Preface

The past decade has seen an extraordinary growth in research interest in neurotrophic factors, and the study of the neurotrophin family has led this activity. Nevertheless, this area of research has often struggled as a result of techniques that were either inadequate or just emerging from other research fields and disciplines. *Neurotrophin Protocols* has brought together many leaders in the neurotrophin field who detail their special expertise in a wide variety of techniques. Though most procedures are valid across many different fields of research, some of those described here have been developed to address particular issues within the neurotrophic factor field. The protocols cover a broad range of biochemical, histological, and biological techniques that are often required by the modern laboratory. However, all have been written with sufficient detail to allow any laboratory to achieve proficiency without need of reference to other texts.

*Neurotrophin Protocol*s is divided into four sections dealing with protein, RNA, recombinant, and in vivo techniques. Protein techniques have in general been less successfully employed than those dealing with RNA or DNA. However, procedures that achieve localization and quantification of the neurotrophins are now being used more extensively. Their inclusion here should assist further studies at the protein level. Transgenic cell lines and animals are commonplace in the scientific research literature, but their inclusion in several chapters in this book provide some novel uses that are not readily available elsewhere. Quantitative and histological methods for the analysis of neurotrophin mRNA are also included. Although radiotracing techniques have become less common, two useful but distinctly different procedures have been included for specialist investigation of neurotrophin transport in both the retrograde and anterograde directions. Other procedures include the increasingly popular use of immunotoxins to study the effects of elimination of a single class of neurons and the essential stereological method for estimation of neuronal numbers.

Because of this wide range of protocols and the extensive contributions of the authors over many years, *Neurotrophin Protocols* should be of high interest and utility to both postgraduate students and established investigators alike.

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Neurotrophin Immunohistochemistry in Peripheral Tissues

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1. Introduction

Neurotrophins are required by a variety of neuronal types for their survival during development and for the maintenance of normal function in mature animals. Within the nervous system, the neurotrophins are synthesized in limited amounts in postsynaptic cells, glia, or neurons. Within neurons, the neurotrophins are transported both retrogradely and anterogradely to neuronal somata or nerve processes, respectively (*see*, for example, **ref. *1***). Because of this mobility, distribution of the corresponding mRNA usually does not match that of the protein. Therefore, to understand their precise function, it is essential to localize both the neurotrophin proteins and their corresponding mRNA. Although immunohistochemistry provides only a view at one instant of time of the antigen's presence at the site of examination, a more dynamic picture can emerge if several time-points are examined after experimental intervention. The movement of the neurotrophins has been studied extensively by the use of nerve ligations, which allows determination not only of the retrograde and/or anterograde movement of the protein, but also can be useful in demonstrating the absence of a neurotrophin in a particular class of nerves *(1)*.

Despite the continual development of the immunohistochemical technique for the localization of neuronal antigens over the past 30 yr, the localization of neurotrophins has proved frustrating *(2)*. Limited success is the result of a number of issues. First, the concentrations of the neurotrophins in most tissues are low, generally in the range of picograms to nanograms per gram wet weight *(3,4)*. Although these levels are generally not below the limits of detection sensitivity for immunohistochemistry, they are often lower than for many other

neuronal antigens. Second, the detection of neurotrophins in certain cell types is sensitive to crosslinking reagents and immunoreactivity is lost after even moderate fixation *(5–7)*. Third, neurotrophins act on responsive cells by binding to specific receptors. This binding leads to dimerization of the high-affinity trk receptors by a mechanism that appears to "engulf" the neurotrophin (*see*, for a review, **ref. 2**). Hence, access to the bound neurotrophin by the detection antibodies is restricted by the surrounding receptor proteins. This concept is supported by several observations such as the inability of immunohistochemistry to localize the neurotrophins present in peripheral nerve terminals, but not the same neurotrophins, at similar or lower concentrations, when they are present in the free state *(3,4,8; see also* **ref. 2**). Thus, whereas the procedures described in this text allow detection of neurotrophins in a variety of peripheral tissues, the experimenter should be aware of this limitation, so that the absence of stain is not necessarily interpreted as an absence of the neurotrophin. Combined use of immunohistochemical and biochemical techniques provides significantly greater strength to any investigation of the neurotrophins.

Despite these considerable limitations, the localization of neurotrophins and their receptors is proving to be increasingly possible and valuable for the study of both normal physiology and disease. A selection of useful references describing localization in peripheral tissues has therefore been included *(9–23)*. Although the focus of neurotrophin research is predominantly directed to understanding their actions on the nervous system, there is also emerging evidence to indicate that these potent proteins are involved in regulation of the metabolism of non-nervous tissues (*see*, for a review, **ref. 24**).

