessary.) (Alternatively, may use 10 mM **sodium citrate buffer**); antigen retrieve for 10 min at low pressure in the pressure cooker (filled between the 6 and 8 mark with MQ water).
      i. When pressure cooker is done *(takes roughly 20 minutes)* – quick release the steam, allowing pressure to normalize

ii. Take off lid, remove Coplin jar(s) – careful! They'll be hot, use oven mitt and set them on bench, remove lids and allow them to cool to ~tepid RT. Wait for water to cool before discarding from the cooker.
iii. Pull Coplin jar out and let cool to warmish RT – 20min maybe?

7. Wash in PBS 1x5 min in Coplin jar, on bench, RT

8. Block for 1-2 hours at room temperature with fresh (no longer than 4 weeks old) 5% BSA/0.3% Triton X-100 (**OR** 10% heat-inactivated Goat Serum with 0.3% TritonX)

9. Incubate samples with primary antibodies overnight (>16 hrs) at 4°C in 1% BSA/0.3% Triton X-100 **OR** 5% HIGS/0.1% Triton X-100. Use 50-200 ul of primary antibody solution per slide (sample size dependent). Best if in a level, moist container (blue slide containers, holds 20 slides each, or use tip boxes with 1 inch MQ water inside)

Primary Antibody	Concentration
Pax 5	1:200
F4/80	1:400
E-cadherin	1:500

****Do NOT add Ab to your Secondary alone control here!**
Instead, incubate in 1% BSA/0.3% Triton X-100 **or** HIGS/Triton o/n

Day 2 (~3.5 hrs-4 hours)

10. Save primary Abs label with concentration and date – use for 3-4wks, keep at 4C

11. PBS wash 5 x 5 min, RT

12. Secondary antibodies for 2 hours at room temperature in 1% BSA/0.3% Triton X-100 **OR** 5% HIGS/0.3% Triton X-100. [50-200 ul per slide] Use moist container and **KEEP DARK with foil. (Most secondary antibodies are diluted 1:500)**

13. PBS wash 5 x 5 min with agitation, RT

14. DAPI stain (from 5000x stock → dilute to 1x in PBS) for 5 min (still protect from light)

15. Coverslip with Prolong Gold Antifade or Fluoromount G (Aqueous, anti-fade mounting medium); Wipe off excess media, Seal with nail polish; Let dry at least 1 hour before imaging; store/ dry away from light