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Neurotrophin Protocols

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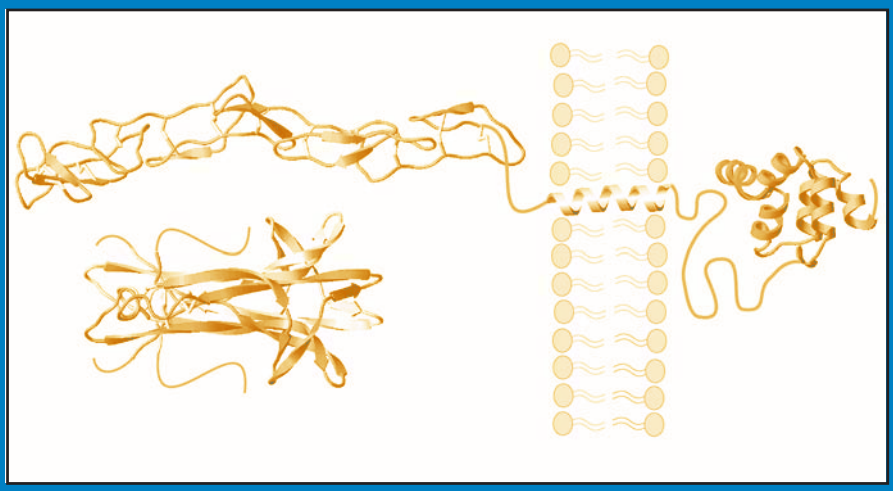

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Localization of Neurotrophin Proteins Within the Central Nervous System by Immunohistochemistry

James M. Conner

1. Introduction

Neurotrophins are a family of growth factor proteins sharing a considerable degree of primary sequence and tertiary structure homology ***(1–4)***. Members of this protein family, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), have been identified as important signals in the developing peripheral nervous system where they serve as survival-promoting and differentiation factors for various populations of neurons ***(5,6)***. The presence of each of these factors within the central nervous system (CNS) has also been confirmed ***(7–12)***, although their physiological role in the CNS has yet to be fully elucidated. Identifying where in the CNS these proteins are made, where they are stored, how they are transferred from one cell to another, what cells they are transferred to, and their final fate are some key steps toward reaching an understanding of endogenous neurotrophin function. One technique for addressing many of these issues is immunohistochemistry. The strength of the immunohistochemical approach lies with its ability to precisely define the distribution of a given protein within a tissue sample obtained under defined experimental conditions. By manipulating the conditions under which a specimen is collected, it is possible to gain a great deal of insight into the nature by which a protein interacts within its environment and to obtain clues regarding its functional role in the animal. Nevertheless, the immunohistochemical approach has distinct limitations that must be considered when interpreting results and drawing conclusions.

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Undoubtedly, one key limitation of the immunohistochemical approach, in general, is that it is a "static" assay being applied to a dynamic biological system. From the moment a protein is synthesized within the endoplasmic reticulum (ER) until the time it is degraded, it will have traveled along a random or defined pathway that may include transfer between various subcellular compartments of the same cell or transfer to another cell entirely. A protein may either move in a continuous fashion or it may temporarily accumulate at one or more sites along a given path (i.e., within one or more cellular compartments). Where, and to what extent, a given protein accumulates can either be a relatively constant property or it may be a highly variable one and dependent on the physiological state of the animal. Thus, when the distribution of a given protein is evaluated in a piece of fixed tissue using immunohistochemistry, the results of this evaluation are actually indicating sites of protein accumulation, unique for the given point in time when the biological system was halted (by fixation). Had the system been halted at some other time or under different conditions, the sites of protein accumulation may have differed (*see* **Note 1**). It is also important to bear in mind that, as a "static" assay, the immunohistochemical technique is incapable of rendering information pertaining to the *rate* of protein production in a given tissue. Robust labeling for a protein at a given location in a tissue does not necessarily indicate that protein production in that tissue occurs at a high level, but it is only indicative that protein storage at that location is relatively abundant. In fact, it should not be assumed that the immunohistochemical identification of sites of protein storage in a tissue are, in any way, related to sites of production. A protein can be transported to, and stored, anywhere along its path of action.

Another general limitation of the immunohistochemical approach is associated with the "detection limit" of the assay, which is the ability of the assay to distinguish between signal and background in the tissue. Without adequate controls (sites within the tissue where known quantities of protein are stored), it is almost impossible to arrive at a quantitative determination of the lower detection limit for an immunohistochemical assay. For this reason, only positive results obtained with the immunohistochemical approach are valid for drawing definitive conclusions. Because a protein may be present at a given location, but in amounts below the detection limit of the assay, it is not appropriate to draw definitive conclusions from negative immunohistochemical results (i.e., one cannot comment on where a protein is *not* located in a given tissue).

Keeping the general limitations for immunohistochemistry in mind, one can consider the special case of applying this technique to look at neurotrophin proteins in the CNS. The first point to be considered is that, unlike the synthetic machinery for neurotrophins (the messenger RNAs), which can be

expected to be found exclusively in association with ribosomes/ER in cells where neurotrophins are produced, neurotrophin proteins can potentially be localized anywhere along the pathway where they act. Based on what is known about neurotrophin actions thus far, these sites may include association with (1) producer-cell ribosomes/ER, (2) producer-cell Golgi apparatus (if the protein is packaged for secretion) ***(13–16)***, (3) secretory vesicles of producer cells, along either axonal or dendritic process (depending on polarity of secretion) ***(13–17)***, (4) presynaptic terminals ***(18–20)***, (5) extracellular space (either diffuse if the protein is soluble, or in association with extracellular matrix components if it becomes bound) ***(13,21)***, (6) postsynaptic cell membranes or synaptic terminals (axonal, dendritic, or somal) ***(16)***, (7) endocytotic vesicles within the postsynaptic cell (if the neurotrophin becomes internalized) ***(22–24)***, (8) lysosomal vesicles (if the neurotrophin is targeted for degradation) ***(25)***, (9) postsynaptic-cell Golgi apparatus (if the neurotrophin is targeted for reuse) ***(16)***, or (10) postsynaptic nuclear membrane (if the neurotrophin is eventually used to regulate postsynaptic gene expression directly) ***(26)***. Once again, localizing neurotrophin proteins anywhere along their functional pathway is dependent on having the protein accumulate at a particular site in amounts in excess of the assay's detection threshold. Thus, a failure to localize a neurotrophin protein to any of these sites using immunohistochemistry does not imply that the protein is not, or has never been, present at that site but must be interpreted simply to mean that at the time the animal was sacrificed, there was not enough protein accumulated at that particular site to permit its detection using this approach.

A second concern pertaining to the immunohistochemical localization of neurotrophin proteins in the CNS has to do with fixation-dependent lability of these antigens. Chemical fixation of the tissue involves crosslinking proteins together and is necessary to stop ongoing biochemical and enzymatic activity in the tissue, to preserve tissue morphology, and to lock proteins, especially the antigen sought after, in their *in situ* locations. In the case of neurotrophins, there have been many reports concerning a loss of antigenicity (the ability of an antibody to recognize a particular antigen) following standard paraformaldehyde or gluteraldehyde fixation ***(24,27–31)***. For this reason, many investigators have resorted to the use of special fixatives that appear to preserve the neurotrophin antigens in such a way as to maximize their interaction with a given antibody preparation ***(18,24,25,32–34)***. The specific fixation protocol included in this chapter is one such example of how this principle has been applied to successfully elaborate the *in situ* distribution of NGF in several different species (including rat, mouse, hamster, monkey, and human) ***(24,34–38)*** and BDNF in rat and mouse ***(18)***. Unfortunately, determining the optimal fixation parameters for any given antigen (and perhaps for any given antibody–

antigen interaction) must be done through an empirical, trial-and-error process. A fixation protocol that is optimal for one antigen may result in the complete loss of staining for another antigen routinely identified using a different fixation protocol. Thus, in instances where multiple antigens are being studied, it may be necessary to prepare more than one set of tissue using various fixation procedures (or alternatively seek out antibody combinations compatible with a single fixation protocol).

Another important issue related to the immunohistochemical localization of neurotrophin proteins is *crossreactivity*. Because neurotrophin family members share a substantial degree of sequence and structure homology, it is possible for antibodies raised using one neurotrophin as an immunogen to recognize more than just that particular neurotrophin as potential antigens, provided the specific epitope to which the antibodies were directed is conserved across proteins ***(39–42)***. Although each investigator should carry out appropriate immunohistochemical controls to check for evidence of crossreactivity (*see* **Note 2**), obtaining definitive proof for the specificity of a given antibody preparation is paradoxically impossible. Proof of antibody specificity in an immunohistochemical assay would require having a positive control tissue known to express the antigen sought after, as well as control tissues known to express every other potential crossreacting antigen, both known and unknown. The paradox arises in that a true positive control tissue would be one in which a given antigen is not only expressed but accumulates at levels that permit its detection by immunohistochemistry. However, such a control cannot be obtained because it is the immunohistochemical technique that is being validated. In light of this apparent irreconcilable uncertainty, is the immunohistochemical approach still a useful tool? Most certainly. First, although absolute proof for antibody specificity may be impossible to obtain, an overwhelming case in favor of specificity may readily be built on experimental evidence. Second, it is important to keep in mind that the results of an immunohistochemical study are usually not sought for their own merit but are most often used to gain insight into how a particular protein interacts *in situ*. Thus, immunohistochemical data are generally considered along with results from other experimental approaches to explore a protein's presence and possible function. In this light, it is important to keep in mind that immunohistochemical data, or any piece of data for that matter, must be consistent with other empirical evidence and should be capable of withstanding further experimental challenge.

One appended note worth including for those who are just beginning to use the immunohistochemical technique in their research has to do with the concept that not all antibody preparations directed against a given antigen can be expected to yield equally good results. Our incomplete understanding of the immune response and of how a single immunogen may elicit different

immune responses in different animals to produce antibody preparations with widely varying properties means that we must rely upon an empirical process to evaluate antibody preparations and identify appropriate conditions for performing an immunohistochemical study. As you will find out in your own investigations, some antibody preparations will yield very good results in a specific assay, whereas others may not work at all or will yield very high background (nonspecific) staining. Common sense and rigorous self-critique are probably two of the most valuable assets a scientist can possess when seeking to use immunohistochemistry for exploring various aspects of protein function.

2. Materials (*see* Note 3)

2.1. Buffers

1. Phosphate buffer stock (0.2 *M*): Add 21.87 g Na_2HPO_4 (anhydrous dibasic; *see* **Note 4**) slowly to 900 mL water while stirring rapidly. Once the dibasic sodium phosphate has dissolved, add 6.35 g $NaH_2PO_4 \cdot H_2O$ (monobasic). Stir until dissolved and add H_2O up to 1 L, pH 7.2–7.4. See **Note 5** for storage conditions.
2. Phosphate-buffered saline (PBS): 0.01 *M* phosphate with 0.9% NaCl. Add 9 g NaCl to 50 mL phosphate buffer stock (0.2 *M*). Add H_2O up to 1 L and stir until dissolved.
3. Tris-buffered saline (TBS): 0.1 *M* Tris with 1.4% NaCl. To 900 mL H_2O, add 13.2 g Trizma hydrochloride ($C_4H_{11}NO_3$·HCl) and 1.92 g Trizma base ($C_4H_{11}NO_3$). Bring up to 1 L with H_2O and stir until dissolved. Add concentrated HCl dropwise (should be 10–15 drops) until pH reaches 7.4.
4. Buffered sucrose solution: 30% sucrose in 0.1 *M* phosphate buffer, pH 7.4. To 500 mL of 0.2 *M* phosphate buffer stock (pH 7.4), add 300 g sucrose. Bring up to 1 L with H_2O and stir until dissolved. Store at 4°C.

2.2. Tissue Fixation (*see* Note 6)

1. Paraformaldehyde: 4% in 0.1 *M* phosphate buffer (*see* **Note 7**). Heat 400 mL H_2O to 50–60°C. Add 40 g paraformalehyde and 10 drops of NaOH (5.0 *M*). Stir until dissolved. Cool and add 500 mL phosphate buffer stock (0.2 *M*) and enough H_2O to bring up to 1 L. See **Note 8** for storage conditions.
2. Parabenzoquinone: 0.4% in H_2O. To 1 L of rapidly stirring H_2O, slowly add 4 g parabenzoquinone. Stir until completely dissolved (may take 10–20 min of continuous stirring). (*See* **Notes 7–9**.)

2.3. Tissue Histology and Immunohistochemistry

1. Dry ice.
2. Sodium azide stock (6%) (*see* **Note 10**): Add 6 g of NaN_3 to 100 mL H_2O and stir until dissolved.
3. Phosphate buffer + sodium azide: 0.1 *M* phosphate buffer + 0.06% sodium azide. Mix 500 mL phosphate buffer stock (**Subheading 2.1., item 1**), 10 mL sodium azide stock (6%), and 490 ml H_2O.

4. TBS + Triton X-100 (Tris-TX): TBS + 0.25% Triton X-100. To 200 mL TBS (**Subheading 2.1., item 3**), add 500 µL Triton X-100. Stir until dissolved. Store at 4°C.
5. Heat-inactivated goat serum (ΔGS) (*see* **Note 11**): To heat inactivate serum, the serum should first be allowed to thaw at room temperature and then placed into a 56°C water bath for 30 min. Serum should then be aliquoted into 3–5 mL volumes under sterile conditions and stored at –20°C.
6. Blocking solution: TBS + 5% ΔGS. For each 9.5 mL TBS (**Subheading 2.1., item 3**), add 500 µL ΔGS. Mix well before using (*see* **Note 12**).
7. Primary antibody (*see* **Note 13**).
8. Secondary antibody (*see* **Note 14**).
9. Avidin–biotin complex (ABC): recommend Vectastain Elite Kit (Vector Labs, Burlingame, CA).
10. Nickel chloride stock (8%): Mix 8 g $NiCl_2$ in 100 mL H_2O and stir until dissolved. Store at 4°C.
11. 30% sodium hydroxide (H_2O_2) solution: Purchased as a 30% solution. Stored at 4°C.
12. Diaminobenzidine (DAB) stock solution (100X) (*see* **Note 15**): Add 400 mg diaminobenzidine tetrahydrochloride to 10 mL H_2O and mix well until completely dissolved. Aliquot out into 100-µL volumes and store at –20°C for up to 1 yr.

2.4. Mounting and Dehydration of Sections

1. Gelatin subbing solution: Heat 200 mL H_2O to 30–40°C and add 2.5 g gelatin (100 bloom). Stir until dissolved and add 300 mL of room-temperature H_2O and 0.25 g of chromium potassium sulfate. Stir until dissolved and let cool to room temperature. (*See* **Note 16**.)
2. Various concentrations of ethanol, including 50%, 70%, 95% (two changes), 100% (two changes): It is more cost-effective to make the 50% and 70% solutions by diluting a 95% ethanol solution than from a 100% ethanol stock.
3. Xylenes (histological grade) or other clearing agents such as Hemo-De (Fisher Scientific, Pittsburgh, PA). Most clearing agents are toxic; handle with care.
4. Xylene-based mounting media such as Pro-Texx (Baxter Diagnostics, Deerfield, IL) or Eukitt (Electron Microscopy Sciences, Fort Washington, PA).

3. Methods

3.1. Fixation

3.1.1. Fixation by Vascular Perfusion

1. Induce a deep anesthesia in the animal and perform a surgical exposure of the heart and aorta.
2. Insert a perfusion cannula through the left ventricle into the aorta and clamp off the cannula to prevent backflow of the perfusion solutions.
3. Cut open the right atrium and begin perfusing the animal with ice-cold PBS (**Subheading 2.1., item 2**). (*See* **Notes 17** and **18**.)

4. Perfuse animal with 2% paraformaldehyde + 0.2% parabenzoquinone mixture (*see* **Notes 18** and **19**).
5. After an appropriate amount of fixative is perfused through the animal, the perfusion pump is stopped and the brain, or other tissues, are dissected.
6. Dissected tissues are then immersion fixed for an additional 2 h in 2% paraformaldehyde + 0.2% parabenzoquinone (*see* **Note 20**).
7. Remove tissues from the fixative, rinse in PBS, and immerse into 50 mL buffered sucrose solution (*see* **Subheading 2.1., item 4**). Allow tissue specimens to remain in buffered sucrose solution at 4°C for between 2 and 3 d (*see* **Note 21**).