Readers are referred to the more extensive discussion of these issues in Chapter 1 as many of the issues addressed for the localization of neurotrophins in the central nervous system are relevant to their localization in peripheral tissues. Issues concerning the quality of available antibodies also can be found in Chapter 1, as well as in other recently published articles *(2,25)*.

2. Materials

All reagents are readily available from a laboratory supply company such as Sigma. Secondary antibodies are available from a large number of companies; we routinely use those from Vector Laboratories.

2.1. Solutions

2.1.1. Perfusion and Fixation

1. 0.2 *M* sodium phosphate buffer, pH 7.4: Dissolve 21.4 g Na_2HPO_4 in 800 mL water, stir, and then add 7.8 g $NaH_2PO_4 \cdot 2H_2O$; when dissolved, make up to 1000 mL.

2. 0.1 *M* sodium phosphate buffer with 1% (w/w) sodium nitrite ($NaNO_2$): Dilute 0.2*M* sodium phosphate buffer 1 : 1 with water and add 10 g $NaNO_2$ per liter.
3. Zamboni's fixative. For 4%, mix 100 mL of 40% (w/w) formaldehyde solution, 150 mL saturated picric acid, 500 mL of 0.2 *M* sodium phosphate buffer, and 250 mL water. (This solution is used for NT-3 and NGF; 2% is used for BDNF.) For 2%, mix 50 mL formaldehyde solution, 150 mL saturated picric acid, 500 mL of 0.2 *M* phosphate buffer, and 300 mL water.
4. 30% Sucrose in phosphate-buffered saline (PBS): Dissolve 30 g of sucrose in 90 mL of PBS, then make up to 100 mL with additional PBS.

2.1.2. Acid Wash

Use 5% acetic acid, pH 3.0.

2.1.3. Immunohistochemistry

1. Ethanol (50%) containing hydrogen peroxide: 50 mL of ethanol is mixed with an equal volume of water and 1 mL of hydrogen peroxide (30%) added.
2. Phosphate-buffered saline (PBS); 20X stock solution: Dissolve 21.4 g Na_2HPO_4 in 800 mL water, stir, and then add 7.8 g $NaH_2PO_4 \cdot 2H_2O$ and 170 g NaCl. When dissolved, make up to 1000 mL.
3. Phosphate-buffered saline, 1X working solution: Take 50 mL of PBS 20X and add 950 mL of water.
4. Phosphate buffer with Tween-20 (PBST): Add 1 mL of Tween-20 to 1000 mL of 1X PBS.
5. Blocking solution: PBS containing 20% (v/v) normal horse serum (NHS) (e.g., 8 mL PBS + 2 mL NHS).
6. Antibody diluent: 2X concentrated PBS containing 0.3% (v/v) Triton X-100 to which 1% (v/v) NHS is added.
7. Secondary antibody solution: Biotinylated affinity-purified anti-host IgG (e.g., Vector Laboratories, raised in goat) diluted 1 : 200 in PBS containing 1% (v/v) NHS.
8. ABC reagent: Vectastain kit (Vector Laboratories) diluted 1 : 100 in PBS. Use according to the manufacturer's instructions (e.g., 100 μL reagent A [Avidin DH] + 100 μL reagent B [biotinylated HRP] + 9.8 mL PBS). This solution should be prepared 30–60 min before use.
9. Tris-buffered saline (TBS): Dissolve 12.1 g of Tris base and 8.5 g of NaCl in 900 mL of water, then adjust pH to 7.4 with dropwise addition of HCl. Make final solution to 1000 mL with water.
10. DAB reagent: 100–120 mg nickel sulfate and one diaminobenzidine (DAB) tablet (10 mg; Sigma) are dissolved in 20 mL TBS. This solution may be stored at 20°C. Hydrogen peroxide is added just before use on the sections (7.5 μL of 30% peroxide per 20 mL, or 2–3 μL per 5 mL).
11. Dehydrating solutions: Ethanol is mixed with distilled water to make 50 and 70% solutions; 100% ethanol and xylene are used straight from the bottle.

