Immunocytochemistry in Free-Floating Sections

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Introduction

Immunocytochemistry comprises a number of methods, where antibodies are employed to localize antigens in tissues or cells for microscopic examination.

There are several strategies to visualize the antibody. For transmitted light microscopy, color development substrates for enzymes are often used. The antibody can be directly labeled with the enzyme. However, such a covalent link between an antibody and an enzyme might result in a loss of both enzyme and antibody activity. For these reasons several multistep staining procedures have been developed, where intermediate link antibodies are used. In this protocol, we use the Vectastain ABC-kit. In the last staining step, the reaction is visualized with a 3-3' diaminobenzidine tetrahydrochloride (DAB).

Stereology

Since tissue sections are two-dimensional samples of three-dimensional structures, correct and efficient morphological analysis should build on well proven mathematical theory leading to unbiased results. Stereology is such a quantitative technique providing the necessary mathematical background to predict the probability of an encounter between a randomly positioned regularly arranged geometrical probe and the structure of interest (Gundersen, 1986). Stereological methods have been introduced in quantitative immunocytochemistry (Janson and Møller, 1993).

Briefly, a color CCD camera (Sony) mounted on an Olympus BH2 microscope with a high precision motorized specimen stage and a microcator to monitor movements in the z-axis are linked to a computer with a stereological software. The entire unit is called a

CAST-system and produced by Olympus, Glostrup, Denmark. The analysis is performed at high magnification using an objective with a high numerical aperture (100x oil immersion, N.A. 1.4), which allows the tissue to be optically dissected in slices of 0.5 µm. This quantitative analysis requires thick sections (40µm) with an even and good penetration of the immunohistochemical staining. These conditions are met using a free-floating immunocytochemical technique.

Materials and Methods

Male C57 B1/6 mice were anesthetized (sodium pentobarbital, 100mg/kg, ip) and perfused transcardially with 10mL 0.9% saline followed by 100mL 4% paraformaldehyde with 1.4% picric acid in 0.1M phosphate buffer (PB, +4°C, pH 6.9, flow rate 20 mL/minute). After removal, the brain was transected coronally through the median eminence and the caudal block was postfixed in the same fixative (+4°C, 90 minutes) and cryoprotected in sucrose (10% for 24 hours followed by 30% for 1-2 weeks) in 0.1M PB. The midbrain was cut in 40µm coronal sections on a cryostat. The sections were sampled systematically throughout the entire substantia nigra with a random start according to the stereological principles (see below). The sections were kept in 30% ethylene glycol with sucrose in 0.1M PB at -70°C until they were taken to immunocytochemistry. All chemicals described above were from Sigma.

Chemicals/Materials

- PBS 0.1M, pH 7.4
- Incubation chamber (flexible PVC, wells with slanted side walls, covered with plastic)
- Polyvinypyrrolidone (PVP)
- BSA grade V
- Triton X-100
- Normal goat serum (Vectastain ABC-kit)
- Rabbit polyclonal antibody to tyrosine hydroxylase (TH, Pelfreeze, Rogers, AR, USA)
- Biotinylated goat anti-rabbit antibody (Vectastain ABC-kit)
- H_2O_2 30% (fresh)
- A and B solutions (Vectastain ABC-kit)
- Tris 0.05M, pH 7.4
- 3-3'-diaminobenzidine tetrahydrochloride (DAB)
- Chrom-alum coated slides
- Coverslips
- Ethanol (70%, 95% and absolute)
- Xylene
- 0.5% cresyl violet acetate in distilled water
- Entellan

Step-by-Step Staining Process

During the staining process, the sections should never be allowed to dry out. Corning® Netwell™ inserts (Cat. No. 3477) that were used were 15mm in diameter and had a mesh size of 74µm.

1. **Rinsing**
   a. The tubes with sections in ethylene glycol were removed out from the freezer.
   b. The sections were transferred to the Netwell inserts and rinsed 8x5 minutes in 0.1M PBS under gentle agitation.

2. **Incubation with normal serum**
   a. The sections were moved to a humidified incubation chamber.

b. The sections were incubated for 40 minutes at room temperature with 4% normal goat serum in 0.1M PBS with 1% PVP, 1% BSA, 0.3% Triton X-100.

3. **Incubation with the primary antibody**
   a. The sections were incubated for 40 hours at +4°C with a rabbit polyclonal antibody to tyrosine hydroxylase diluted 1:400 in 0.1M PBS with 1% BSA and 0.3% Triton X-100.

4. **Rinsing**
   a. The sections were gently transferred from the incubation chamber to the Netwells and rinsed 8x5 minutes in 0.1M PBS under gentle agitation.

5. **Incubation with the secondary antibody**
   a. The sections were moved to an incubation chamber (humidified).
   b. The sections were incubated for 2 hours at room temperature with a biotinylated anti-rabbit antibody made in goat diluted 1:200 in 0.1M PBS.

6. **Rinsing**
   a. The sections were gently transferred from the incubation chamber to the Netwells and rinsed 3x10 minutes in 0.1M PBS under gentle agitation.

7. **Quenching endogenous peroxidase**
   a. The sections were incubated in 0.3% H_2O_2 for 10 minutes at room temperature diluted in 0.1M PBS.

8. **Rinsing**
   a. The sections were rinsed 4x10 minutes in 0.1M PBS under gentle agitation.

9. **Biotin-avidin complex**
   a. The sections were moved to a humidified incubation chamber.
   b. The sections were incubated for 2 hours at room temperature with A and B solutions diluted 1:100 in 0.1M PBS.

10. **Rinsing**
    a. The sections were gently transferred from the incubation chamber to the Netwells and rinsed 2x10 minutes in 0.1M PBS and 1x10 minutes in 0.05M Tris under gentle agitation.
    b. While sections were rinsing, fresh 0.03% DAB in 0.05M Tris was prepared and filtered.

11. **Color development**
    a. H_2O_2 was added to a final concentration of 0.003% in the filtered DAB substrate solution
    b. The Netwells with the sections were incubated in the DAB/H_2O_2 solution for approximately 10 minutes (the development process was checked under microscope for an optimal signal-to-noise ratio).

12. **Rinsing**
    a. The sections were rinsed in 2x10 minutes in 0.05 M Tris.
    b. The sections were mounted on chrom/alum-coated slides

c. The sections were dried overnight

13. **Counterstain**
    a. The slides with the sections were dehydrated in increasing concentrations of ethanol and xylene
    b. The slides were again hydrated, counterstained with cresyl violet and dehydrated
    c. The slides were cover slipped in Entellan.

References

1. Gundersen HJG (1986) J Microsc. 143, 3

2. Janson AM, Moller A (1993) Neuroscience 57, 931

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