3.1.2. Fixation by Immersion (see **Note 22***)*

1. Dissect out fresh tissue and block into pieces no more than 1 cm^3.
2. Drop tissue blocks into freshly prepared 2% paraformaldehyde + 0.2% parabenzoquinone (*see* **Note 19**).
3. Leave tissue blocks immersed in fixative, preferably on a rotating shaker set at moderate speed, for approx 1 h for each 2-mm tissue thickness (i.e., an 8 mm × 8 mm × 8 mm block would remain immersed for approx 4 h; a tissue piece that is 4 mm thick, 12 mm wide, and 14 mm tall would be immersion fixed for only 2 h because the thickness is 4 mm). For tissue culture preparations, add fixative to plated cells for 1–2 h. Remove the fixative solution, rinse cultures briefly with PBS two or three times, and store in phosphate buffer + sodium azide (**Subheading 2.3., item 3**) until beginning the immunohistochemical procedure.
4. Remove fixed tissue blocks from the 2% paraformaldehyde + 0.2% parabenzoquinone solution, rinse in PBS briefly, and cryoprotect in buffered sucrose solution (**Subheading 2.1., item 4**). No cryoprotection is required for tissue culture preparations.

3.2. Histology and Immunohistochemistry

1. Cut tissue sections (30–40 µm thickness) and store in phosphate buffer + sodium azide (**Subheading 2.3., item 3**). (*See* **Note 23**.)
2. Transfer freshly cut or previously stored tissue sections to a net-bottomed well, or other small-volume dish for carrying out immunohistochemical processing (*see* **Note 24**), containing TBS (**Subheading 2.1., item 3**). Sections should be rinsed in TBS for at least 10 min, but can be kept in TBS until all sections have been selected for the immunohistochemical run.
3. If nonspecific endogenous peroxidase activity is a problem, tissue sections can be incubated for 30 min in TBS containing 0.6% H_2O_2 (*see* **Note 25**). Although rat brain tissue does not tend to contain a great deal of endogenous peroxidase activity, including a peroxidase-quenching step is important if the animal was not perfused with PBS because erythrocytes in the blood contain peroxidase activity and will catalyze the DAB reaction. For primate tissue, a peroxidase-quenching step should be included.
4. Transfer sections to Tris-TX (**Subheading 2.3., item 4**) and incubate them on a rotary shaker for 20 min (*see* **Note 26**). Using a rotary shaker set on a slow to

moderate speed improves the circulation of tissue sections in the various solutions; however, shaking sections at higher speeds can result in extensive damage to the tissue, rendering it unusable for microscopic examination.

5. Transfer sections to blocking solution (**Subheading 2.3., item 6**) and incubate on a rotary shaker for 1 h.
6. Immediately before needed, dilute the primary antibody in Tris-TX containing 5% ΔGS (**Subheading 2.3., item 5**) (*see* **Note 27**). Transfer sections to primary antibody solution and allow to incubate for 48–72 h at 4°C (*see* **Note 28**).
7. Sections can be poured out of the microcentrifuge tubes into net-bottomed wells containing TBS (**Subheading 2.1., item 3**). Sections should be washed three times in TBS, for a minimum of 10 min each wash.
8. Sections are then incubated with the biotinylated secondary antibody diluted in a solution of Tris-TX containing 5% ΔGS for 3 h at room temperature (*see* **Notes 14** and **29**). For neurotrophin staining, a dilution of goat anti-rabbit secondary antibody (BA-1000, Vector Laboratories) of 1 : 1000 has been found optimal.
9. Rinse sections in TBS three times, for 10 min each rinse.
10. Incubate sections in ABC solution (*see* **Note 30**) for 90 min at room temperature. For neurotrophin staining, a dilution of ABC solution of 1 : 500 (each part A and B) has been found optimal.
11. Remove sections from the ABC solution and rinse three times in TBS. While sections are in their final TBS rinse, prepare the DAB solution as follows: For each 10 mL DAB working solution needed, add 100 µL DAB stock solution (**Subheading 2.3., item 12**) to 9.8 mL TBS. Stir and add 75 µL nickel chloride stock (**Subheading 2.3., item 10**) and 2 µL of 30% H_2O_2. Put sections into DAB working solution and incubate on a rotating shaker at a moderate speed. Incubate sections approx 10 min or until a desired staining intensity is reached (*see* **Note 31**).
12. Rinse section at least two times in 0.1 *M* phosphate buffer for 10 min each rinse.
13. Mount sections onto gel-subbed glass slides (**Subheading 2.4., item 1**) and allow to dry for several hours or overnight.
14. Dehydrate sections through a graded series of alcohols, rinse in an appropriate clearing agent, and cover slip with a xylene-based mounting media (**Subheading 2.4., item 4**). (*See* **Note 32**.)

4. Notes

1. A good example of the how a protein may be localized to different sites depending on the conditions under which the tissue was obtained comes from immunohistochemical studies of NGF in the adult rat CNS ***(24,37)***. Under normal conditions, NGF immunohistochemistry failed to detect NGF protein within cell populations producing NGF (as determined by *in situ* hybridization). However, after treating animals with colchicine, which disrupts microtubule-assisted transport, immunohistochemistry did reveal the presence of NGF protein in the soma of all cell populations known to produce NGF. These results were interpreted as indicating that NGF is normally made and transported away from the producer cell body at a rate that permits little NGF to accumulate within the soma. Follow-

ing colchicine treatment, the rate of NGF production is not altered, but the rate at which newly synthesized NGF protein is transported away from the cell dramatically decreases. The net result is an accumulation of NGF within the producer cell body at levels detectable by the immunohistochemical technique. Conversely, NGF staining normally found within basal forebrain cholinergic neurons, which are known to be NGF consumers, was reduced or absent following colchicine administration. These results were again taken to suggest that NGF protein found within basal forebrain cholinergic neurons, under normal conditions, is derived by retrograde axonal transport, and the rate of NGF transport and accumulation must exceed NGF degradation within the basal forebrain cell. Colchicine disrupts this balance by interfering with retrograde NGF accumulation while permitting the normal process of degradation to continue. Over time, NGF degradation exceeds NGF accumulation and the overall level of NGF within basal forebrain neurons eventually falls below the threshold of detection.

2. The extent to which an antibody may or may not crossreact with various potential antigens is highly dependent on the assay conditions under which crossreactivity is evaluated. An investigator should always seek to evaluate antibody crossreactivity in an assay, and under conditions that closely approximate the experimental conditions under which the antibody will be used ***(42)***.
3. All reagents may be obtained from Sigma (St. Louis, MO) unless otherwise noted.
4. May substitute 41.27 g $Na_2HPO_4 \cdot 7H_2O$ in lieu of anhydrous dibasic sodium phosphate.
5. All solutions may be stored at room temperature unless otherwise noted.
6. Parabenzoquinone and paraformaldehyde solutions give off toxic vapors and should be prepared in a hood or in a well-ventilated area. Animal perfusions should also be performed in a hood or well-ventilated area.
7. For each rat perfused, 150 mL paraformaldehyde and 150 mL parabenzoquinone solution will be required. For adult rhesus monkeys, approx 8 L of fixative are needed (4 L paraformaldehyde + 4 L parabenzoquinone); for adult mice, approx 100 mL fixative is required. For other species, the volumes of perfusate should be determined empirically, but may be estimated as 1 L of fixative per kilogram of body weight.
8. All fixative solutions should be freshly made on the day they are used and should be chilled to 4°C prior to using. Do not mix the two fixative solutions until immediately prior to perfusion, otherwise the parabenzoquinone will oxidize too rapidly.
9. Solid parabenzoquinone is light sensitive and should be stored desiccated, in the dark, at 4°C. Parabenzoquinone solutions will oxidize over time, turning the yellow solution to dark brown. Exposing the solution to bright light for extended periods of time will speed up the oxidation process. Parabenzoquinone solutions with a basic pH also tend to oxidize rapidly. If the parabenzoquinone solution turns dark brown as it is being made, chances are the solution was too basic. Before remixing a new parabenzoquinone solution, check the pH of the water; it should be neutral or slightly acidic. Even slight soap residues left on the glass-

ware will raise the pH and cause the solution to oxidize rapidly. Although some oxidation always occurs, turning the solution a little brownish, it is not advisable to use the parabenzoquinone solution if it has turned dark brown, as this often indicates the solution has been made improperly.

10. Sodium azide is very toxic and should be handled with caution. Also, because sodium azide can react with copper and lead to form an explosive, solutions containing sodium azide should not be discarded down the sink.
11. If alternate detection methods are used, employing secondary antibodies from a species other than goat, then the species of serum used throughout the procedure should be changed to match the species in which the *secondary* antibody was raised. A full range of serum products can be obtained from Gemini BioProducts Inc. (Calabasas, CA).
12. Mix up only as much blocking solution as you will need for a single immunohistochemical run. It is not recommended that you store leftover blocking solution because it will tend to grow bacteria unless kept under sterile conditions.
13. For our studies, we have consistently used affinity-purified polyclonal antibodies produced in rabbits, and the protocol described here will assume that a rabbit polyclonal primary antibody will be used. The production and characterization of primary antibodies (polyclonal and monoclonal) is beyond the scope of this chapter (however, *see* **refs. *43–45***. In general, it is recommended that a primary antibody be sought that has been thoroughly characterized and demonstrated to yield good results in immunohistochemical studies. Antibodies are often available from commercial sources or can be obtained from the investigators originally producing them. (In the latter case, the antibody may be more thoroughly characterized, but details of the characterization will have to be requested.) It should not be assumed a particular antibody preparation that works well in one type of an assay will work well in immunohistochemistry, nor should data pertaining to cross-reactivity obtained in one assay be taken to reflect antibody crossreactivity in other types of assays ***(42)***.
14. The secondary antibody can come from any species other than the one in which the primary was raised and should be different from the species in which the study is being conducted. For studies in rats, it is recommended that a biotinylated goat anti-rabbit (BA-1000, Vector Labs, Burlingame, CA) be used. For primate studies, a biotinylated donkey anti-rabbit (cat. no. 711-065-152, Jackson Immunoresearch, West Grove, PA) has typically been used.
15. Bulk powdered preparations of diaminobenzidine tetrahydrochloride (DAB; Sigma D-5637) have been found to yield the best results in neurotrophin-staining protocols and tend to generate the least amount of nonspecific background staining. DAB is a potential carcinogen and should be handled with extreme caution, using appropriate protective gear at all times. DAB and solutions containing DAB are light sensitive and should be kept in the dark.
16. Make only enough gel subbing solution for a single set of slides and discard after using. To sub slides, place slides in a rack and rinse for >1 h in running tap water.

Remove slides from the water and dip them into the subbing solution. Drain the slides on a paper towel and dry them overnight in a 37°C oven. On the following day, remove the slides from the oven, dip them into the gel subbing solution, and place them back in the drying oven overnight. On the next day, remove the slides from the drying oven, cool, and store covered so they do not collect dust. Subbed slides can be store indefinitely at room temperature.

17. Phosphate-buffered saline is used prior to perfusing with fixative to flush out blood because erythrocytes contain endogenous peroxidase activity that may interfere with the DAB–peroxidase reaction (*see* **Subheading 3.2., step 3**). Animals should be flushed with a volume of PBS at least as great as their blood volume but can be perfused with as much as 50–100 mL for an adult rat.
18. The perfusion pressure should be approximately equal to the normal blood pressure in the tissue to be fixed. For an adult rat, this typically means a perfusion rate of approx 25 mL/min. A peristaltic pump can be used to maintain a constant perfusion rate.
19. The 2% paraformaldehyde + 0.2% parabenzoquinone solution is prepared for each animal immediately before it is perfused by mixing an equal volume of 4% paraformaldehyde (*see* **Subheading 2.2., item 1**) and 0.4% parabenzoquinone (*see* **Subheading 2.2., item 2**). Once again, a total volume of 300 mL fixative is needed for each adult rat; 250 mL will be used for perfusing the animal and 50 mL will be used for postfixation of the tissue once it has been dissected.
20. The postfixation time is extremely critical and should not exceed 2 h. Our own studies with neurotrophin staining have indicated that antigens are rapidly lost with longer postfixation times. For instance, with NGF, postfixation for even 4 h causes a marked reduction in staining intensity and extended postfixation for 20 h or more causes a complete loss of staining!
21. Tissue is immersed in the buffered sucrose solution (**Subheading 2.1., item 4**) for cryoprotection purposes (to eliminate tissue damage upon freezing), so if the tissue will be cut on a vibratome at room temperature or 4°C, this step may be omitted. For larger tissue specimens, such as primate brains, it may be necessary to extend the time in the buffered sucrose solution. It is generally a good practice to leave specimens in the buffered sucrose solution until they sink. Cryoprotection of the tissue will take place faster if the specimens are placed on a shaker table while in the buffered sucrose solution. In general, tissue antigens will not be damaged if it is left in the buffered sucrose solution for longer periods of time, but if the tissue will not be cut the during the first week following the perfusion, it should be cryoprotected, removed from the sucrose solution, and stored at –70°C. When tissue is ready to be cut, it can be removed from the –70°C freezer and brought to an appropriate temperature for sectioning on either a cryostat or sliding microtome.
22. Fixation by vascular perfusion is definitely the preferred method of preparing the tissue for histology, because it generally produces the most consistent results and often is better at preserving tissue morphology. However, in cases where vascu-

lar perfusion is not possible, such as is the case with human tissue, embryonic tissue, and cell culture preparations, immersion fixation may be used.

23. Tissue sections are usually cut on a sliding microtome with the tissue frozen on dry ice. However, sections can also be cut on cryostat (frozen to approx –15°C) or a vibratome (with the tissue immersed in 0.1 *M* phosphate buffer at 4°C). Once again, it is beyond the scope of this article to discuss techniques for cutting tissue sections on the various instruments. Cut sections may be stored at 4°C in phosphate buffer + sodium azide (**Subheading 2.3.**, **item 3**) for several years without any appreciable loss of antigenicity, although care must be taken to prevent buffer from evaporating. One way to accomplish this is to store sections in an airtight humidified chamber, such as a sealed Tupperware container lined with moist paper towels.
24. Tissue prepared with the paraformaldehyde–parabenzoquinone fixative can be fragile and hard to process through an entire immunohistochemical protocol without damaging its integrity. One technique for minimizing tissue damage is to process sections in net-bottomed wells, which eliminate the need for transferring sections using a glass hook. Net-bottomed wells can be constructed from modified 50-mL conical tubes and nylon mesh (such as that used to strain house paint) or can be purchased from a commercial source (Costar, Cambridge, MA).
25. It is not recommended that other protocols be used for quenching endogenous peroxidase activity. For instance, diluting the hydrogen peroxide in methanol for use in peroxidase quenching has been shown to result in a complete loss of NGF staining.
26. The use of nonionic detergents, such as Triton X-100, in immunohistochemical processing is apparently necessary to permeabilize membranes and improve antibody penetration. This step is especially critical for antigens that are primarily localized to intracellular compartments. The omission of Triton X-100 from the preincubation step (**Subheading 3.2., step 4**) or from the primary antibody and secondary antibody solutions (*see* **Subheading 3.2., steps 6** and **8**) has been found to cause a dramatic reduction in neurotrophin-staining intensity. One drawback of using detergents for immunohistochemical processing is that they often result in extensive damage at the ultracellular level, making it difficult to carry out electron microscopy studies for neurotrophin antigens in the tissue.
27. The appropriate dilution for any given primary antibody must be determined empirically. Even if an antibody preparation has been used by other investigators, it is best to optimize its use under your own experimental conditions. If a concentration has been suggested in the product literature or by other investigators, it is best to try this concentration and then experiment with the antibody diluted twofold to fourfold more and twofold to fourfold less than what was originally suggested. If no prior knowledge of what an appropriate antibody concentration may be, it is best to try a full range of dilutions. This is best done by beginning with the antibody at a low dilution (1 : 20–1 : 100 for instance) and then titrating the antibody 1 : 1 with equal parts Tris-TX + 5% ΔGS until a final dilu-

tion of approximately 1 : 30,000 (i.e., 1 : 100, 1 : 200, 1 : 400, 1 : 800, 1 : 1600, etc.). This initial screening may seem difficult at first glance, but it will allow the investigator to find an antibody concentration that will yield optimal immunohistochemical results and minimize antibody use.