3. Methods

3.1. Perfusion and Fixation

1. Anesthesia: Nembutal (approximately 0.8–1.0 mL) is injected intraperitoneally into a 300- to 350-g rat. When the rat is deeply anesthetized, perfusion can begin.
2. Routine fixation (*see* **Note 1**): Expose the heart by surgically opening the chest. The rat is perfused via the left ventricle into the aorta. When the perfusion cannula is in place and the fixative is clearly moving through the vasculature, clamp the needle onto the heart muscle with a pair of hemostats and cut open the right atrium. Begin the perfusion with sodium nitrite in 0.1 *M* phosphate buffer for sufficient time to clear the bulk of the blood from the rat. This usually is between 50 and 100 mL per rat.
3. Start fixative perfusion and continue until a minimum of 500 mL of Zamboni's fixative has been used. Good fixation should take between 500 and 1000 mL of fixative over 20–30 min for an adult rat. Perfuse no longer than 30 min.
4. The tissues to be examined are immediately dissected and postfixed for 1–2 h in Zamboni's fixative (*see* **Note 2**).
5. Rinse tissues with PBS and transfer to approx 50 mL of 30% sucrose in PBS at 4°C until sectioned (at least overnight). Tissues can be stored cold for months if sterile or antibacterial agent is present.

3.2. Acid Wash Perfusion (see Note 3)

Following the onset of anesthesia, rats are briefly perfused with 50 mL of 5% acetic acid (pH 3.0), followed by the perfusion protocol described in **Subheading 3.1., step 2**.

3.3. Immunohistochemistry

3.3.1. Cryostat Sectioning and Prewash

1. Section tissues at 30 µm and collect in PBS at room temperature in plastic containers. (Alternatively, sections can be collected sequentially in separate wells of a 24-well tissue culture plate.)
2. Wash sections by resuspension in 50% ethanol containing H_2O_2 three times, for 15 min in each wash. Each time the bulk of the solution is removed from the wells using a glass pipet attached to a suction flask, then the remaining solution is aspirated manually with a glass pipet. New solution is added forcefully to disturb the sections from the bottom of the well and provide better washing. Do not allow the sections to dry (*see* **Note 4**).
3. The final resuspension should be in PBS or TBS. Sections can be stored for weeks at 4°C, but the addition of 0.2% sodium azide is advisable.

3.3.2. Blocking and Primary Antibody Incubations (D 1)

1. Tissue sections are transferred to a 24-well plate using a glass pipet. Where controls and experimental samples are to be compared, these should be processed in

parallel for all subsequent steps, so that exposure times to secondary antibodies and to enzyme substrate is identical for all sections.

2. PBS is aspirated from wells using a glass pipet and then sufficient blocking solution is added to cover sections so they are free to float. This is 0.4 mL per well of a 24-well plate. Leave the plate at room temperature for at least 1 h (preferably 2 h) to give adequate blocking.

   This step is critical to reduce the background noise to an absolute minimum. Primary antibody solutions can be prepared during this time.

3. Primary antibody solutions are prepared using antibody diluent. The appropriate dilution is dependent on the particular antibody being used (usual range is from 1:200 to 1:10,000; monoclonal or affinity-purified polyclonal antibodies are usually used at approx 0.5–2.0 µg/mL). (*See* **Note 5**.) Gentle agitation, such as with a mechanical platform shaker, is helpful to improve uniform staining.

4. The blocking solution is aspirated with a pipet and immediately replaced by the diluted primary antibody solution. Any air bubbles are removed, as they can attach to the sections and prevent antibody access. The plate is incubated overnight at room temperature (*see* **Note 6**).

3.3.3. Secondary Antibody and Color Development Incubations (D 2)

1. After removing all traces of primary antibody solution with a glass or plastic bulb pipet, sections are washed three times for 15 min each with PBST as follows.
2. Standard wash method: Squirt the washing buffer forcefully from the wash bottle or bulb pipet to fill wells, causing sections to be gently disturbed from the bottom. After each wash, remove the bulk of the solution from each well using a pipet attached to a suction line and waste flask (*see* **Note 7**).
3. After the final wash, all solution is aspirated and the secondary antibody solution is added. Incubate at room temperature for 2 h (*see* **Notes 8** and **9**).
4. Wash sections three times for 15 min each time with PBST, after removing all traces of secondary antibody solution with a pipet, using the "standard wash method."
5. After the final wash, all solution is aspirated and the ABC reagent is added. Incubate at room temperature for 2 h.
6. Remove all traces of the ABC reagent with a pipet. Sections are then washed three times for 15 min, using the "standard wash method" (*see* **Note 10**).
7. Add at least 0.5 mL of DAB solution to all wells (*see* **Note 11**). After the DAB reagent is added, the sections are incubated at room temperature until the bluish/brown color of the substrate develops (*see* **Note 12**). When the desired level of reaction intensity is achieved, the reaction must be terminated quickly to minimize background, nonspecific color development. This is achieved by removing the DAB reagent with a pipet and immediately flushing each well with PBST. When all wells have been stopped, wash all sections again with one final wash of PBST.

