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Protocol for Immunohistochemistry

Sacrifice and Tissue Processing

Rats are deeply anesthetized with pentobarbital sodium (Pental). Brain is fixed by transcardial perfusion, first with 50 ml of phosphate buffered saline (0.02M PBS, pH 7.4) containing heparin (5U/ml), then with 220 ml of ice-cold 4% paraformaldehyde in 0.1M PBS, pH 7.5 containing sucrose 4%. Brain is cut in coronal blocks and further fixed by immersion in the same fixative, refrigerated, for 1-2 hours. Brain blocks are transferred to 10% sucrose in 0.1M PBS and sectioned in a cryostat within 3 days. Brain sections, 30 um thick, are floated in 0.1M PBS and then preserved in a cryopreservation buffer at -20×C. The cryopreservation buffer contains 40% ethylene glycol and 1% polyvinylpyrrolidone in 0.1M potassium acetate buffer, pH 6.5.

Staining procedure

1. Floating sections are rinsed in 0.02M PBS, 2x 5 minutes.
2. Endogenous peroxidase activity is quenched by incubation with 0.2 % hydrogen peroxide in 0.1M phosphate buffer pH 7.3 containing 0.2% Triton X-100 for 25 minutes at room temperature.
3. Sections are rinsed in 0.02M PBS, 2x 5 minutes.
4. Sections are incubated with the primary antiserum in a medium containing 0.3% Triton X-100, 0.05% Tween 20, 4% normal donkey serum (NDS), for 1 hour at room temperature and then overnight refrigerated.
5. Sections are rinsed in 0.02M PBS, containing 4% NDS, 2x 5 minutes.
6. At this point, staining may proceed with various types of secondary antibodies. Two alternative procedures are described here:

   Protocol A. Sections are incubated with biotinylated donkey anti-rabbit (from Chemicon USA, catalog number AP182B) diluted 1:400 in 0.02M PBS, containing 0.3% Triton X-100, 0.05% Tween 20, and 4% NDS, for 1 hour at room temperature and then overnight refrigerated.

   Protocol B. Sections are incubated with horseradish peroxidase labeled donkey anti-rabbit (from Chemicon USA, catalog number AP182P), 1:400 in 0.02M PBS, containing 0.3% Triton X-100, 0.05% Tween 20, and 4% NDS.

7. Sections are rinsed in 0.02M PBS containing 4% NDS.
8. If sections have been processed through 6A, they are incubated with extravidin-peroxidase (Sigma Catalog number E2886) diluted 1:100 in 0.02M PBS, for 45 minutes at room temperature.
9. If sections have been processed through 6B, they are rinsed in 0.02M PBS, 3x 5 minutes.
10. If sections have been incubated with extravidin-peroxidase they are rinsed as in step 9.
11. Sections are incubated with a solution of diaminobenzidine (Sigma catalog number D5637) at the concentration of 0.0125% and containing 0.05% nickel ammonium sulfate for 10 minutes at room temperature.
12. Sections are transferred to the same DAB solution but with added hydrogen peroxide at a final concentration of 0.0015%. Duration of incubation should be adjusted by the end user.
13. Sections are rinsed in 0.02M PBS, 4x 10 minutes.
14. Sections are mounted on glass slides (gelatinized or coated by any other type of adhesive material) and allowed to dry.
15. Sections are dehydrated in ascending series of ethanol concentrations (70%, 90%, 100%, 5 minutes in each), delipidated in xylene (10 minutes) and coverslipped in Permount (or any other xylene diluted adhesive).