NOTE: To conserve primary antibody, which may be limited in availability or very costly, it is possible to incubate sections in very small volumes of primary antibody working solution. One method of doing this is to place sections in 1.5-mL microcentrifuge tubes containing between 300 and 1000 µL of working primary antibody solution. For a tube containing 750 µL of primary antibody working solution, 15–20 coronal rat brain sections can be incubated without incurring problems related to antibody penetration.

28. Shorter incubation times may be used if sections are incubated in a higher primary antibody concentration or at high temperatures (room temperature or 37°C); however, background staining may occur under these conditions. If incubation times longer than 3 d are used, 0.06% NaN_3 should be added to the primary antibody solution to prevent bacterial growth.
29. Sections can be incubated in secondary antibody in either the net-bottomed wells or in 1.5-mL microcentrifuge tubes. The choice of a secondary antibody (*see* **Note 11**) as well as a secondary antibody dilution range and incubation time should be determine empirically; however, it is generally not necessary to examine a broad of range of dilutions, as was done for the primary antibody. It is often acceptable to use the manufacturer's recommended dilution or to try the antibody diluted 2- to 10-fold more than what is suggested, especially if cost or nonspecific background staining are a problem.
30. As recommended by the manufacturer (Vector Labs), the ABC working solution should be made 30 min prior to using. Therefore, it is a good practice to make this solution before beginning the rinses in **Subheading 3.2., step 9**. Once again, it is best to determine the optimal ABC concentration empirically, beginning with the manufacturer's recommended dilution (1 : 100) and then further diluting out the ABC solution stepwise to a final concentration of 1 : 1000. Regardless of what final concentration is chosen, equal parts A and B should be used in preparing the working solution.
31. Incubation times in the DAB solution can range from as little as 30 s to 30 min. If the incubation time in the DAB solution is less than 3 min, the primary antibody should be further diluted. Unfortunately, the time in DAB must be determined empirically so that sections have the optimal amount of specific staining but do not containing nonspecific background staining. If longer incubation times are used (>10–15 min), sections should be placed in the dark because the DAB solution is light sensitive and will darken more rapidly with constant exposure to intense light.
32. A standard dehydration sequence would consist of passing slides containing mounted sections through the following series of solutions for the designated times: 0.1 *M* phosphate buffer for rehydration (3–5 min); H_2O (15 s); 50% EtOH (3 min); 70% EtOH (3 min); 95% EtOH #1 (3 min); 95% EtOH #2 (3 min); 100%

EtOH #1 (3 min); 100% EtOH #2 (5 min); clearing agent #1 (5 min); clearing agent #2 (5–10 min).

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2

Neurotrophin Immunohistochemistry in Peripheral Tissues

Robert A. Rush and Xin-Fu Zhou

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

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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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3

Extraction and Quantification of the Neurotrophins

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1. Introduction

Neurotrophins are a family of neuronal survival and differentiation factors ***(1,2)***. The mammalian neurotrophin family consists of four members sharing 50–55% amino acid identity: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and NT-4/5 ***(2,3)***. Significant interest surrounds the possible use of neurotrophins for the potential treatment of patients with various neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease, and for lesioned neurons after traumatic injury ***(4)***. However, successful therapy requires more knowledge of the physiology of the neurotrophins, and precise, reliable determinations of endogenous neurotrophin protein levels are essential for progress in this area.

Immunoassay has been used for quantitative measurement of neurotrophins for over three decades. However, this apparently simple task has proved elusive and requires continuous improvements. For example, early competitive one-site radioimmunoassays for NGF were shown later to be erroneous because of the risk of false-positive determinations, which resulted from the binding of receptors or other binding molecules to the exogenous radioactively labeled NGF ***(5–9)***. Although the competitive one-site assay was replaced later by two-site immunoassays ***(10–14)***, these assays run the risk of underestimation of the neurotrophins if they exist as binding molecule–protein complexes and are unable to be accessed by antibodies. For example, it has been reported that treatment of tissue extracts with an extreme pH increased the measurable amounts of NGF ***(15,16)***, BDNF ***(17)***, and transforming growth factor β ***(18)*** although different results have been found for NT-4/5 ***(19)***. It has been proposed that it is the association of the neurotrophins with their respective high-

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affinity receptors (e.g., trkA for NGF and trkB for BDNF) that is responsible for masking their interaction with antibodies in immunoassays ***(20–22)***.

In addition to the disturbance of binding proteins, there are many other issues that influence the results obtained in an immunoassay, resulting in variations of, for example, BDNF concentrations (in rat hippocampus: 5–256 ng/g wet weight) among laboratories ***(23–26)***. These include quality and concentrations of components/reagents such as antibodies, conjugates and substrates, buffer compositions and pH, assay methods and conditions, and wash methods. It is our experience that the quality and matching of the antibodies are the most critical of these variables. Some major scientific companies market immunoassay kits or reagents that produce large false-positive results. In this chapter, we provide researchers with detailed protocols and experiences for reliable extraction and ELISA (enzyme-linked immunosorbent assay) estimation of neurotrophins.

2. Materials

2.1. Neurotrophin Extraction

1. Homogenizer.
2. Bench-top centrifuge with a centrifugal force of 20,000*g*.
3. Homogenization buffer: 100 m*M* Tris-HCl, pH 7.0, 1 *M* NaCl, 4 m*M* EDTA, 2% bovine serum albumin (BSA), 2% Triton X-100, and 0.01% Thimerosal. Store at 4°C, 1–2 mo (*see* **Note 1**).
4. Protease inhibitors (Sigma): Antipain (0.5 µg/mL), aprotinin (5 µg/mL), benzamidine (157 µg/mL), pepstatin A (0.1 µg/mL), and phenylmethylsulfonyl fluoride (17 µg/mL) (*see* **Note 2**).
5. 1 *N* HCl.
6. 1 *N* NaOH.

2.2. Neurotrophin Quantification

1. A 96-well microplate (*see* **Note 3**).
2. Plate sealer such as Parafilm.
3. Pipets capable of accurately delivering volumes of 0.5–1000 µL.
4. Multichannel pipet.
5. Microplate shaker.
6. Microplate reader.
7. Wash bottle, or a large reservoir (e.g., 20-L bottle) connected to an eight-channel Immuno Autowash Nozzle (Nunc), or an automatic plate washer.
8. Wash buffer: 50 m*M* Tris-HCl (pH 7.0), 1 m*M* EDTA·Na_2, 0.5 *M* NaCl, and 0.1% Triton X-100. A 20X Tris containing EDTA stock solution may be prepared, which is then diluted and combined with salt and detergent for use.
9. Coating buffer: 25 m*M* sodium bicarbonate and 25 m*M* sodium carbonate. Adjust the pH to 9.6 using 1 *N* HCl or 1 *N* NaOH. The buffer can be stored at 4°C for at least 3 mo. (*See* **Note 4**.)

10. Blocking buffer: Coating buffer containing 1% BSA and 0.1% NaN_3 (*see* **Note 5**). The buffer can be stored at 4°C for 1–2 mo.
11. Standard diluent: Homogenization buffer, but containing 0.2% Triton X-100. The diluent can be stored at 4°C for 1–2 mo (*see* **Note 6**).
12. Conjugate diluent: Same as the standard diluent
13. Antibodies. Capture antibodies: polyclonal antibodies to neurotrophins produced from sheep (NGF, NT-3, and NT-4/5) or rabbit (BDNF) and purified on a protein G or protein A column, respectively; detector antibodies: monoclonal antibodies to neurotrophins raised from mouse, which have been purified and conjugated to peroxidase (HRP) (*see* **Note 7**).
14. Substrate: Tetramethylbenzidine (TMB) Peroxidase EIA Substrate Kit (Bio-Rad).
15. Stop solution: 1 *M* H_2PO_4 by combining 6.76 mL of 85% H_2PO_4 and deionized water to 100 mL. Carefully add acid to water.

3. Methods

3.1. Neurotrophin Extraction

1. Rats are anesthetized with an overdose of inhalational halothane. Tissues are rapidly dissected, weighed, and homogenized. Alternatively, tissue samples may be snap-frozen in liquid nitrogen and stored at –70°C for several months until use.
2. Add protease inhibitors in ice-cold homogenization buffer immediately prior to homogenization.
3. Combine an appropriate amount of the homogenization buffer with tissue sample. Recommended dilutions (g:mL):

Central nervous system (CNS) tissues	1:10–20
Peripheral nervous system (PNS) tissues	1:50–100
Peripheral organs	1:20–50

   (*See* **Note 8**.)
4. Homogenize the tissue.
5. Add 1 *N* HCl to pH 3–4. Check with pH paper (*see* **Note 9**).
6. Incubate for 15 min at room temperature.
7. Centrifuge at 20,000*g* for 30 min.
8. Collect the supernatant.
9. Add 1 *N* NaOH to neutralize the extract to pH 7 prior to assay. The supernatant may be snap-frozen in liquid nitrogen and stored at –70°C for at least 3 mo. Repeated freeze–thaw cycles should be avoided.

3.2. Neurotrophin Quantification

3.2.1. Sandwich ELISA Principle

The ELISA technique is based on the ability of an antibody to bind to its antigen. First, a primary capture antibody specific to the object molecule is bound to the microplate surface to create the "solid phase." Samples and standards are then incubated with the solid-phase antibody, which "captures" the

antigen of interest. The solid-phase bound molecule is then incubated with a secondary detector antibody that has been conjugated to enzymes such as HRP. This antibody binds to a different epitope of the molecule being measured, completing the antibody (Ab)–antigen (Ag)–Ab "sandwich." Alternate forms of the assay use three antibodies (*see* **Note 7**).

The remainder of the assay involves a detection system. After washing, the amount of specifically bound antibody–HRP is then detected by a color reaction with a chromogenic substrate. The color change is measured using a microplate reader. The amount of antigen in the samples is proportional to the color generated in the reaction. Results are calculated in reference to the standard curve.

3.2.2. ELISA Protocol

We present a detailed protocol for the estimation of NT-4/5. This protocol is applicable to all other neurotrophins. The assay has been optimized to ensure the highest sensitivity. It might be modified for a particular purpose (e.g., shorten the incubation time to speed an assay).

Day 1

1. Dilute the capture antibody to a concentration of 2 µg/mL in coating buffer (*see* **Note 10**).
2. Coat a 96-well polyvinyl Costar plate by adding 100 µL of the capture antibody per well. Leave the first column empty as the "blank," which will be filled with substrate and stop solution only.
3. Seal and incubate at 4°C overnight on a shaker.

Day 2

4. Empty the plate and wash it three times with wash buffer (*see* **Note 11**).
5. Block the plate by adding 150 µL of blocking buffer per well (*see* **Note 12**).
6. Seal and incubate at room temperature for 1 h.
7. Prepare duplicate standards during the incubation. Concentrations: 0, 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 pg/mL. Accurately dilute stock standard solution in sample buffer to achieve a concentration of 500 pg/mL. Make a total volume of 450 µL.
8. Wash as in **step 4**.
9. Add 200 µL of the standard (500 pg/mL) to the top two wells in the two columns designated for the standard curve, and 100 µL sample buffer to other wells in both columns. Add 100 µL sample buffer to three wells designated as "spiked buffer" for calculating the recovery.
10. Using a multichannel pipet, immediately perform serial twofold dilutions from the top two wells down the plate in columns designated for the standard curve until 7.81 pg/mL is reached (*see* **Note 13**).
11. Add 100 µL of each sample to four wells, of which two are used as the "spiked sample" for estimating the recovery (*see* **Note 14**).

12. Add 10 pg (approx 2 µL) of NT-4/5 in the wells of "spiked buffer" and the "spiked sample" for estimating the recovery (*see* **Note 15**).
13. Seal and incubate at 4°C overnight on a shaker.

Day 3

14. Wash five times as in **step 4**.
15. Add 100 µL of diluted monoclonal antibody–HRP (2 µg/mL) per well (*see* **Note 10**).
16. Seal and incubate at room temperature for 6 h.
17. Wash five times as in **step 4**.
18. While washing, prepare the substrate according to the manufacturer's instructions. For the TMB substrate, immediately before use, combine nine parts TMB solution A with 1 part solution B; mix gently and thoroughly. Keep the solution protected from light.
19. Add 100 µL/well (including the "blank") of substrate.
20. Incubate at room temperature on a shaker for 8–15 min (*see* **Note 16**).
21. Adding 100 µL/well (including "blank") of 1 *M* H_2PO_4 to stop the reaction.
22. Read the plate at 450 nm to obtain optical density (OD) values.

3.2.3. Calculations

Subtract the background value (OD of the zero standard) from all values and perform calculations as follows:

1. Manual plotting. Plot the standard curve on linear–linear axes. Known concentrations of the standard (e.g., NT-4/5) are plotted on the x-axis and the corresponding OD on the y-axis (**Fig. 1**). The straight line of the standard curve shows a direct relationship between NT-4/5 concentrations and the corresponding ODs. The concentration of NT-4/5 in unknown samples may be determined by plotting the sample OD on the *y*-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the *x*-axis at the concentration of NT-4/5 in the unknown sample. If ODs of unknown samples do not fall into the linear range of the standard curve, dilute and reassay these samples. Do not forget to include dilution factors when calculating results.
2. Plate reader software. Plate reader software or other software allows use of various curve-fitting programs. The data may best fit a linear regression, semilog, log/log, or 4-parameter.
3. Correction for the recovery. Concentrations of neurotrophins estimated from unknown samples should be corrected for the recovery. Recovery is calculated as follows:
   i. Calculate the difference between ODs of unknown samples and their respective "spiked sample."
   ii. Calculate the ratio of the OD from **step i** to the OD of the "spiked buffer" (*see* **Note 17**).

   If the concentration from an unknown sample is 7 ng/g wet weight and the recovery is 0.7, then the corrected concentration is 7/0.7=10 ng/g wet weight.

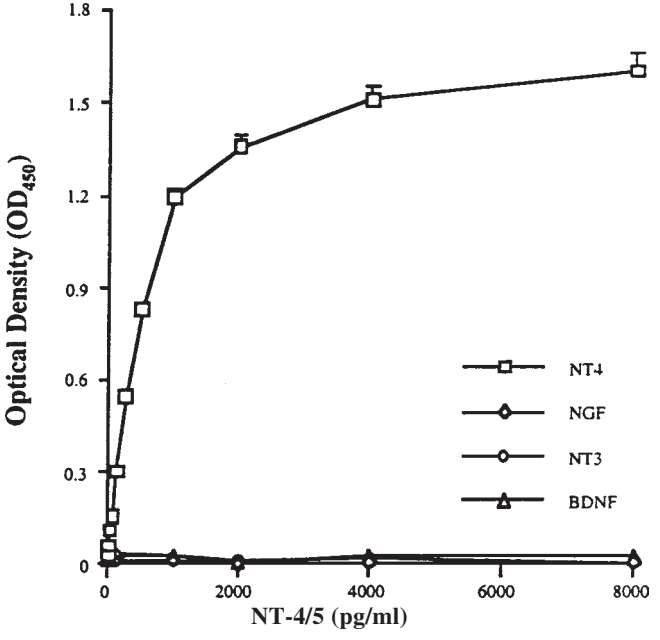

Fig. 1. Standard curve of the two-site immunoassay for NT-4/5. The assay system is highly specific for NT-4/5 with a sensitivity of 1 pg/mL. When the closely related neurotrophins NGF, BDNF, and NT-3 were substituted for NT-4/5 standards, no crossreactivity was detected. Data are mean ± SD values from seven independent assays with interassay and intraassay variation coefficients of 6.3% (n = 8) and 3.0% (n = 6), respectively.

3.2.4. Troubleshooting

Problems	Possible causes	Comments
No signal at all	Reagents incorrectly prepared, missing, or added in an incorrect order	Repeat assay. Check reagents and their calculations. Follow assay protocol.
Poor replicate	Insufficient washing	*See* **Note 11**.
	Technique problems	Mix reagents thoroughly. Add stop solution to wells in the same order as substrate.
	Poor plate quality	*See* **Note 3**.
Poor reproducibility run in assays	Variations in protocol	Maintain the same protocol from to run.
	Poor replicate	As stated above.

