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Protocol

A sequential fluorescence method for neurotransmitter-specific retrograde tracing in the central nervous system of the rat; utilizing True Blue and immunohistochemistry in combination with computer-assisted photography

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Abstract

Aiming to map the distribution of spinally projecting, hypothalamic neurons containing neuronal nitric oxide synthase (nNOS), True Blue (TB) is injected into the rat spinal cord. After survival times of 7–14 days the animals are anaesthetized and perfused transcardially with a solution containing paraformaldehyde and sucrose. After dissection, the injection site is further fixed for 4–8 h, cut in a cryostat, and documented by computer-assisted digital photography. The brain region of interest is fixed for 4 h, rinsed in phosphate buffer for 48 h, sectioned, and photographically documented utilizing filter settings for visualization of TB. The brain sections are then immunohistochemically processed using a primary antibody against nNOS and a Texas Red (TR)-labelled secondary antibody and once again photographically documented, now using filter settings for visualization of TB and TR, respectively. Utilizing the Photoshop program, the TB containing cells can then be exactly aligned and the presence of TB and/or TR fluorescence in the same cell bodies are evaluated. This method for neurotransmitter-specific retrograde tracing derives its high sensitivity from the optimization of fixation/rinsing parameters, the use of appropriate fluorophores, and sequential digital microphotography.

Theme: Cellular and molecular biology
Topic: Staining, tracing, and imaging techniques

Keywords: Digital photography; True Blue; Fluorescence; Retrograde tracer; Spinal cord; Immunohistochemistry

1. Introduction

The combination of retrograde tracing and immunohistochemistry allows for mapping of axonal projections of cell bodies containing a defined neurotransmitter or transmitter-related enzyme. Such methods were described already in the mid-seventies utilizing conventional light-microscopy and photography. The technique initially relied on the different distribution and morphology of horseradish peroxidase, whether bound to an antibody for visualizing the transmitter, or retrogradely transported from a distal injection site in order to demonstrate the neuronal projection [7]. A simpler and apparently more sensitive technique was later provided by the combination of a natively fluorescent retrograde tracer [6] with either fluorescence histochemistry [3] or immunofluorescence [10] utilizing fluorophores with non-overlapping spectral characteristics [5]. A protocol for the latter method was provided by Sawchenko and Swanson [10], who used the fluorescent retrograde tracer True Blue (TB) in combination with immunofluorescence utilizing a fluorescein

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isothiocyanate (FITC)-labelled secondary antibody. Although these methods were shown to be highly useful, they are somewhat cumbersome to use, mainly because conventional photography is time consuming and sometimes difficult to perform because of insufficient fluorescence intensity of the tracer. Furthermore, the sensitivity of the technique sometimes appears less than optimal probably due to partly overlapping spectral characteristics, diffusion, and fading of the retrograde tracer during tissue processing and the consequent need for long photographic exposure times when using conventional photography. These drawbacks are largely overcome by the present protocol, which utilizes a modified procedure for tissue processing in combination with fluorophores with non-overlapping spectral characteristics, and takes advantage of the recent developments for digitalized photography which allows for short exposure times and subsequent, but immediate, computerized image analysis and processing. Utilizing TB in combination with a neuronal nitrergic oxide synthase (nNOS) antibody, the nitric projections originating in the hypothalamic paraventricular nucleus (PVN) was investigated [9]. In this protocol retrogradely transported TB and Texas Red (TR) bound to the secondary antibody are selectively visualized using amino methylcoumarin acetic acid (AMCA) and TR filter settings, respectively.

2. Type of research

Neuroanatomical studies concerned with the origins, projections, and terminations of neuronal systems containing a specified neurotransmitter.

3. Time required

Seven to 14 days for retrograde tracing. Approximately 8 h for preparation of sections and photography session I. Approximately 24 h for immunohistochemical processing and photography session II. Thus, the complete protocol can be executed in approximately 9–16 days.

4. Materials

4.1. Animals

Female virgin Sprague–Dawley rats weighing 200–300 g (B&K Universal, Sollentuna, Sweden) are housed, up to four animals together in each cage, under standard laboratory conditions. The rats have free access to water and standard pellets and are housed under controlled conditions at 22 ± 2 °C with 12 h of light and 12 h of darkness (the experimental protocol was approved by the Animal Ethics Committee, University of Lund).

4.2. Special equipment

- Camera: SONY DKC-5000, 3CCD digital camera
- Computer: PC, Windows 98 (Compliq, Lund, Sweden)
- Cryostat: Leica CM 3050 (Leica Microsystems Nussloch GmbH, Nußloch, Germany)
- Injection equipment: Hamilton microsyringe (10 μl) equipped with beveled steel cannula, with an outer diameter of 0.47 mm (Hamilton Company, Reno, NV, USA)
- Microscope: Olympus BX 40 (HBO 103W/2 mercury lamp) equipped with objective (Uplan Apo 10×/0.40) and filter settings for AMCA fluorescence (U-MNU, no. 37274, excitation: 360–370 nm, dichroic mirror: 400 nm, barrier filter: 420 nm, injection site and brain area), TR fluorescence (U-MWIY, no. 37273, excitation: 545–580 nm, dichroic mirror: 600 nm, barrier filter: 610 nm, brain area), and FITC fluorescence (U-MWB, no. 37266, excitation: 450–490 nm, dichroic mirror: 500 nm, barrier filter: 515 nm, injection site)
- Operating microscope: Zeiss OPMI-6H (Carl Zeiss, Jena, Germany)
- Soft ware: Image Access (Digital Arts and Science, Alameda, CA, USA) for image analysis, PC version of Adobe Photoshop® (4.0) for camera control
- Stereotaxic apparatus (Stoelting, Wood Dale, IL, USA)

4.3. Retrograde tracer

-TB (EMS-POLYLOI GmbH, Groß-Umstadt, Germany) A 3% weight/volume (w/v) suspension of TB is prepared by suspending 3 μg of TB in 0.1 ml distilled water and sonicating for 10 min (sonicator; Model B-30, Branson Sonic Power, Danbury, CT, USA). This suspension may be stored in a refrigerator for at least 6 months without appreciable loss of fluorescence intensity.

4.4. Antibodies

- Rabbit nNOS antiserum (code no. 9224, Euro-Diagnostica, Malmö, Sweden)
- TR-conjugated $F(ab)_2$ fragment donkey anti-rabbit IgG (1:80; code 711-076-152, Jackson ImmunoResearch, West Grove, PA, USA)

4.5. Chemicals

- Etylene Glycol-bis (*b*-aminoethylether)-*N*,*N*′-tetra acetic acid (EGTA; Merck KGaA, Darmstadt, Germany)
- Gelatin (Kebo, Stockholm, Sweden)
- Glacial acetic acid (17,4M; Merck Eurolab, Darmstadt, Germany)
- Glycerol (Merck)
- HCl (Merck)
- Heparin (Lövens, Malmö, Sweden)
- $KCr(SO_4)_2 \times 12\ H_2O$ (Merck)