IMMUNOSTAINING (USING NEN BIOTINYL TYRAMIDE AMPLIFICATION) PERKIN ELMER #NEL700A
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Reagents and materials:

- TBS (100 mM Tris pH7.5, 150 mM NaCl)
  - Make 500 ml of 1X TBS (100 mM Tris pH 7.5, 150 mM NaCl)
    - 50 ml of 1 M Tris pH 7.5
    - 15 ml of 5 M NaCl
    - Fill to 500 ml w/ dH_2O
- TNB (0.5% blocking reagent in TBS)
  - Add 0.05 g NEN block reagent to 10 ml of 1x TBS
    - heat TBS on stir plate to dissolve blocking reagent; aliquots, store at -20°C degrees)
- Prepare humid chambers (with Whatman paper and 1X TBS or dH_2O)
- TNT (100 mM Tris pH7.5, 150 mM NaCl, 0.05% Tween-20)
  - Make 500 ml of 1X TNT
    - 50 ml of 1 M Tris Ph7.5
    - 15 ml of 5 M NaCl
    - 2.5 ml of 10 % Tween-20
    - Fill to 500 ml w/ dH_2O (mix well)

Staining process: (use PerkinElmer TSA Biotin kit, #NEL700A)

1. Incubate slides at 60°C on slide warmer for at least 1hr.

2. Deparaffinize and hydrate (use clean solutions!)
   - Xylene, 2x or 3x 3 min
   - 100% EtOH, 90% EtOH, 70% EtOH, H_2O, 2x 2 min

3. Incubate in 1X TBS, 5 min RT

4. (Optional): Digest the sections with 1 mg/ml Hyaluronidase in PBS for 45 min at 37°C.

5. Perform antigen retrieval, if necessary:
   - For 250 ml of NaCitrate pH 6 buffer:
     - 4.5 ml 0.1 mM citric acid
       i. to make 500 ml of 0.1 mM citric acid (MW192.12), add 9.61 mg to 500 ml dH_2O
     - 20.5 ml 0.1 M Na-citrate
       i. to make 500 ml of 100 mM Na-citrate (MW294.1), add 14.71 g to 500 ml dH_2O
     - 225 ml dH_2O
   - For embryos < P0
     - Microwave buffer alone, 2 min on high
     - Put slides in container and heat in 95 °C water bath, for 15 min
     - Let solution and slides cool to RT (~30 min)
   - For pups > P0
     - Microwave buffer alone, 2 min on high
     - Put slides in container and heat in 95 °C water bath, for 2-5 min
     - Let solution and slides cool to RT (~30 min)

6. Quench in 3% H_2O_2/ MeOH 10min RT
   - 25 ml 30% H_2O_2 + 225 ml MeOH

7. Incubate in dH2O, 5min, RT

8. Circle sections with Pap-pen, rinse in 1X TBS

9. To block, incubate with TNB (200-300 μl per slide) at RT in moist chamber for 30 min.

10. Pour off TNB, and add 1° antibody diluted in TNB (200-300 μl per slide). Incubate at RT in moist chamber for 1hr or O/N at 4 °C

11. Wash: 1X TNT, 3x 5min at RT (on rocking platform)

12. Add Biotinylated 2° Ab diluted in TNB (200-300 μl per slide). Incubate in moist chamber for 30 min
    - Dilute 1/250 (~8 ug/ml for Invitrogen antibodies (biotin-xx anti-mouse B2763; biotin-xx anti-rabbit B2770))

13. Wash: 1X TNT, 3x 5min at RT (on rocking platform)

14. Add SA-HRP (from kit) diluted in TNB 1/100 (200-300 μl per slide). Incubate for 30min RT in moist chamber

15. Wash: 1X TNT, 3x 5min at RT (on rocking platform)

16. Add Biotynil Tyramide (kit), diluted 1/50 in **amplification diluent** (200-300 μl per slide). Incubate 3-10 min RT in moist chamber. **NOTE: NEED TO THAW BIOTYNIL TYRAMIDE REAGENT BEFORE USE**

17. Wash: 1X TNT, 3x 5min at RT (on rocking platform)

18. For fluorescence:
    - Add Streptavidin-Alexa555 or 488 (from Invitrogen: S32355 for 555; S32354 for 488) diluted in TNB 1/250 (~8 ug/ml; 200-300 μl per slide). Incubate for 30min RT in moist chamber
    - Wash: 1X TNT, 3x 5min at RT (on rocking platform)
    - Incubate sections with DAPI (from Invitrogen, D1306) (**1:1000** in TNT) for 10-15 min at room T
    - Wash: 1X TNT, 3x 5min at RT (on rocking platform)
    - Mount sections with Gel/Mount (Biomeda Fluorogel, Fishersci cat # NC9034735) and apply coverslip, and store slides in moist chamber, in the dark, at 4 degrees. (signal lasts at least few days)

19. For IHC
    - Add SA-HRP (from kit) diluted in TNB 1/100. Incubate for 30min RT in moist chamber
    - Wash: 1X TNT, 3x 5min at RT (on rocking platform)
    - For HRP detection, use DAB (VECTOR kit)

      <u>Vector DAB</u>

      To 5 ml dH2O:
      - add 2 drops buffer; mix well
      - add 4 drops DAB; mix well
      - add 2 drops H_2O_2mix well
      - add 2 drops NiCl (optional, if not using hematoxylin counterstain turns DAB color from brown to black)

      i. Incubate until color develops (~2-10min.)
      ii. Wash 5 min in dH2O.
      iii. Counter stain with methyl green or Hematoxylin QS (Vector Labs, H3404)…follow Vector protocol
      iv. Dehydrate and coverslip with histomount (Eukitt mounting media, Electron Microscopy Sciences, Cat#15320)