Problems	Possible causes	Comments
High background	Insufficient blocking	0.5–1% BSA in coating buffer or PBS is commonly used for blocking. If BSA interferes with the assay, casein or gelatin could be used.
	Insufficient washing	*See* **Note 11**.
	Too high concentrations of reagents such as antibodies or conjugates	*See* **Note 10**.
	Cross reactivity of antibodies/ conjugates	Use affinity-purified antibodies. Choose absorbed conjugates. Increase ionic strength in buffers, which helps to eliminate the "crossreacted," weakly bound, molecules.
	Buffer: pH, ionic strength, composition	For primary antibody, the pH value should be one to two units higher than the isoelectric point (p*I*) value of the antibody molecule, the ionic strength ought to be low, and the composition be as simple as possible. For other incubations, buffers should have a neutral pH and a low to medium high ionic strength.
	Color reaction time too long	Shorten the time.
Poor sensitivity	High background	As stated above.
	Insufficient incubation	Overnight coating is recommended in order to achieve a successful immobilization of the primary antibody. In all other steps, incubation time must not be too short.
	Too low concentrations of reagents	*See* **Note 10**.

4. Notes

1. Other commonly used buffers are phosphate-buffered saline or borate buffer. The correctly designed buffers should include factors that make an object molecule stable and that could possibly eliminate the chance of having an elevated background or even false-positive results (*see* **Note 6**).

2. Protease Inhibitor Cocktail Tablets (Boehringer Mannheim) are commercially available. Follow manufacturer's instructions for use.
3. Do not use tissue/cell culture plates. For the best well-to-well accuracy and low background, a high-quality, name brand immunoassay plate is recommended. We are satisfied with polyvinyl Costar plates. The binding efficiency of plates from the same manufacturer may vary from lot to lot. We recommend to test plates from several batches and then purchase a large quantity with a single lot number.
4. Carbonate buffer of 25–50 m*M*, pH 9.6, is a generally accepted coating buffer. If an optimization of a buffer for each coating molecule is desired, there is a rule of thumb stating that the pH of the buffer be one to two units higher than the p*I* of the molecule and that the ionic strength of the buffer not be high.
5. If BSA interferes with assay or is not sufficient for blocking the free spaces, which can be determined by an elevated background, other molecules such as casein, gelatin, or appropriate detergent could be used.
6. Buffers used for preparing standards should be the same as those used for the preparation of the sample in order to control the nonspecific effects. For example, if extracts of tissue samples are to be assayed, the extraction buffer should be used to prepare the curve. If running cell culture supernatants, prepare the standard curve in cell culture medium that has not been exposed to cells. Also, it would be beneficial to include in the buffer the molecules that have been used previously on the solid surface (e.g., the blocking molecules) except those molecules that are expected to bind.
7. Quality and matching of antibodies are critical to a successful immunoassay. Some major scientific companies market immunoassay kits or reagents that produce an enormous false-positive reaction with tissue samples. To check the specificity of the ELISA system, we recommend including negative control wells by replacing the primary capture antibody with appropriate molecules such as normal Ig in the Ab–Ag–Ab–*E* (enzyme) assay system (*see* **Subheading 3.2.1.**); and in the case of Ab–Ag–Ab–anti-species Ig–*E* system, replacing the secondary detector antibody with dilution buffer. Also, it is necessary to screen prospective conjugates from various manufacturers in order to choose one that is specific in the assay. Chemicon Int. (Temecula, CA) has produced neurotrophin ELISA kits in an Ab–Ag–Ab–*E* format, whereas Promega International (Madison, WI) markets the kits in an Ab–Ag–Ab–anti-species IgG–*E* format.
8. Dilution factors should be adjusted according to the level of the neurotrophin in a particular tissue. A pilot experiment would help to determine the dilution factor.
9. The effect of acid extraction is different for each neurotrophin and dependent on tissue type. Acid extraction may result in an increased measurable amount of one neurotrophin such as NGF ***(15,16)***, but a decreased amount or false-positive absorbance reading of another such as NT-4/5 in some rat tissues ***(19)***. Therefore, we recommend a pilot experiment to find out whether or not acid extraction is needed, and, if needed, to determine the optimal pH and incubation time for a particular tissue.

10. The best way to determine the optimal concentrations of both primary/capture and secondary/detector antibodies is to perform a "grid" titration. To form the grid, divide a 96-well plate into two halves. Use a constant concentration of the antigen on one (e.g., left) half and nonantigen on the other (right) half. In an identical fashion on both halves of the plate, titrate the primary antibody down the plate and the secondary antibody across the plate. The left half gives a "signal" resulting from each of the many antibody pair concentrations, and the right half gives the respective "noise" or background value. Choose the paired concentrations that produce the highest signal-to-noise ratio with an acceptable background. Antibodies should be aliquoted, and can be stored at 4°C for 1–2 mo if 0.1% NaN_3 is added, or at –70°C for several years. Repeated freeze–thaw cycles should be avoided.
11. Sufficient washing is one of the most important aspects of running a successful ELISA. If using a wash bottle or Immuno Autowash Nozzle, it should have a good pressure. If using an automatic plate washer, all ports must be clean and free of obstructions. After the final wash, blot using paper towels, and "bang" the plate onto the towels to ensure that all liquid has been removed. When no liquid remains, proceed to the next step.
12. Take care not to touch or scratch the bottom or sides of the wells with the pipet tips. All solutions should be allowed to come to room temperature prior to use. After blocking, the plate can be inverted and allowed to air-dry at room temperature, wrapped in plastic, and stored at 2–8°C for a maximum of 1 wk. Coated plates with a guaranteed shelf life of 1 yr are available commercially (Chemicon Int., Temecula, CA).
13. Mix thoroughly in each well during dilution but avoid creating bubbles.
14. For each tissue sample, several dilutions should be assayed to ensure a parallel relationship with the linear part of the standard curve.
15. The amount of exogenous neurotrophin added should be small, but sufficient for easy detection. The volume added should be less than 5 µL, in order not to increase significantly the total volume in the spiked wells.
16. Observe the color development in wells. Pay particular attention to the color formation in the wells used for the standard curve. Alternatively, read the plate at 655 nm to monitor the color change.
17. Alternatively, a recovery experiment may be performed by mixing exogenous neurotrophins (*see* **Note 15**) with an aliquot of homogenate before acid extraction or centrifugation. In this case, recovery should be caculated as the ratio of A (the difference between the amount measured in unknown sample and the sample mixed with the exogenous) to B (the amount added).

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4

Identifying Novel Proteins in Nervous Tissue Using Microsequencing Techniques

Selena E. Bartlett

1. Introduction

Identifying signaling molecules that participate in pathways leading from receptor activation to changes in gene expression in cells is the fundamental focus of many research endeavors. For example, neurotrophins interacting with their trk receptors mediate a number of cellular effects, including cell differentiation, neurite outgrowth, and neuronal survival ***(1–4)***. This results from specific protein–protein interactions in different signaling pathways. The primary aim of many research efforts is to elucidate these signaling pathways by identifying the proteins that participate in them. The most common method for isolating protein–protein interactions is by coimmunoprecipitation of protein complexes from cell lysates, where an appropriate antibody is available. Isolated proteins are separated using polyacrylamide gel electrophoresis and then transferred from the gel to a membrane support and identified using immunoblotting. These techniques have been well documented (*see* **refs. *5–8***). The major drawback of this technique is its inability to find novel proteins. Currently, the best technique available for identifying novel proteins is by chemical or enzymatic fragmentation of isolated proteins in a gel matrix following one- or two-dimensional electrophoresis or after electrotransfer onto a suitable membrane ***(9,10)***. In most cases, reverse-phase high-performance liquid chromatography (RP-HPCL) is used to separate the cleavage fragments. This is achieved by ultraviolet (UV) absorbance at a wavelength that detects the peptide bond. The HPLC fractions containing the separated peptides are then automatically collected for both mass spectrometric analysis and N-terminal peptide sequencing. Peptide masses obtained are entered into a protein data-

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base. These masses are then compared with accurate masses of specific cleavage fragments of proteins already in the database, and a protein is predicted ***(11)***. As many genes are now being sequenced before their respective gene products are discovered, mass spectroscopy enables protein identification with greater sensitivity than current protein/peptide sequenators provide. If there are no corresponding peptide masses in the database, suggesting it is a novel protein, N-terminal peptide sequencing is then used to identify it.

2. Materials

2.1. Equipment

1. Standard gel electrophoresis apparatus (Hoeffer Scientific Instruments, AMRAD Pharmacia Biotech, Boronia, Victoria, Australia).
2. SMART HPLC system to separate the cleavage fragments obtained after enzymatic digestion of proteins and a Sephasil C18 SC 2.1/10 reverse-phase column (AMRAD Pharmacia Biotech, Boronia, Victoria, Australia).
3. Procise 494 Sequenator (Applied Biosystems Division, Perkin Elmer Corporation, Foster City, CA, USA) for N-terminal peptide sequencing. If this equipment is not available, the protein may be sent, in the gel, to a facility equipped to identify it.
4. VG TofSpec mass spectrometer fitted with a 337-nm nitrogen laser (Fisons Instruments, NSW, Australia). Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry is an excellent instrument for mass analysis; however, it is also very expensive. It is worthwhile going to a multiuser facility that houses this piece of equipment. However, this equipment is not essential for identifying proteins.

2.2. Reagents

1. Sample buffer: 10% glycerol, 3% sodium dodecyl sulfate (SDS), 2.5% dithiothreitol (DTT), and 80 m*M* Tris-HCl, pH 6.9, made on the day of the experiment.
2. Resolving acrylamide gel solution 9%, the method of **ref. *12***:

Tris-HCl, pH 8.8	6.25 mL
Acrylamide solution:	
Acrylamide 40%	
bis-Acrylamide 1.5%	5.626 mL
SDS (10%)	0.25 mL
Water	12.75 mL
Ammonium persulfate (APS) (10%)	125 µL
N,*N*,*N*′,*N*′-tetramethylethylene-diamine (TEMED)	6.5 µL

3. Stacking acrylamide gel solution (5%), the method of **ref. *12***:

Tris HCl, pH 6.8	4 mL
Acrylamide solution	2 mL
SDS (10%)	80 µL
Water	9.6 mL
APS (10%)	160 µL
TEMED	16 µL

4. Coomassie blue solution: 0.2% Coomassie blue R-250 in 50% methanol and 10% acetic acid, stored at room temperature.
5. Coomassie destaining solution: 30% methanol, stored at room temperature.
6. Desilvering solution: copper-based oxidizing solution, $copper^{II}$ tetraammonium chloride solution, 32 m*M* NaCl, 8 m*M* $CuSO_4$; add NH_4OH until the solution is saturated.
7. AMBIC solution 1: 0.2 *M* ammonium bicarbonate (AMBIC) and 50% acetonitrile, pH 8.9, stored at room temperature.
8. AMBIC solution 2: 0.2 *M* ammonium bicarbonate, stored at room temperature.
9. TFA solution: 10% trifluoroacetic acid (TFA), stored at room temperature.
10. Peptide extraction solution: 0.1% TFA, 60% acetonitrile, stored at room temperature.
11. Solvent A: 0.09% TFA in deionized, degassed MilliQ water.
12. Solvent B: 0.09% TFA in deionized, degassed 70% acetonitrile/ MilliQ water.
13. Standards for mass spectrometry: 3 pmol of bovine insulin (MH^+ 5734.5) and 15 pmol of Gramicidin S (MH^+ 1142.5) made up in MilliQ water and stored at 4°C.
14. Polybrene solution: 50 mg/mL in 50% methanol, stored at 4°C.

3. Methods

3.1. Sample Preparation

Proteins are isolated in gels using either one- or two-dimensional gel electrophoresis and then visualized using either Coomassie blue dye or a silver-stain technique. The main limiting factor is the quantity of protein available. This has been overcome, in some instances, by using silver stain, which is more sensitive than Coomassie blue dye to visualize the protein, then combining the silver-stained protein gel pieces, and using a concentration gel apparatus to concentrate the protein.

3.2. Protein Visualization

Proteins are visualized using either Coomassie blue or a silver-stain technique. The lower limits of detection for these stains depend on the protein itself. Rules of thumb are, for Coomassie blue protein detection, approx 100 ng of a 40-kDa protein, and for silver-stain protein detection, approx 1–10 ng of a 40-kDa protein.

3.2.1. Coomassie Stain

1. After electrophoresis, place the gel in 50 mL of Coomassie blue solution for 30 min.
2. Place the gel in 20 mL of Coomassie destaining solution, swirl the solution over the gel, and remove the solution.
3. Add 50 mL of Coomassie destaining solution and leave the gel in this solution until the protein bands of interest appear.
4. Remove the destaining solution and leave the gel in deionized water for 24 h. The gel may be kept in a sealed bag at 4°C.

3.2.2. Silver Stain

Silver stain is one of the most sensitive methods for visualizing proteins in polyacrylamide gels and is up to 100 times more sensitive than Coomassie blue stain ***(13,14)***. It was thought that micropurification and N-terminal sequencing techniques would not be possible following silver staining. However, it has been shown that it is possible to identify silver-stained proteins using conventional micropurification and N-terminal sequencing techniques by desilvering the protein after visualization.

1. Fix the gel using a 20% trichloroacetic acid solution for 30 min. After each step in this procedure, it is important to wash the gel thoroughly with double-distilled water.
2. Remove excess SDS by washing the gel twice in 100 mL of 10% ethanol and 5% acetic acid for 15 min.
3. Incubate the gel with 100 mL of 3.4 m*M* potassium dichromate and 3.2 m*M* nitric acid for 15 min.
4. Place the gel in 12 m*M* silver nitrate solution for 20 min.
5. Develop the gel using 100 mL of 0.28 *M* sodium carbonate solution containing 100 µL of formaldehyde.
6. Stop the developing by adding a 3% acetic acid solution.
7. After the proteins are visualized, the protein band of interest is excised and then soaked in deionized water.
8. The bands are then desilvered using the copper-based oxidizing solution, copper^{II} tetraammonium chloride.
9. The gel pieces are then combined and concentrated using concentration gel electrophoresis.

3.3. Concentration Gel Electrophoresis

1. The gel pieces are soaked in deionized water (200 µL) for 30 min and then the water is removed.
2. Sample buffer (50 µL) is added and the mixture boiled for 4 min at 100°C.
3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is performed using a 9% resolving gel below a 5% stacking gel with a central well that had the dimensions 2 cm × 1 cm × 1.5 mm, as described by in detail in **ref. *12***.

4. Gel pieces are placed in the central well, and the gel is run at 150 V until the sample buffer has begun to run into a stacking gel. The central spacer is placed in between the two other spaces and the voltage is increased to 250 V until the solvent front outside the central well reaches the end of the vertical spacers. Then, the central spacer is removed, and the gel run until the sample buffer front is running about halfway down the resolving gel.
5. After electrophoresis, the resolving gel is stained with Coomassie blue solution and then destained until the protein band of interest appears using the Coomassie destaining solution.

3.4. Protein Identification

3.4.1. In-Gel Protein Digestion and Peptide Extraction

The following procedures are adapted from methods described in **refs. *9*** and ***10***:

1. Remove the Coomassie-stained protein spot obtained either after one- or two-dimensional gel electrophoresis or after concentration gel electrophoresis from the polyacrylamide gels using a scalpel blade.
2. Wash the gel piece with AMBIC solution 1 (400 µL) and incubate for 60 min at 37°C with mixing during this time.
3. Completely dry the gel piece under a stream of N_2.
4. Rehydrate the gel piece for approx 30 min in 5 µL solution containing 0.5 µg of modified trypsin (Promega) in AMBIC solution 2. It is important at this step that the gel piece absorbs the enzyme after the solution is added to it. (*See* **Note 1**.)
5. After the gel piece has taken up the trypsin, slowly rehydrate the gel piece by adding small aliquots (5–10 µL) of AMBIC solution 2, until the gel piece is completely rehydrated.
6. Cut the gel pieces into smaller pieces and incubate overnight in 100 µL of AMBIC solution 2 at 37°C.
7. Add TFA solution (10 µL) to stop the reaction. The supernatant is removed and placed into a clean microfuge tube. The peptide fragments are extracted by adding 100 µL of the peptide extraction solution to the original mixture, which is then incubated at 37°C for 40 min, the supernatant is removed; this step is repeated. Then the combined supernatants are concentrated by rotary evaporation in a spinvac to approx 100 µL.

3.4.2. Chromatographic Separation of the Peptide Fragments Using RP-HPLC

The SMART HPLC system also comes with detailed instructions on its use.

