General Protocol for Immunostaining on Paraffin Sections

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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 (from Perkin Elmer TSA kit #NEL700A) to 10 ml of 1x TBS
        - heat TBS on stir plate to dissolve blocking reagent; aliquot and store at -20°C
- Prepare humidified 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)

Procedure:

I. Heat slides on slide warmer at 60 °C for 30 min - 1 hour

II. Deparaffinize and hydrate (use clean solutions!)

- Xylene, 3x 2 min
- 100% EtOH, 2x 2 min
- 90% EtOH, 70% EtOH, H_2O, 1x 2 min

III. Antigen retrieval.

A. For matricellular proteins: Digest the sections with 1 mg/ml Hyaluronidase (Sigma H3506) in PBS for 45 min at 37°C.

B. Boil samples in citrate buffer (10 mM sodium citrate pH 6.0):

- 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)

IV. Incubate the samples in dH_2O for 5 min at room T. Circle samples with PAP Pen, rinse 1X TBS

V. Block the sections.

A. Incubate the sections for 30 min with TNB (from Perkin Elmer TSA kit #NEL700A)

VI. Apply primary antibody.

A. Incubate the sections for 1 hr to overnight with primary antibody diluted in TNB at 4 °C (~200 µl per slide).

B. Wash the sections in TNT (100 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 3 times, 5 min for each.

VII. Apply secondary antibody.

A. Add Biotinylated 2° Ab diluted in TNB (200-300 µl per slide). Incubate in moist chamber for 30 min

   i. Dilute 1/250 (~8 ug/ml for Invitrogen antibodies (biotin-xx anti-mouse B2763; biotin-xx anti-rabbit B2770))

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

C. 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

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

E. Incubate sections with DAPI (**1:1000** in TNT) for 10-15 min at room T

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

G. 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)