IMMUNOFLUORESCENCE

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A- Paraffin Blocks

1 - Dissect the tissue and place in PBS on ice (record weight, number, sex and genotype). Wash once in PBS.

2 - For one adult pancreas, incubate in 8 mL of Paraformaldehyde (PFA) 4% en PBS (in 15 mL Falcon tubes) O/N at 4C on a rotator table. The ratio tissue/PFA, in volume, should be 1 to 10.

Make paraformaldehyde 30 minutes before - should be dissolved with hot agitation (12 min for 200 mL and allowed to cool at 4C another 15 min).

3 - Dehydrate the tissue:

- Remove the PFA and incubate in 50% EtOH for 10 min at RT (repeat 3 times).
- Repeat this procedure with 70% EtOH.
  The samples can be stored at 4C in 70% EtOH for up to a week.
- The samples are delivered to processing and parrafin embedding.

B- Cut the paraffin embedded tissues on a microtome (3-6 uM)

C- Preparing paraffin sections

1 - Dewaxing and rehydration:

- To remove the paraffin, place the slides in a bath (200 mL) with xylene or histoclear for 15 min then change to another bath with xylene or histoclear for 15min.
- Remove excess liquid and place the slides in absolute ethanol for 5 min (+/- 1min).
- Remove excess liquid and place the slides in 95% ethanol for 5 min (+/- 1min).
- Remove excess liquid and place the slides in 70% ethanol for 5 min (+/- 1min).
- Remove excess liquid and place the slides in distilled water for at least 1 min.

If necessary, samples can be maintained rehydrated in buffer at 2-8C up to 18h.
Bring to room temperature before staining.
NOTE!! Xylene damages/dissolves plastic.
If you clean the glass jar of Xylene or absolute EtOH, the bowl must be completely dry.

2 - Antigen retrieval/unmasking by heating:

- Boil twice for 5 min in the microwave in Citrate buffer (10mM, pH=6).
  To prepare stock solution:
  Solution A:
  - 0.1M citric acid solution: dissolve 21g citric acid, monohydrate ($C_6H_8O_7.H_2O$) in 1000ml dH_2O.
  Solution B:
  - 0.1M sodium citrate solution: dissolve 29.4g trisodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$) in 1000ml dH_2O
  Working Solution:
  Add 9ml of stock solution A and 41ml of stock solution B to 450ml dH_2O. Adjust pH to 6.

- Let cool for 5 min then remove the slides with the tissue preparation.

3 – Permeabilization:

- Incubate 5 min in PBS, 30 min in PBST (1% Triton) and PBS again 5 min at room temperature.

4 – Blocking:

- Dry the slide with cellulose paper (use a DAKO hydrophobic pen to draw safe area around the tissue).
- Incubate for at least 30 min. with serum at 3% (of the same species in which the secondary antibody is made), in DAKO blocking solution (# S3022) or PBS +0.1% BSA in a moist chamber.
- Add 50-100ul of this solution per tissue area.

5 - Remove the blocking solution shaking the slide laterally on cellulose paper.

6 - Primary Antibody:

- Incubate overnight at 4C with primary antibody in a humid chamber.
- Dilute the antibody in 3% serum in DAKO blocking solution (#S3022) or PBS +0.1-1%. BSA and/or 0.1% triton X-100 or Tween 20 (PBST).

7 - Remove the antibody and wash 5 min in PBS, PBST (0.2% Triton) and PBS again.
NOTE that incubations/washes must be in the dark once the fluorochrome is present.

8 - Bridge or Secondary Antibody:
Incubate for 40 min. in a humid chamber with antibody + avidin + fluorochrome in 3% serum in blocking solution (DAKO # S3022) or PBS + 0.1% BSA
Dilution of the antibody: 1/500. Add DAPI to the antibody solution 1:50000.

9 - Remove excess solution and wash 5 min in PBS, PBST (0.5% Triton) and PBS again.

10 - Mount dako fluorescent mounting medium (DAKO # S3023) or in glycerol / PBS (9:1) or Aquatex (if hematoxylin-eosin staining).

- Dry the edges of the slide, put 1-2 drops of dako fluorescent mounting medium and gently place the cover slide avoiding bubbles. Save the slide in box avoiding light and look at specimen in the fluorescence microscope.
NOTE if you want the dye last longer mount with dako fluorescent mounting medium.

E-Immunohistochemistry in paraffin with TSA amplification system (Perkin Elmer)

1. Rehydrate as in section C-1
2. Heat in 10 mM citrate buffer pH6 for 2x5 min in a microwave
3. Incubate in 0.3% methanol-hydrogen peroxide for 30 min at RT (inactivates endogenous peroxidases)
4. Wash in 1x PBS for 5 min at RT
5. Permeabilize in PBS-Triton 1% for 30min at RT and 1x PBS for 5min
6. Block in TNB blocking solution for 30 min at RT
7. Add primary antibody diluted in TNB buffer O/N at 4C
8. Wash 5min each PBS / PBST / PBS
9. Add biotinylated antibody 1/500 (against the species of the 1st Ab) for 45 min at RT
10. Wash 5min each PBS / PBST / PBS
11. Add streptavidin-HRP 1/100 45 min at RT
12. Wash 5min each PBS / PBST / PBS
13. Add Substrate 1/50-1/75 TSA-Cy3 (diluted in buffer amplifier) for 4-8 min at RT
14. Wash 5min each PBS / PBST / PBS
15. Mount