1. Separate the tryptic cleavage fragments using a Sephasil C_{18}-bonded stationary-phase SC 2.1/10 column.
2. Run a 5-mL gradient of 0–40% acetonitrile in 0.065–0.05% TFA at a flow rate of 100 µL/min using solvents A and B. A column with a diameter of < 2 mm and

eluent rates of < 200 µL/min ensures that the peptides are detected in amounts of the order of 10 pmol.
3. After the column has been equilibrated, inject 100 µL of the tryptic fragment solution into the loop of the injector, which is then automatically loaded onto the column. Peptide elution is followed by UV absorbance at three different wavelengths, 215 nm, 280 nm, and 595 nm, for Coomassie-related compounds.
4. Automatically collect fractions into 0.5-mL microfuge tubes. Take a small sample, 1 µL, and prepare for mass analysis. (*See* **Note 2**.)
5. Add polyvinylidene difluoride (PVDF) membrane to fractions that appear to contain one peptide, determined either from the chromatogram or after mass spectroscopy.

3.4.3. Matrix-Assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) is performed using a VG TofSpec mass spectrometer. All spectra are recorded with the analyzer in linear mode at an accelerating voltage of 20 kV, using a 15-well sample slide as the probe.

1. Add 1 µL of the fraction collected from the HPLC to 1 µL of the premade saturated matrix solution containing α-cyano-4-hydroxycinnamic acid in 40% acetonitrile and 0.09% TFA to one well of the sample slide.
2. Then, add to one well of each sample slide, 1 µL of the standards for mass spectrometry, outlined above, and 1 µL of the premade saturated matrix solution, this is to externally calibrate the spectra.
3. Air-dry the sample slide until all the solutions have evaporated.
4. Place the sample slide into the mass spectrometer for mass analysis.
5. Calibrate the instrument externally using the standards prepared on one well of the sample slide.
6. As each instrument is different, refer to the manual for detailed instructions on its use.
7. After obtaining the peptide masses, enter the peptide masses into a mass searching program, which is accessed through the Internet at the World Wide Web site http://cbrg.inf.ethz.ch/ ***(15)***. The results will be returned by e-mail and will list the potential proteins from which these peptides were generated.

3.4.4. N-Terminal Sequencing

1. After mass analysis and if the HPLC solution contains one peptide, add PVDF membranes (10 mm^2) to the remaining HPLC fraction, to passively adsorb the peptide fragment for 24–48 h at room temperature.
2. After 24–48 h, remove the remaining solution.
3. Treat the remaining PVDF membrane with the polybrene solution prior to sequencing. (*See* **Note 3**.)

4. The sequence of the peptides is then determined by N-terminal sequencing using a Procise 494 Instrument or by sending the appropriate peptides to a facility for sequencing.

4. Notes

1. When rehydrating the polyacrylamide gel pieces, ensure that the modified trypsin is added first in the minimum volume to ensure that is taken up into the gel.
2. When separating the cleavage fragments using reverse-phase HPLC, keep all fractions, as some may contain peptides that are not visible in the chromatogram.
3. Polybrene treat the PVDF membranes added to the fractions, to ensure that the peptides remain adsorbed to these membranes during peptide sequencing.

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5

Quantification of mRNA Levels Using Northern Blotting

Paul Fernyhough

1. Introduction

Quantification of mRNA levels can be performed using polymerase chain reaction (PCR)-based techniques (e.g., competitive reverse transcription PCR; RT-PCR), RNase protection, or Northern blotting. Northern blotting is less sensitive than the other techniques; however, the methodology is less complex and quantification, certainly compared with RT-PCR, is more straightforward. Aliquots of total RNA or mRNA are separated according to size in an agarose gel; the separated species are then transferred to a filter where the RNA transcripts are immobilized. The filter is then probed with a labeled single-stranded DNA probe specific for a certain mRNA species. The immobilized RNA species on the filter maintain an ability to anneal to single-stranded DNA to form stable heteroduplexes or hybrids. The resulting hybrids are detected using a technique related to the specific type of label attached to the DNA probe. There are now several approaches available for labeling DNA probes and detecting resulting hybrids. The use of ^{32}P as a label will be described in this chapter. There are other procedures for labeling of DNA that involve the use of other detectable compounds (e.g., fluorochromes, colorimetric, and antibody-based techniques). Although these procedures are beneficial in that they circumvent the need to use radioactivity, this author strongly recommends the use of ^{32}P to workers new in the field because of its ease of detection, superior sensitivity, and greater capacity for accurate quantification (the result of phosphorimaging devices now being available).

The single most important facet to be considered when using Northern blotting to quantify mRNA expression is that all data derived from the blot will be

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dependent on the levels of total RNA immobilized on the blot, and, therefore, equal loading of amounts of total RNA samples is paramount and some element of internal standardization is extremely important. Because Northern blots can be reprobed sequentially with up to eight different DNA probes, it is important to plan a strategy that includes probing with at least one DNA probe that detects a housekeeping gene or a gene that is constituitively expressed and relatively resistant to experimentally induced variation. Detecting signals for such "control" mRNA expression allows for variations in RNA loading on the gel to be adjusted for. Typically used "control" DNA probes include probes for the genes coding for actin, glyceraldehyde-3-phosphate dehydrogenase, and histone 3.3 ***(1)*** (a probe specific for the pseudogene product of histone constituitively expressed in all cells).

Finally, all procedures described herein relate to the use of total RNA as the source of transcripts to be analyzed. It is feasible to prepare mRNA from total RNA using a poly-oligo d(T) column and to subject this enriched population of transcripts to Northern blotting. However, the purification of this mRNA population is very difficult to perform in a quantitative manner and, consequently, loading equal amounts of mRNA samples onto an agarose gel is fraught with difficulties.

2. Materials

All chemicals should be of molecular biology grade.

2.1. RNA Isolation

1. Guanidine thiocyanate (GTC) solution: 4.215 *M* guanidine thiocyanate, 25 m*M* sodium citrate, pH 7.0, 0.5% *N*-lauroyl sarcosine, 0.1 *M* β-mercaptoethanol. Filter through 45 μ*M* mesh and store at 4°C for 1 mo. **PRECAUTIONS:** Guanidine thiocyanate and β-mercaptoethanol are highly toxic and should be handled with gloves and while wearing a mask.
2. Guanidine hydrochloride (GHCl) solution: 6 *M* guanidine hydrochloride, 0.1 *M* sodium acetate, pH 5, 5 m*M* dithiothreitol (DTT). Filter and store at 4°C for 1 mo). **PRECAUTIONS:** Guanidine hydrochloride is highly toxic and should be handled wearing gloves.
3. Sodium acetate solution: Add 18 mL of 2 *M* sodium acetate to 82 mL of 2 *M* acetic acid; prepare at pH 4 and pH 5. Autoclave and store indefinitely at 4°C.
4. Phenol solution: Purchase Tris-buffered phenol solution. Store at 4°C for 1 mo. (*See* **Note 1**.) **PRECAUTIONS:** Highly toxic, wear gloves.
5. RNase-free water: Diethylpyrocarbonate (DEPC)-treated or ultrapure water should be used, autoclaved and stored in dark at 4°C for up to 1 mo. (*See* **Note 2**.)

2.2. Northern Gel and Transfer

1. Running buffer (10X): 200 m*M* **3**-*N*-(morpholino)propanesulfuric acid (MOPS), pH 7.0, 50 m*M* sodium acetate, 10 m*M* EDTA. Prepare fresh.

2. RNA sample solution: 50% formamide (*see* **Note 3**), 2.2 *M* formaldehyde, 1X running buffer, RNA (up to 40 µg), water to 20 µL. Combine just prior to running gel. **PRECAUTIONS:** Formaldehyde and formamide are toxic.
3. Bromophenol blue buffer (10X): 50% glycerol, 1.0 m*M* EDTA, pH 8.0, 0.25% bromophenol blue. Store at 4°C indefinitely.
4. Ethidium bromide solution: 0.5 µg/mL ethidium bromide in 0.1 *M* ammonium acetate made up in 500 mL double-distilled water. **PRECAUTIONS:** Ethidium bromide is highly carcinogenic. Wear gloves and a mask.
5. Denaturation solution: 50 m*M* NaOH, 150 m*M* NaCl (500 mL volume). Use fresh.
6. Neutralization solution: 0.1 *M* Tris-HCl, pH 7.5, 150 m*M* NaCl (500 mL volume). Use fresh.
7. 20X SSC: 1.5 *M* NaCl, 150 m*M* sodium citrate, pH 7.0. Autoclave and store at room temperature for 1 mo.

2.3. Labeling of DNA: Random Primer Labeling

1. 5X Transcription buffer: 250 m*M* Tris-HCl, pH 8.0, 25 m*M* $MgCl_2$, 10 m*M* DTT, 1.0 *M* HEPES, pH 6.6, and 26 A_{260} units/mL random hexadeoxyribonucleotides.
2. Random primer reaction: 1X transcription buffer, 20 µ*M* each dNTP, 25–50 ng template DNA, 400 µg/mL nuclease-free bovine serum albumin (BSA), 333 n*M* [α-^{32}P] dCTP, and 100 U/mL Klenow enzyme.
3. NAP-5 buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0, 100 m*M* NaCl.

2.4. Labeling of DNA: Asymmetric Polymerase Chain Reaction

1. 10X PCR buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 9.0, 1% Triton X-100 ($MgCl_2$-free).
2. APCR reaction conditions: 50 m*M* KCl, 10 m*M* Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.78 m*M* $MgCl_2$, 20 µ*M* dNTP (no dCTP), 10 µ*M* dCTP (unlabeled), 100 ng template DNA, 20 n*M* sense primer, 1 µ*M* antisense primer (gives 1 : 50 ratio), 2.5 U Taq polymerase, 100 µCi [α-^{32}P] dCTP (3000 Ci/mmol); total volume of 50 µL.

2.5. Filter Hybridization

2.5.1. Mahmoudi and Lin (2) Procedure

1. Sodium phosphate solution: 1 *M* $Na_2HPO_4 \cdot 7H_2O$, pH to 7.0 with 85% H_3PO_4. Autoclave and store at room temperature. This solution can precipitate at low temperatures, therefore, it may require warming prior to use.
2. Prehybridization solution: 0.5 *M* Na_2HPO_4, pH 7.0, 7% sodium dodecyl sulfate (SDS), 1% BSA (fraction V), 1 m*M* EDTA, 100 µg/mL salmon sperm DNA. (DNA should be boiled for 5 min and placed on ice for 5 min before addition; this ensures that the DNA is single stranded.)
3. Hybridization solution: As in **item 2** with ^{32}P-labeled DNA probe.
4. 25X PPi: 0.5 *M* sodium phosphate, 1.5% pyrophosphate, pH 6.9. Autoclave and store at room temperature indefinitely.

5. 2X Wash solution: 2X SSC, 1X PPi, 0.05% SDS. Prepare fresh.
6. Stripping solution: 1% SDS in 1X SSC.

2.5.2. Hybridization at 42°C: Hybridization Solution 2

1. Prehybridization solution: 50% formamide, 5X SSC, 2X Denhardt's, 5 m*M* EDTA, 0.2% SDS, 1X PPi, 100 µg/mL salmon sperm DNA (boiled previously; e.g., single stranded).
2. Hybridization solution: 50% formamide, 5X SSC, 10% dextran sulfate, 1X Denhardt's, 3 m*M* EDTA, 0.16X PPi, 0.125% SDS, 100 µg/mL salmon sperm DNA (single stranded) and radio-labeled probe.
3. 100X Denhardt's solution: 2 g BSA (fraction V), 2 g polyvinyl pryrrolidone (360K mol. wt.), 2 g Ficoll (400K mol. wt.). Make up in 100 mL of water, filter, and store in aliquots at –20°C.
4. 50% Dextran sulfate: Dissolve 25 g in 50 mL water, heat until homogeneous solution, filter, and store at 4°C indefinitely.

3. Methods

3.1. Total RNA Isolation (3) (see Note 4)

1. All work must be performed on ice. Homogenize tissue in 0.8 mL of GTC. (*See* **Note 5**.)
2. Add 80 µL of sodium acetate (2 *M* sodium acetate, 2 *M* acetic acid, pH 4) and then 0.8 mL of saturated phenol and 160 µL of chloroform/isoamyl alcohol (24 : 1)—in this order. Vortex and then place on ice for 15 min.
3. Separate aqueous/nonaqueous with 11,000*g* spin for 15 min at 4°C.
4. Collect the aqueous phase and precipitate RNA with equal volume of isopropanol, leave at least 2 h, preferably overnight, at –20°C. (*See* **Note 6**.)
5. Spin at 11,000*g* for 15 min at 4°C to precipitate RNA and discard supernatant.
6. Redissolve precipitate in 0.5 mL of guanidine hydrochloride, gently vortex and leave on ice for 15 min.
7. Add 250 mL of absolute ethanol and store overnight at –20°C.
8. Spin as earlier and discard supernatant; wash pellet successively with 0.5 mL of 70%, 80%, and absolute alcohol.
9. Discard the final ethanol wash and dry sample in a hood under red light and then resuspend pellet in 40 µL of water, gently vortex and heat to 60°C for 10 min, then freeze and store at –20°C. Remove 4 µL for optical density (OD) measurement. (*See* **Note 7**.)
10. Add 4 µL of RNA sample to 0.4 mL of water in quartz cuvet and read OD at 260 nm and 280 nm.
11. The OD ratio of 260/280 nm gives an index of purity of the RNA sample; a ratio of >1.8 is acceptable and suggests little contamination with protein. The 260-nm value is used to calculate the concentration of RNA in the sample. Use the relation; 1.0 OD = 40 µg/mL RNA. Remember to adjust for the 100X dilution.

3.2. Northern Gel and Transfer (4)

1. The gel should be prepared and run in a hood. For a 1.2% agarose gel, combine 2.4 g of agarose with 147 mL water (add to cold water then gradually warm until boiling, shake frequently).
2. Add 20 mL of 10X running buffer.
3. Allow to cool to 60°C, add 33 mL of concentrated formaldehyde, pH 3.6 (gives 2.2 *M* formaldehyde). **PRECAUTION:** Do not add formaldehyde until temperature is at 60°C, otherwise there is a potential for explosion.
4. Pour gel.
5. RNA sample preparation. Add up to 40 µg total RNA to each tube. Add, in sequence, components of the sample solution: water (required volume to make final volume 20 µL), 10X running buffer (2 µL), formamide (10 µL), and formaldehyde (3.0 µL). Vortex and heat to 60°C for 10 min. Vortex and place on ice immediately. Add 2 µL of 10X bromophenol blue solution. (*See* **Note 8**.)
6. Add the sample to each lane of the gel. Do not submerge the gel at this juncture. (*See* **Note 9**.) Apply voltage (e. g., 100 V, until samples have entered the gel, then submerge gel with running buffer and continue separation until the dye front is approx 8 cm from wells. Recirculate running buffer during the separation. (*See* **Note 10**.)
7. Remove gel and stain with ethidium bromide in 0.1 *M* ammonium acetate for 30–60 min (in 500 mL), gently shaking. Destain for 30–60 min in water and photograph ethidium bromide staining on UV transilluminator using a Polaroid camera. This is an important procedure with regard to quality control. The rRNA bands at 18S and 28S (in eukaryotic cells) should be clearly visible. If these bands are unclear or absent, it suggests that degradation of total RNA has occurred. Additionally, if equal amounts of total RNA were subjected to Northern transfer, then the intensity of the rRNA bands should be equivalent. The Polaroid photograph should be saved and used as documentation confirming the quality of the gel.
8. Denature gel for 45 min in 500 mL of denaturation solution. (*See* **Note 11**.)
9. Neutralize in 500 mL of neutralization buffer for 45 min.
10. Apply gel to blotting device to transfer separated RNA to nitrocellulose ***(5)***. Use 10X SSC as the transfer medium. (*See* **Note 12**.)
11. Crosslink RNA to nitrocellulose and store filter at room temperature protected in a sheet of 3M paper. (*See* **Note 13**.)

3.3. Labeling of DNA Probes: Random Primer Labeling (see Note 14)

1. Add 1–4 µL (25–50 ng) of pure insert DNA to water to final volume of 35 µL. Denature by boiling for 2 min. Rapidly chill on ice for at least 5 min to prevent renaturation. (*See* **Note 15**.)
2. Add the following in order at room temperature: 5X transcription buffer, mixture of unlabeled dNTPs, nuclease-free BSA, [α-^{32}P] dCTP (50 µCi, 3000 Ci/mmol), and 5 U of Klenow enzyme.