3.4. Mounting of Stained Sections

1. Sections are transferred with the aid of a pipet to coated glass microscope slides (*see* **Note 13**) and arranged using a fine paintbrush on a slide that is premoistened

with PBST. Excess liquid is carefully poured off (the wet sections can be observed under a dissecting microscope if required). Slides are left at room temperature to air-dry by resting the slide in a tilted position or transferring to a drying rack.

2. Dried sections are dehydrated by sequential immersion in 70, 90, and 100% (two times) ethanol, 5 min in each solution. The sections are then clarified in xylene (twice) for 3–5 min. A xylene-based mounting compound is spread carefully over all sections as each slide is removed from the xylene, and the cover slip is applied, being careful to exclude all air bubbles. Each slide is then blotted face down on absorbent paper and left face up to dry in air.

3.5. In Vivo Absorption (see **Note 14**)

1. Rats are injected with either neurotrophin antiserum or nonimmune serum at a dose of 10 µL/g body weight.
2. Every other day, inject, subcutaneously, antiserum or nonimmune serum at a dose of 10 µl/gram body weight.
3. On the d 8, the rats are anesthetized and perfusion fixed as described in **Subheading 3.1.**). Sections of ganglia are then examined immunohistochemically for both the corresponding antigen and another neurotrophin (*see* **Note 15**).

4. Notes

1. We routinely use tubing with an 18-gage needle secured in the end.
2. We use screw-capped plastic specimen containers or glass scintillation vials for large quantities or 24-well culture plates for smaller volumes. Although it is possible to postfix some tissues for longer than 2 h, we have found that localization is rarely improved by longer postfixation periods, but is often adversely affected by extended fixation.
3. This procedure has been found to enhance staining of all neurotrophins in sympathetic and sensory ganglia. Benefit has also been seen with neurotrophin receptor (trk's) localization. No benefit has been found in any other tissues, including the central nervous system. A brief perfusion is made with either a high- or low-pH buffer prior to fixation to unmask several of the growth factors and their receptors in peripheral ganglia, including NGF, NT-3 and BDNF. We routinely use the acid perfusion described here as greater uniformity has been achieved at low-pH buffers compared to the use of high-pH buffers. The mechanism of this unmasking is unclear, but it appears most useful for neurotrophins that have been retrogradely transported to the neuronal somata (or on the distal side of a nerve ligation), suggesting that it might involve endosomal disruption. The procedure improves the localization of neurotrophins in both sensory and sympathetic ganglia.
4. Sections must NEVER be allowed to dry. Poor staining and high background always result.
5. Valuable primary antibodies that are either expensive or in short supply can been kept at 4°C and reused between two and four times.

6. Incubations up to 1 wk at 4°C can sometimes enhance staining in difficult situations.
7. After removing the bulk of the wash buffer with a pipet connected to a vacuum line, aspirate the remaining solution manually and gently with a hand pipet. By tilting the plate sideways, the tissue sections tend to fall together, allowing most of the liquid to be aspirated. The plate is then laid flat to remove the last traces of liquid. A black background, such as a sheet of black Perspex, is useful for visualization of the sections during washes.
8. It is good practice to determine the best dilution of each new batch of secondary antibody that gives the best signal-to-noise ratio.
9. Ensure that the host species is compatible with the primary antibody being used.
10. The final step should be performed in batches for each primary antibody used, as the color development time will vary for each antibody and each dilution.
11. Diaminobenzidine is very hazardous and must be inactivated after use with commercial bleach.
12. This step can be performed with a few wells at a time to allow better control of the reaction, as the time necessary to achieve optimal staining varies considerably depending on primary and secondary antibody concentrations as well as the antigen concentration (and other unknown mysterious conditions!). During the development step, it is useful to follow the reaction under a dissecting microscope, as the reaction product can be seen forming within the tissue sections.
13. Sections can be left in wash buffer for long periods of time, but we prefer to mount immediately, as some deterioration of the stain can occur with time. Slides are previously coated with polyornithine or gelatin. (See Chapter 1, Note 16.)
14. This is a method that we have found useful for demonstrating the ability of a particular antibody to block the action of a neurotrophin in vivo. As it involves the use of antibody delivered sytemically to bind and remove extracellular neurotrophin prior to internalization, we have termed the procedure "in vivo absorption" *(1,8)*
15. This requires that the antibodies used for immunohistochemistry are from a different species than those used for the neutralization in vivo. We have combined the use of sheep and rabbit antibodies, but any combination is possible so long as the secondary detection antibodies are tested to verify specificity. The absence of the corresponding antigen, together with the presence of another neurotrophin indicates that the antiserum has specifically neutralized the antigen if staining for the other neurotrophin remains unaffected. We have used this technique successfully for NGF, NT-3, and BDNF *(1,21,22)*.

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