3. Vortex gently and leave at room temperature for 2–4 h.
4. Add 450 µL of NAP-5 buffer and apply to NAP-5 column (preequilibrated with 20 mL of NAP-5 buffer). Allow to enter column, then position collection tube and add 0.8 mL of NAP-5. Collect 0.8 mL eluate; this contains the probe (check incorporation using counter).
5. Boil for 5 min and place on ice immediately. Store for at least 5 min on ice before adding to hybridization solution.
6. The specific activity of the probe should be routinely measured. Take a 1-µL aliquot of probe prior to separating on the NAP-5 column and apply to a piece of DE-81 chromatography paper. Take a 1-µL aliquot from the eluate of the NAP-5 column and similarly apply to DE-81 paper. Allow to dry; then wash sequentially with 0.5 *M* sodium phosphate, water, and absolute ethanol. Allow to dry; then subject to scintillation counting. The percentage incorporation can thus be determined. This value can then be used to determine the specific activity of the probe (specific details can be found in the methodologies accompanying kits). Values $>10^8$ cpm/µg DNA are acceptable.

3.4. Labeling of DNA Probes: Asymmetric Polymerase Chain Reaction (*see* Note 16)

1. Perform asymmetric polymerase chain reaction (APCR) on target DNA (plasmid or purified insert). Use 50–100 ng of DNA and add 10X Taq transcription buffer, $MgCl_2$, dNTPs (no dCTP), dCTP (unlabeled), forward and reverse primers with 50:1 ratio for antisense:sense, 100 µCi [α-^{32}P] dCTP (3000 Ci/mmol), and 5 U Taq polymerase. (*See* **Note 17**.)
2. The PCR conditions will depend on the DNA to be amplified; however, 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C and usually for 40 cycles is often productive. Separate DNA species on agarose minigel. Run unlabeled product as double- and single-stranded species to allow identification of ^{32}P-labeled single-stranded DNA product generated by APCR. (Prepare single-stranded species by boiling for 3 min and then placing immediately on ice.)
3. Cut band of interest from gel and melt agarose for 2 min at 95°C. Check that the probe is radioactive using a hand-held monitor and add molten agarose directly to the hybridization solution (keep volume down to 0.5 mL).

3.5. Filter Hybridization (2) (*see* Note 18)

1. Soak filter in 0.5 *M* sodium phosphate, pH 7.0, for 5 min, then prehybridize filter for 4–6 h at 65°C in prehybridization solution. (*See* **Notes 19** and **20**.)
2. Remove prehybridization solution and add hybridization solution containing ^{32}P-labeled DNA probe. Incubate at 65°C for 16–18 h. (*See* **Note 21**.)
3. Remove hybridization solution and wash filter successively at 68°C with 2X SSC/phosphate wash solution for 15 min three times (perform in the hybridization oven using 100 mL for each wash), once with 2X wash solution for 30 min (remove from oven for this wash, place filter in sealed plastic container in a 68°C gently shaking water bath, and use 300 mL of wash from this point on), 1X wash

solution twice for 45 min, and once with 0.5X wash for 30 min. Drain the filter, place on 3MM paper, and cover with cling film to prevent drying out (*see* **Note 22**).
4. Stripping of filters can be achieved by boiling for 10 min in stripping solution. Do not allow to dry out following stripping (*see* **Note 22**).

3.6. Detection of Hybrids

1. Hybrids can be detected using X-ray autoradiography. Following hybridization, the filter is placed against a piece of X-ray film, which, in turn, is placed against an enhancing screen inside a light-tight cassette. The film should be placed at –70°C and exposed for up to 3 wk (for very rare transcripts). Moderately abundant transcripts should be detectable within 1–2 d. The film is then developed and the signal quantified by densitometry. This procedure is inexpensive, but X-ray film has a dynamic range of only two orders of magnitude and large differences between signals may be underestimated. Furthermore, detection of rare transcripts may involve exposure for up to 1 mo—limiting turnaround time within the laboratory.
2. The technique of choice now involves the use of a phosphorimager. Filters can be exposed to sensitive screens for up to a week, for rare transcripts, and the level of radioactivity is then quantified using a scanner. The dynamic range of such machines can be as much as four to five orders of magnitude. Although no more sensitive than X-ray film, results can be generated more quickly because of reduced turnaround times, and, overall, data acquisition and quantification are more reliable and accurate. Biorad and Molecular Dynamics both make superb phosphorimaging machines, but they are expensive.

3.7. Studies with Neurotrophins and Their Receptors

Studying the expression of the neurotrophins is difficult in tissues of adult vertebrates because of the extremely low levels of expression. For success, especially when attempting to detect NGF and NT-3 transcripts, high levels of total RNA (i.e., up to 40 µg) must be separated and probes must be labeled using APCR. **Table 1** presents a list of neurotrophin-related probes and recommended sources and labeling approaches. Please note that NT-4 is not included; detection of NT-4 by Northern blotting is problematical and the author recommends the use of RNase protection ***(6)***.

4. Notes

1. A source of molecular-biology-grade saturated phenol is essential. The phenol should come as a solution that is Tris-buffered (i.e., saturated with an aqueous solution) and sealed under argon. For long-term storage, the phenol should be frozen at –20°C immediately upon delivery. Liquid phenol can be stored at 4°C for a maximum of 1 mo. Prior to use, or following thawing out, add 100 mg of 8-hydroxyquinoline to 100 mL of liquid phenol. Shake well; this will dissolve the 8-hydroxyquinoline but it will also ensure that the phenol is fully saturated

Table 1
Neurotrophin and Neurotrophin Receptor Probes for Quantification of Transcripts

	Species/size	Labeling method[a]	Ref.
NGF	Mouse/0.92 kb	APCR	*7*
NT-3	Rat/0.8 kb	APCR	*8*
BDNF	Rat/1.1 kb	RPL	*8*
trkA	Rat/1.5 kb	RPL	*9*
trkB	Rat/2.9 kb	RPL	*10*
trkC	Rat/0.79 kb	APCR	*10*
$p75^{NTR}$	Rat/3.4 kb	RPL	*11*

[a]APCR: asymmetric polymerase chain reaction; RPL: random primer labeling.

with Tris buffer. Long-term exposure of phenol to air will lead to oxidation and generation of free radicals that degrade RNA (8-hydroxyquinoline being a reducing agent protects against this process).

2. When resuspending RNA pellets, the purest RNase-free water should be used. DEPC treatment followed by autoclaving is usually sufficient. Unfortunately, DEPC is carcinogenic. The best option is to buy ultrapure DEPC-treated water in small volumes and keep in the dark at 4°C for 1 mo maximum. All other solutions should be prepared in double-distilled water (or with water derived from a purifying system). Always try to use freshly distilled or purified water that has been autoclaved.
3. Freeze formamide in aliquots at –20°C. Formamide will generate free radicals that degrade RNA if exposed excessively to light and/or air.
4. RNA is degraded by RNases; these are ubiquitous enzymes that can only be circumvented with strict laboratory practice. Always wear gloves; all pipet tips and tubes must be autoclaved and kept covered. All solutions should be of molecular-biology-grade materials, prepared in pure fresh water and, in most cases, autoclaved.
5. Use of a polytron, with a small head, is recommended. Tissue should be placed in GTC and homogenized immediately. Soft tissues will homogenize easily; however, more fibrous tissues (e.g., muscle and skin) should be finely minced and then added to GTC. Once homogenized, freeze immediately. GTC is a chaotropic agent that inhibits RNase activity; however, at 4°C, there may still be some residual activity. Therefore, never allow tissues in GTC to sit on ice indefinitely.
6. When collecting the aqueous phase, take care not to touch the interface where high concentrations of DNA and protein reside.
7. Carefully dry the pellet under a lamp (free of UV light) in a hood. RNA pellets can be difficult to resuspend; therefore, add 40 µL of ultrapure water and heat to 60°C for 10 min to aid resuspension and vortex gently before measuring the OD.

8. A maximum of 40 µg of total RNA is usually loaded onto the gel; this figure is derived from the binding capacity of most forms of nitrocellulose. To keep volumes to be loaded to a minimum, the RNA to be separated should be precipitated overnight (in 2 vol of absolute ethanol and 0.2 *M* sodium acetate at –20°C) and pelleted in the morning of the gel with a 11,000*g* spin for 30 min at 4°C; carefully remove all the supernatant with a pipet (the pellet should be clearly visible as a white or opaque disk) and then resuspend in sample solution. Heating to 60°C and gentle vortexing of the sample is important to aid resuspension of the pellet—absolutely crucial if you wish to perform quantitative Northern blotting.
9. Do not submerge the gel prior to adding the sample, this can lead to sample loss, which will hinder later attempts for quantification.
10. Recirculate the buffer, otherwise a pH buildup will occur, resulting in acidic pH at one pole, and acid conditions can result in degradation of RNA.
11. Denaturation of the separated RNA will aid transfer of RNA molecules to the nitrocellulose. The conditions described result in most RNA molecules being transferred in a state that can be detected later. If you are studying the expression of a particularly small RNA molecule (e.g., <500 bases), you may need to lower the NaOH concentration to prevent overdenaturation.
12. There are several means of transferring RNA to nitrocellulose, overnight capillary-action blotting will work; however, we find a positive pressure device gives the best results and is certainly quicker. (The Stratagene posiblotter is recommended.)
13. Choice of nitrocellulose will determine the manner of crosslinking. Nylon membranes are durable and RNA can be crosslinked to the membrane using UV light. Nytran-N nitrocellulose (Schleicher & Schuell) is a good choice that gives minimal background signal and can be reprobed successfully up to eight times.
14. Use of a kit is recommended, the Prime-a-Gene system from Promega is very good and routinely gives specific activities of labeling of DNA of $>10^9$ cpm/µg DNA.
15. For success in detecting rare transcripts, the template DNA must be pure (e.g., free of plasmid DNA). The target template DNA should be gel separated from plasmid DNA, purified, and carefully quantified (it is important that only 25–50 ng DNA is added to labeling reaction). Removal from plasmid DNA by restriction enzyme can be performed; however, PCR of the insert of interest followed by gel purification is the most convenient technique. The size of the insert to be labeled is also an important factor, in our hands, random primer labeling only labels DNA in a suitable fashion if the template is >500 bp. Smaller DNA molecules (e.g., <400 bp), are efficiently labeled, but such probes do not detect mRNAs with any degree of specificity. For small DNA inserts, APCR labeling of single-stranded DNA should be used (*see* **Notes 16** and **17**).
16. In cases in which sensitivity is paramount, or where the target DNA is <400 bp, the use of APCR to label DNA is recommended. Sensitivity is increased because a single-stranded antisense DNA molecule is being used for detection of transcripts; therefore, there is no possibility of homoduplex formation between labeled complementary strands of DNA (as is the case with random-primer-

labeled probes). This procedure also allows small DNA probes to be generated that have maintained specificity. The APCR reaction can be performed on plasmid or purified DNA.

17. The specific labeling conditions for any given probe will vary depending, in large part, on the size of the probe to be generated. For efficient labeling, the concentration of unlabeled dCTP must be kept to a minimum; too little dCTP and the yield of product will be poor. In most cases, an unlabeled dCTP concentration of about 10 μ*M* gives good results. However, for each probe, a standardization for dCTP concentration using reactions without ^{32}P is recommended before commencing. Finally, given that lower levels of dNTPs are being used, compared with standard PCR, it follows that a lower concentration of $MgCl_2$ is required. (This is because dNTPs bind Mg^{2+}.)
18. The Mahmoudhi and Lin procedure ***(2)*** works well for the majority of probes; however, small probes (<400 bp) can sometimes prove difficult to work with because of the high annealing temperature, 60–65°C. Under these circumstances, hybridization should be performed at 42°C with hybridization solution 2 (*see* **Subheading 2.**).
19. Use of a hybdridization oven is recommended. Filters should be carefully laid against nylon sheets, excluding air bubbles, rolled, and positioned inside glass tubes. The Hybaid range of ovens is recommended.
20. When preparing the SDS-based hybridization solution carefully, heat the solution on a hot plate to 60°C and stir until a clear solution appears and keep at 60°C until ready to use. Heating the solution above 70°C will denature the BSA/SDS and result in the deposition of material, which will give severe background problems on the filter.
21. When attempting to detect rare transcripts, lowering the hybridization temperature to 60°C may prove beneficial. It is not possible to reduce the temperature any lower because the SDS precipitates. The level of washing can also be modulated; for rare transcripts the final 0.5X SSC wash can be omitted.
22. Do not allow the filter to dry out, because subsequent attempts to strip the filter of the labeled probe will prove futile.

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6

Quantification of mRNA Levels Using Ribonuclease Protection Assay

Pirkko Henttu

1. Introduction

Several methods are available for the quantification of neurotrophin mRNA levels. This chapter describes the RNAse protection assay (RPA) first publishcd by Zinn and co-workers ***(1)*** and developed further by Melton and co-workers ***(2)***. In this method, a radioactively labeled antisense RNA probe is hybridized in solution with a purified RNA sample. The labeled probe forms double-stranded RNA–RNA hybrids with complementary mRNA molecules. Separation of RNA–RNA hybrids from free probe and sample RNA is achieved by digestion with RNAses that specifically degrade single-stranded RNA. After these RNAses have been inactivated by proteinase K digestion, protected RNA fragments are separated in ultrathin acrylamide gels and visualized with autoradiography. The labeled antisense RNA probe is derived from a plasmid construct that has a bacterial RNA polymerase promoter such as SP6, T3, or T7 downstream of a sequence that encodes a portion of neurotrophin mRNA. This DNA fragment can either be part of a cDNA clone or a genomic subfragment, but it should be selected so that the plasmid construct can be digested with a specific restriction enzyme prior to transcription, to yield a labeled RNA species from 100 to 400 nucleotides long (*see* **Fig. 1**). As RNAses can detect mismatches of one basepair, the probe has to be derived from the same species as the RNA being studied, or if it is heterologous, it has to correspond to a region where its nucleotide sequence is fully conserved between the two species.

Compared to other methods that are used in the quantification of mRNA levels, RNAse protection assay offers higher sensitivity than Northern blot-

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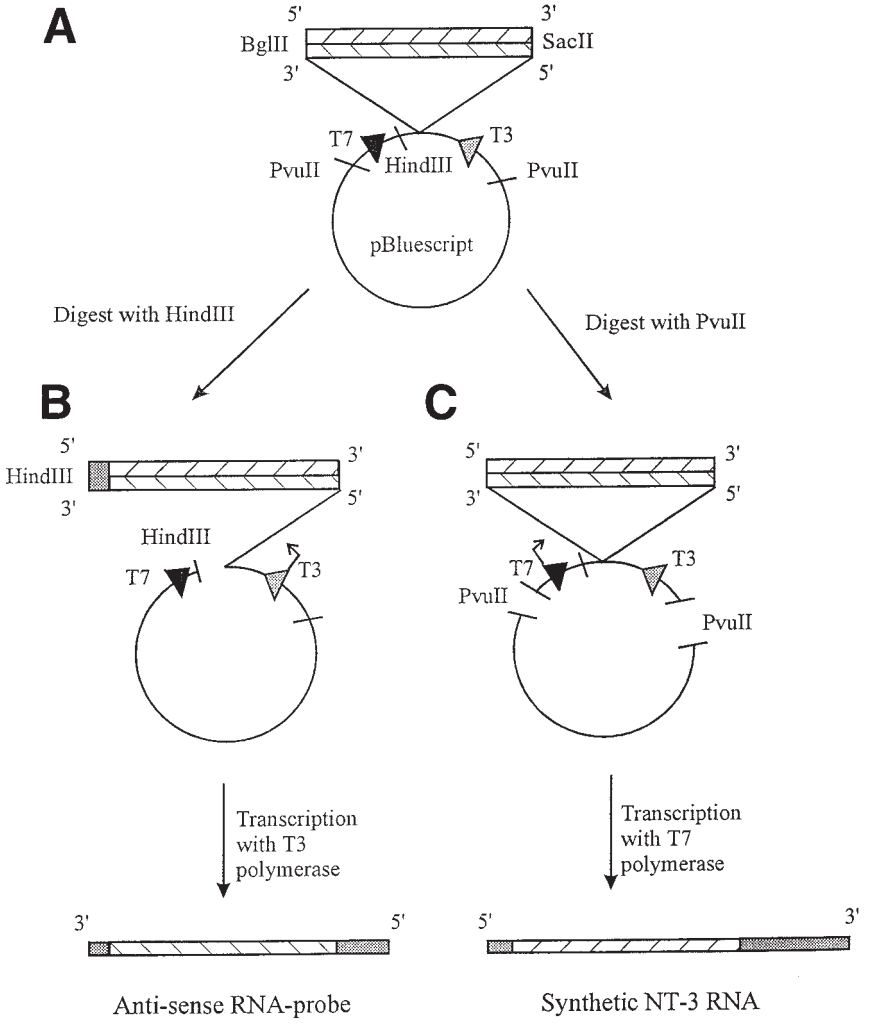

Fig. 1. Generation of antisense RNA–probe and synthetic mRNA from plasmid construct. **(A)** An internal BglII–SacII fragment of the human NT-3 cDNA was cloned into the pBluescript vector between the T3 and T7 RNA–polymerase promoters. Note that the two promoters are oriented so that opposite DNA strands will be transcribed into RNA. **(B)** To synthesize the antisense probe, the plasmid is cut with *Hind*III within the polylinker. Transcription of this linearized plasmid with T3 RNA polymerase will result in the noncoding strand being synthesized. The antisense probe has some vector-derived sequences at both 5′ and 3′ ends (shown by the gray color). **(C)** To prepare synthetic NT-3 mRNA, the plasmid is cut with *Pvu*II, which cuts twice in the plasmid backbone. The presence of the vector-derived *Pvu*II fragment will not interfere with transcription with T7, as it has no promoter sites. The coding mRNA strand is synthesized by T7 RNA–polymerase. The synthetic mRNA will have some vector-derived sequences at both ends (shown by the gray color).

ting, but it is less sensitive than reverse transcription–polymerase chain reaction (RT-PCR). However, as the detected signal in RNAse protection assay is generated from a fully complementary RNA–probe hybrid, the specificity of this method is high and the problems encountered in Northern hybridizations of generating signal from the probe that hybridizes with some partially complementary RNA species or amplification of contaminating DNA by RT-PCR are eliminated. On the other hand, establishing a new RNAse protection assay for any specific mRNA species requires more work than other methods. However, when successfully established, the RNAse protection assay can be used to analyze several samples simultaneously, and it is also possible to use several specific probes in the same hybridization to detect multiple mRNA species. This means that in addition to quantitating the mRNA species of interest, another probe detecting transcripts from a housekeeping gene can be included in hybridizations as an internal control. As the amount of neurotrophin signal is dependent on the total amount of sample RNA included in the hybridization, it is important to ensure that the specific signal is correlated to the abundance of some constant mRNA species to eliminate errors arising from uneven mRNA amounts or losses during manipulations. Suitable control mRNAs species include β- or γ-actins, glyceraldehyde-3-phosphate dehydrogenase (GADPH), and 18S ribosomal RNAs or any other transcript that is resistant to the manipulations being studied.

The genes encoding the nerve growth factor family members contain several promoters and are transcribed into multiple RNA species whose expression is regulated in a tissue-specific fashion by cellular signaling pathways. The RNAse protection assay can be tailored to detect several mRNA species in one hybridization by choosing a probe that will fully protect one neurotrophin mRNA species and partially protect others. In this manner, it is possible to study, for example, whether all neurotrophin mRNA species are regulated similarly by pharmacological agents. In addition to the quantification of mRNA levels, RNAse protection assay is also a useful method for mapping exons and transcription start sites. By using RNA probes derived from genomic fragments, it is possible to localize transcription start sites within a few nucleotides and confirm the presence of alternatively spliced exons.

2. Materials

All chemicals should be of molecular biology grade. Unless stated otherwise, buffer and salt solutions can be stored at room temperature. All enzymes and enzyme buffers should be stored at –20°C and kept on ice while setting up reaction mixes.

RNA is highly susceptible to degradation by RNAses from various sources, such as water, microbes, hands, and contaminated surfaces. Therefore, when

working with RNA, always wear disposable gloves and change them when you think they may have come into contact with RNAses. All solutions used in RNA work should be prepared using sterile water that has been treated with diethylpyrocarbonate (DEPC) and either autoclaved of filter-sterilized. It is advisable to aliquot reagents and change aliquots regularly in order to minimize contamination while working. Use only autoclaved plastic tubes to contain RNA samples. Finally, keep the lab bench clean and free of dust.

2.1. Preparation of Template DNA for RNA Probe

1. DEPC-treated water: Add 50 µL DEPC to 100 ml double-distilled water. Let stand at room temperature overnight and autoclave. **PRECAUTIONS:** DEPC is a carcinogen and should be handled in a fume hood while wearing gloves.
2. Phenol/chloroform/isoamyl alcohol: Purchase Tris-saturated phenol and store it at –20°C for long-term storage. To thawed phenol, add 100 mg of 8-hydroxyquinoline to 100 mL phenol and mix well. Mix Tris-saturated phenol, chloroform, and isoamylalcohol in 25:24:1. Store in dark at 4°C. If color changes, discard.
3. 3 *M* sodium acetate, pH 5.2: Dissolve sodium acetate in water and adjust pH to 5.2 with glacial acetic acid and sterilize by autoclaving.
4. 70% ethanol: Mix 30 mL sterile water and 70 mL absolute ethanol. Do not autoclave.

2.2. Preparation of a High-Specific-Activity RNA Probe

1. 5X transcription buffer: This is usually supplied with the RNA polymerase.
2. 200 m*M* dithiothreitol (DTT): Dissolve DTT in water. Aliquot and store at –20°C.
3. 4 m*M* ATP, CTP, GTP: Dilute stock solutions of ribonucleotides (usually 100 m*M*) with water so that the concentration of all three nucleotides is 4 m*M*. Store at –20°C in small aliquots.
4. 100 µ*M* UTP: Dilute from stock solution. Store at –20°C in small aliquots.
5. RNAse inhibitor: Placental RNAse inhibitor such as RNAsin from Promega. RNAsin binds to RNAses, thus inhibiting their action. RNAsin requires DTT for binding and is destroyed by heating to 95°C.
6. RNA polymerase SP6, T3, T7: Select the correct one for your probe. Store at –20°C and keep on ice while in use.
7. RNAse-free RQ1 DNAse: Purchase ready made. Store at –20°C and keep on ice while in use.
8. 2.5 *M* ammonium acetate: Dissolve ammonium acetate in water. Filter sterilize.
9. tRNA solution: Make up 10 mg/mL solution of yeast tRNA in water. Aliquot and store at –70°C for long-term storage. An aliquot can be kept at –20°C for a couple of weeks.

2.3. Alternative Method for Purifying RNA Probe

1. Qiagen nucleotide removal kit: This kit is supplied by Qiagen (Hilden, Germany and branches worldwide), and it contains all necessary buffers to perform this step.

2.4. Gel Purification of Full-Length Probe

1. Loading buffer: Mix 9.2 mL formamide, 800 µL of 0.25 *M* EDTA, with a trace of bromphenol blue and xylene cyanol.
2. 40% Acrylamide : bis (19 : 1): This can be purchased as a ready-to-use solution, which eliminates the need to handle acrylamide powder. Store at room temperature. **PRECAUTIONS:** Unpolymerized acrylamide is very toxic. Wear goggles and protective clothing.
3. 10% Ammonium persulfate (APS): Weigh out 60–100 mg aliquots of ammonium persulfate into microcentrifuge tubes. Prepare a fresh solution each time by dissolving the contents of an aliquot in water. Store undissolved APS at room temperature.
4. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED): Can be purchased from various sources.
5. 10X TBE: Dissolve 108 g Tris base, 55 g boric acid, and 9.3 g disodium–EDTA in a final volume of 1 L of double-distilled water. Dilute to 1X TBE before use. This solution does not need to be autoclaved.
6. Sensitive X-ray film: Kodak X-ar 5 is recommended, but other sensitive films are also suitable.
7. Elution buffer (2 *M* ammonium acetate, 1% sodium dodecyl sulfate [SDS] and 25 µg/mL tRNA): Dissolve 1.54 g ammonium acetate and 1 mL of 10% SDS in 10 mL water. Prior to use, add 1 µL 10 mg/mL tRNA per 400 µL. This solution usually precipitates at room temperature, so warm it at 37°C to dissolve completely before use.

2.5. Preparation of a Low-Specific-Activity Probe

1 m*M* UTP: Dilute from UTP stock solution and store at –20°C.

2.6. Hybridization with RNA Samples

1. 5X Hybridization buffer: Make by mixing 10 mL of 1 *M* PIPES (pH 6.4), 20 mL of 5 *M* sodium chloride, and 1 mL of 250 m*M* sodium–EDTA and adding water to 50 mL. Dilute to 1X hybridization buffer by mixing four parts of formamide and one part of 5X hybridization buffer. A 1X hybridization buffer is 200 m*M* PIPES (pH 6.4), 2 *M* sodium chloride, and 5 m*M* sodium–EDTA, and 80% formamide. Use molecular-biology-grade formamide. Prepare 1X buffer freshly for each experiment. The 5X buffer is stable at room temperature.
2. Digestion buffer: Prepare by mixing 0.5 mL of 1*M* Tris-HCl (pH 7.5), 3 mL of 5 *M* sodium chloride, and 1 mL of 250 m*M* sodium–EDTA and adding water to 50 mL.

3. RNAse A and RNAse T1: Use the purest quality you can get (*see* **Note 1**).
4. 10% SDS: Prepare by dissolving 10 g SDS in 100 mL sterile water. Do not autoclave, as this causes the detergent to precipitate in storage. Store at room temperature; it can precipitate if the temperature falls too low.
5. Proteinase K: Make a 10-mg/mL solution in water, aliquot, and store at –20°C.

2.7. Gel Analysis of Protected RNA Fragments

1. Acrylease: Ready-to-use spray sold by Stratagene (La Jolla, CA, USA).
2. Solution A: Mix 50 mL absolute ethanol with 50 µL γ-methacryloxypropyltrimethoxysilane.
3. Solution B: Mix 10 mL water with 1 mL glacial acetic acid. For treating glass plates, mix 3 mL of solution A and 90 µL of solution B.
4. Urea. It is important to use molecular-biology-grade urea; lower qualities will result in unsatisfactory resolution on gels.
5. 40% Acrylamide (AA):bis (19:1): You can also purchase a ready-to-use gel mix containing AA:bis and urea.
6. 10% Acetic acid: Make up in double-distilled water. This solution can be reused up to three times.

2.8. Preparing Labeled DNA Molecular-Weight Markers

10 m*M* dCTP, dGTP, dTTP: Dilute from stock solutions and store at –20°C in small aliquots.

3. Methods

3.1. Preparation of Template DNA for RNA Probe

1. Clone a suitable neurotrophin cDNA or genomic fragment into a plasmid containing a bacterial RNA polymerase promoter. There are several suitable cloning vectors: the Bluescript (Stratagene, La Jolla, CA, USA), pSP (Promega, Madison, WI, USA), and pGEM (Promega, Madison, WI, USA) series plasmids being the most widely used. Either isolate a fragment from an existing plasmid by restriction digestion or use RT-PCR to amplify a fragment from RNA (*see* **Note 2**). Always verify the nucleotide sequence and the orientation of the insert by sequencing (*see* **Note 3**).
2. Digest 10 µg of template plasmid DNA with a restriction enzyme that cuts *once* 100–400 bp downstream of the RNA polymerase site to be used (*see* **Fig. 1**). Into a microcentrifuge tube, add 5 µL of 10X enzyme digestion buffer supplied with the enzyme, 10 µg of DNA in water, 30 U restriction enzyme, and water to make up the total volume to 50 µL. Incubate at 37°C overnight. This is to ensure complete digestion, as an uncut template will result in extended RNA probes. Analyze a 1-µL aliquot in an agarose gel to check that the digestion is complete (*see* **Note 4**).
3. Heat the digest to 65°C for 20 min to denature the restriction enzyme.
4. Extract with 50 µL phenol/chloroform/isoamyl alcohol.

5. Transfer the aqueous phase into a new microcentrifuge tube, add 5 *M* to l 3 *M* sodium acetate and 150 µL absolute ethanol. Incubate on dry ice for 10 min and centrifuge at 16,000*g* for 10 min (*see* **Note 5**).
6. Remove solution and wash the pellet with 100 µL of 70% ethanol. Centrifuge as in **step 5** for 5 min.
7. Dry the pellet and dissolve it in 20 µL DEPC-treated water. The digested DNA template can be stored at –20°C for several years.

3.2. Preparation of a High-Specific-Activity RNA Probe

PRECAUTIONS: As the preparation of RNA probes involves the use of large amounts of radioactive nucleotides, all work should be performed behind Perplex screens in a designated area. Before starting these experiments, you should familiarize yourself with local safety regulations and follow whatever safety rules exist in your institute. Protective clothing, such as lab coats and disposable gloves, should be worn during all of the following procedures. This is not only to protect the worker from radiation hazards but also to protect the RNA samples from contaminating RNAses from the environment. While working, monitor your hands and the work area regularly and clean up any contamination immediately. Counts-Off (Du Pont-NEN) is recommended for cleaning up contaminated surfaces and equipment.

1. At room temperature, pipet into a microcentrifuge tube in this order: 2 µL of 5X transcription buffer, 0.5 µL of 200 m*M* DTT, 1 µL of 4 m*M* ATP, CTP, GTP mix, 1 µL of 100 µ*M* UTP, 0.5 µL of RNAse inhibitor RNAsin (20 U), 4 µL of α-^{32}P-UTP (10 mCi/mL, 3000 Ci/mmol) (*see* **Note 6**), 0.5 µL linearized DNA template at 500 ng/µL, and 0.5 µL RNA polymerase (*see* **Note 7**). The transcription buffer contains spermidine, which may precipitate the DNA template in the cold.
2. Mix the contents of the tube carefully and incubate for 1 h at 37°C, or 40°C if using SP6 polymerase.
3. Transfer the mix to 90°C for 3 min and cool on ice to separate RNA from the DNA template (*see* **Note 8**).
4. Add 2 µL RNAse free DNAse I (2 U) and incubate at 37°C for 15 min to digest the DNA template. The undigested DNA template will interfere with the hybridization reaction, so it should be removed carefully.
5. Add 1 µL of 10 mg/mL tRNA solution as carrier RNA and 40 µL DEPC-treated water to the mix.
6. Extract with 50 µL phenol/chloroform/isoamyl alcohol. Separate phases by centrifuging for 1 min at 16,000*g* at room temperature.
7. Transfer the aqueous phase into a clean tube.
8. Add 200 µL of 2.5*M* ammonium acetate and 750 µL absolute ethanol. Mix well.
9. Incubate 5 min on dry ice and centrifuge in a microcentrifuge at 16,000*g* for 10 min at room temperature.
10. Remove liquid carefully and redissolve the probe in 50 µL water.

11. Repeat the precipitation from **step 7** twice.
12. Dissolve the probe in 100 µL of hybridization buffer. The probe can be stored at –20°C for up to 7 d. Autodegradation will occur during storage and its extent is determined by the composition of the probe.
13. Determine activity from 1 µL with scintillation counting (*see* **Note 9**).

3.3. Purifying RNA Probe with Spin Chromatography

As an alternative method for purifying labeled RNA probes from free nucleotides, Qiagen nucleotide removal kit (Qiagen) can be used (*see* **Note 10**).

1. To the probe-labeling mix from **Subheading 3.2., step 6**, add 500 µL buffer PN. Mix well and transfer into a Qiagen spin column that has been inserted into a collection tube and centrifuge for 1 min at 3,500*g*. Labeled RNA will bind to the column.
2. Discard eluate and transfer column into a clean tube.
3. Add 500 µL wash buffer PE into the column containing the RNA probe and centrifuge for 1 min at 3,500*g* at room temperature.
4. Discard eluate, add 500 µL of wash buffer and centrifuge for 1 min at 3,500*g*.
5. Discard eluate and recentrifuge for 1 min at 3,500*g*.
6. Transfer the column into a clean microcentrifuge tube.
7. Add 50 µL water, let stand for 1 min, and collect probe by centrifugation for 1 min at 3,500*g*.
8. Add 50 µL of 2X hybridization buffer and store at –20°C.
9. Determine activity from 1 µL with scintillation counting.

3.4. Gel Purification of Full-Length Probe

Sometimes, obtaining a full-length probe may be difficult and the presence of shorter probe fragments may give rise to a high background. The probe can be purified on an acrylamide gel to eliminate shorter contaminating molecules. This will decrease the yields of probe, but sufficient amounts for up to 50 hybridizations can still be generated. If gel purification is to be done, extreme care must be taken to minimize the radiation hazards to the worker. All steps should be performed behind perplex screens and the handling time of radioactive material should be kept to the minimum (*see* **Note 11**).

1. Prepare labeled RNA probe as usually, DNAse I treat it, extract with phenol/chloroform/isoamyl alcohol and precipitate as in **Subheading 3.2., steps 1–9**.
2. Dissolve probe in 10 µL loading buffer.
3. Prepare acrylamide gel by mixing 10 mL of 40% acrylamide : bis (19 : 1), 5 mL of 1X TBE, and water to 50 mL. Add 500 µL of 10% ammoniumpersulfate and 75 µL TEMED. Cast a 15 × 15-cm gel using 1-mm spacers and a comb. When the gel has set, assemble gel electrophoresis apparatus and prerun the gel for 15 min at 300 V using 1X TBE as running buffer.

4. Heat the dissolved probe at 85°C for 5 min to denature RNA and transfer it onto ice.
5. Load the samples to the gel carefully. Electrophorese the gel at 300 V until the bromphenol blue dye is one-third from the bottom of the gel.
6. Remove the gel from the tank and lift the longer gel plate away from the gel with a spatula. Wrap the gel in cling film and place in a Perplex box.
7. In a dark room under a safe light, expose a sensitive X-ray film to the gel for 1 min. Use a sharp scalpel to make a couple of cuts through the film to the underlying gel. These cuts will make it possible to orientate the processed film with the gel. Develop the film.
8. Cut a small hole into the film where the full-length probe is visible as a black area. Orientate the film with the gel and cut out the part of the gel containing the probe. Transfer the gel piece into a microcentrifuge tube that has 400 µL of prewarmed elution buffer. Incubate at 37°C overnight using a shaker, if available.
9. Add 1 mL of absolute ethanol, incubate on dry ice for 5 min, and centrifuge at 16,000*g* for 10 min at room temperature.
10. Discard solution and dry the probe.
11. Dissolve the probe in 50 µL of hybridization buffer and use 1 µL to determine activity with scintillation counting at the ^{32}P channel (*see* **Note 12**).

3.5. Preparation of a Low-Specific-Activity Probe

Because neurotrophin transcripts are present at low amounts in most tissues and cells, it is often necessary to use several micrograms of RNA in RNAse protection assays. This may cause a problem with the intensity of the signal derived from the internal control probe, such as β- or γ-actin, glyceraldehyde-3-phosphate dehydrogenase (GADPH). In order to circumvent this problem, it is advisable to prepare control probes that have lower specific activity than the neurotrophin probe. This is achieved by altering the ratio of labeled to cold UTP used in the labeling reaction.

At room temperature, pipet the following into a microcentrifuge tube in this order: 2 µL of 5X transcription buffer, 0.5 µL of 200 m*M* DTT, 1 µL of 4 m*M* ATP, CTP, GTP, 1 µL of 1 m*M* UTP, 0.5 µL RNAse inhibitor RNAsin (90 U), 2 µL of α-^{32}P–UTP (10 mCi/mL, 3000 Ci/mmol), 4 µL DEPC-treated water, 0.5 µL linearized DNA template 500 ng/µL, and 0.5 µL RNA polymerase. Continue as in **Subheading 3.2.**

3.6. Hybridization with RNA Samples

1. Aliquot RNA samples into microcentrifuge tubes and dry them down in vacuum concentrator. Alternatively, add water to bring the volume of RNA solution to 50 µL, add 5 µL of 3 *M* sodium acetate and 150 µL absolute ethanol. Incubate on dry ice for 10 min and collect RNA by centrifugation at 16,000*g* for 10 min at room temperature. Remove liquid and let the pellet air-dry for 10–15 min (*see* **Note 13**).

2. For each RNA sample, prepare 30 µL of hybridization mix containing (5–10) × 10^4 cpm of neurotrophin probe and (5–10) × 10^3 cpm of internal control probe in 1X hybridization buffer (*see* **Note 14**).
3. Pipet 30 µL of the above probe mix into the tubes containing the dry RNA.
4. Dissolve the RNA by vortexing for a few seconds, then allow the solution to stand for a couple of minutes before vortexing again. Repeat once. This is to be certain that all RNA will dissolve.
5. Incubate the hybridization mix at 85°C for 10 min to denature RNA.
6. Transfer immediately to a 45°C water bath and incubate overnight (*see* **Note 15**).
7. Add to each hybridization reaction 350 µL digestion buffer containing 1 U RNAse A and 40 U RNAse T1 (*see* **Note 1**).
8. Incubate at 30°C for 30–60 min.
9. Add 20 µL of 10% SDS and 5 µL of 10 mg/mL proteinase K (*see* **Note 16**).
10. Incubate at 37°C for 15 min.
11. Extract with 200 µL phenol/chloroform/isoamyl alcohol.
12. Carefully transfer 400 µL of the aqueous phase into a clean tube containing 1 µL of 10 mg/mL tRNA. Add 1 mL absolute ethanol and mix well. If necessary, the samples can be stored at –20°C for a few days at this stage. Incubate for 10 min on dry ice and centrifuge at 16,000*g* for 10 min at room temperature.
13. Air-dry the pellet and dissolve in 4 µL of loading buffer.

3.7. Gel Analysis of Protected RNA Fragments

The protected fragments are analyzed in a standard sequencing gel. For best results, use a gel that is 0.4 mm thick and at least 35 cm long. If you have access to a slab gel drier, the gel glass plates do not need to be pretreated. Alternatively, you can dry the gel onto a glass plate in an conventional oven, but then it is necessary to treat the plates to ensure that the gel sticks to only one plate.

1. Carefully wash two glass plates that fit the sequencing eleectrophoresis tank. Rinse the plates with ethanol and wipe them dry. No drying marks should be visible on the plates. Spray the longer of the plates with a small amount of Acrylease™. Spread evenly with a paper towel. Let dry and wipe the glass plate with a damp paper towel. To the shorter plate, apply a freshly prepared mix of 3 mL of solution A and 90 µL of solution B. Spread evenly on the plate by rubbing with a paper towel. Let polymerize for 5 min and wipe excess of silane away with a paper towel dampened with ethanol (*see* **Note 17**).
2. Assemble the two plates with a 0.4 mm spacer and tape the bottom and sides of the assembly. Avoid trapping any air bubbles under the tape, as the gel may leak.
3. In a beaker, mix 31.5 g urea, 7.5 mL of 10X TBE, 11.25 mL of 40% acrylamide:bis (19:1) and about 30 mL water. Warm the solution in a 37°C water bath to dissolve the urea. Transfer the clear solution into a measuring cylinder and add water to bring the volume to 75 mL. Return the gel mix to the beaker, add 500 µL of 10% ammonium persulfate and 75 µL TEMED. Mix by

swirling. Pour the gel between the two glass plates (*see* **Note 18**). Insert shark-tooth combs between the plates so that the straight sides are inside the plates. Clamp plates together with spring clips, two on each side and three holding the combs in place. Let the gel polymerize for 30 min with the comb edge raised about 10 cm. **PRECAUTION:** Wear safety glasses at this step.

4. When the gel has set, remove spring clips and tape. Pull the combs out. Transfer the gel into the electrophoresis tank and fasten in place. Fill buffer tanks with 1X TBE. With a syringe and an 18- to 19-gage needle, wash the hollow where the combs were inserted to remove all remains of loose acrylamide. Insert the combs, this time with the teeth well in contact with the top of the gel.
5. Pipet 1 µL of loading buffer into every other well to be certain that the wells are not leaking. Electrophorese the gel up to 2000 V, 45 mA for 15–30 min to warm it.
6. To prepare loading standards for the gel, take an aliquot of the labeled RNA probes and dilute them with loading buffer to 4 µL. Also aliquot 1–4 µL of labeled DNA or RNA molecular-weight standards into a microcentrifuge tube and bring the volume to 4 µL with loading buffer.
7. Heat the protected RNA samples and loading standards at 95°C for 3 min, transfer to ice, and load into individual wells. Electrophorese the gel at 2000 V, 45 mA for 60–90 min or until the bromphenol blue in the loading buffer has reached the bottom of the gel. The gel should not be too hot to touch. Reduce the current if the gel heats up too much.
8. Remove the gel from the electrophoresis tank and lift the longer plate off in one movement using a thin spatula or a palette knife.
9. Fix the gel on the shorter plate by incubating it in 10% acetic acid for 15 min or until the bromphenol blue has turned yellow. Wash the gel with tap water for 10 min to remove urea, which will otherwise quench the signal.
10. Either dry the gel onto the glass plate in an 80°C fan oven for 15–20 min or use a sheet of Whatmann 3MM filter paper to lift the gel off the plate and transfer into a slab gel drier. Set heating to 80°C and let the gel dry for 10–15 min.
11. Transfer the gel into an autoradiography cassette and expose a sensitive X-ray film for 1–5 d. Alternatively, use a phosphorimager to visualize the protected fragments (*see* **Note 19**).

3.8. Preparing Labeled DNA Molecular-Weight Markers

Both DNA and RNA molecular-weight markers can be used in RNAse protection gels. If it is important to determine the exact length of protected fragments, the use of RNA markers is recommended, as DNA and RNA have different mobilities in these gels. The size of RNA fragments is overestimated by 5–10% if DNA markers are used.

1. In order to prepare DNA markers, digest 5 µg Bluescript-plasmid DNA with 10 U *Hinf*I restriction enzyme, 2 µL of 10X digestion buffer as supplied with the enzyme, and water to 20 µL. Incubate at 37°C for 2 h or more. Heat to 65°C for 15 min. (*See* **Note 20**.)

2. To 2 µL of digested plasmid–DNA, add 3 µL of 10X Klenow buffer, 0.5 µL of 10 m*M* dCTP, dGTP, dTTP, 3 µL of α-^{35}S-dATP (10 µCi/µL, 3000 Ci/mmol) 10 U Klenow, and water to 30 µL (*see* **Note 21**).
3. Incubate at room temperature for 30 min.
4. Add 3 µL of 3*M* sodium acetate and 90 µL absolute ethanol. Incubate on dry ice for 5 min and centrifuge at 16,000*g* for 10 min.
5. Dry the pellet and dissolve in 100 µL of loading buffer.

3.9. Preparing Labeled RNA Molecular-Weight Markers

Cut the template DNA that is used to generate the neurotrophin probe with three or four different restriction enzymes in different tubes. These enzymes should be selected so that they cut approximately 50, 100, 200, and 300 bp downstream of the RNA polymerase binding site. After digestion, combine the digests and precipitate with 1/10 volume of 3 *M* sodium acetate and 3 vol of absolute ethanol. Dissolve the pellet in a suitable amount of water to yield a DNA concentration of 500 ng/µL. Use this template mix to generate a low-specific-activity probe. This marker RNA need not to be rigorously purified; precipitation with ethanol is sufficient. Dissolve the labeled RNA marker in 50 µL loading buffer and store in –20°C.

4. Notes

1. Besides a good quality probe, this is perhaps the second most critical step in RNAse protection assays. It is of paramount importance to use good quality RNAses. This author recommends the use of Ambions RNAse A and T1 that have been validated for RNAse protection assay. As a starting point, use 1 U of RNAse A and 40 U of T1 in the digestion step. (These units refer to those specified by Ambion. Other suppliers may express the activity of their RNAses in other units. If unsure how much RNAse to use, test several concentrations to find the optimal range.) If this results in excessive background, which makes it impossible to discern correctly protected fragments, and if you have background signal in the tRNA lane, use more of the RNAses to titrate optimal amounts to be used with your probe and samples. Using too much RNAses is not advisable, as it will result in the degradation of RNA–RNA hybrids and decrease correct signal. On the other hand, if tRNA lane is clean, but sample lanes have a high background and little correctly protected fragment, it is best to test RNAse T1 alone. Probes that have a high A+T content may form RNA–RNA hybrids that can breath during digestion. This will result in internal cleavage and reduction in the sensitivity of the assay. The extent of this problem depends on the sequence of the probe. If the undigested probe shows predominantly shorter than full-length fragments and sample lanes contain high background, it is necessary to gel-purify the probe. It may be necessary to optimize the RNAse digestion step for each probe used.

2. It is beyond the scope of this chapter to give detailed instructions on how to clone a suitable fragment and how to purify plasmid DNA. If the reader is unfamiliar with general molecular-biology methods, *Current Protocols in Molecular Biology* ***(3)*** and *Molecular Cloning: A Laboratory Manual* ***(4)*** describe basic techniques. Another good source of information is *The Source of Discovery* ***(5)***.
3. This is important whether you made the construct yourself or received it from elsewhere. The success of RNAse protection assay is critically dependent on the use of a fully complementary antisense probe.
4. Do not use restriction enzymes that leave protruding 3′ ends, as these can lead to the generation of a sense probe that will result in a high background. Consult the catalog of your restriction enzyme supplier for more information on the enzyme you have chosen. A good length for a probe is from 100 to 400 bp. As probes typically contain some vector sequences, this will yield a protected fragment of about 80–380 bp. Fragments of this length are easily separated in standard sequencing gels. If multiple probes are to be used or one probe will generate several protected RNA species, make sure that these fragments differ by at least 20 bp. It is advisable to make the internal control probe the shortest, as this reduces the likelihood of it masking lower abundance signals derived from neurotrophin transcripts. Also, longer protected fragments contain proportionally more labeled nucleotides, which aids in the detection of rare transcripts.
5. If no dry ice is available, incubate at –20°C for 30 min. All centrifugations in these protocols can be performed at room temperature.
6. Other labeled ribonucleotides except ATP can be used. ATP results in poor labeling.
7. This reaction can be scaled up if necessary. The specific activity of the probe is determined by the ratio of labeled to cold UTP in the labeling mix. If necessary, the reaction can be performed in a volume of 20 µL using 10 µL of α-^{32}P–UTP (10 mCi/mL, 3000 Ci/mmol), leaving out the cold UTP and doubling the volume of the other reagents. Adjust the final volume to 20 µL with water. Under these conditions, the total amount of RNA synthesized is less than in the standard protocol, but its specific activity is higher as all incorporated UTP is labeled. It is worth noting that the probe has to be in excess of the mRNA species being measured, so this modification is only recommended for situations when it is difficult to obtain a visible signal with standard probes.
8. This step is included to reduce the appearance of fully protected probe in RNAse protection gels after digestion. Newly synthesized RNA may stay hybridized with a small portion of the template DNA, which is then protected from DNAse I digestion.
9. A typical probe will be $(1–10) \times 10^5$ cpm/µL.
10. Although this author prefers the Qiagen columns for their ease of use, other spin or free-flow chromatography columns that have been designed to separete separate nucleotides from RNA can be used here and can be obtained from several molecular-biology-grade reagent suppliers.
11. First, try purifying the probe with either ethanol precipitations or column chromatography and test it in the RNAse protection assay. If the probe yields a high

background when RNAse digestion has been optimized, it is necessary to gel purify the probe, as this will clean up the signal considerably.

12. It is difficult to elute all probe from the gel slice, but all eluted probe should be precipitated. Only a fraction of the original activity will be recovered, but this should still be enough for up to 50 hybridizations.
13. The RNA that is used as samples in RNAse protection assays should be of good quality. Several methods exist for obtaining RNA from cells and tissues. Fernyhough (Chapter 5) has described one method that is suitable for isolating total RNA from both tissue samples and cultured cells and does not require ultracentrifugation. As RNAse protection assay requires that only a small portion of the mRNA molecule is intact, it can tolerate some degradation of RNA without appreciable decrease in signal, unlike Northern blotting. All RNA samples should be stored at –70°C. Varying amounts of RNA can be used in RNAse protection assay. Whereas it is possible to detect abundant mRNA species in less than 1 µg total RNA, low-abundance mRNA species such as neurotrophins may require up to 100 µg of total RNA. As sample RNA gets degraded before gel analysis, using large amounts of RNA is not a problem. Therefore, isolation of poly(A)–RNA is not usually required.

    Always include tRNA as a negative control sample, which will show you how well the RNAse digestion worked. If sample RNA containing the neurotrophin of your interest is in short supply, it is a good idea to make synthetic neurotrophin mRNA that can be used to set up hybridization and digestion conditions. If the cloning vector for the template DNA that was used to generate the neurotrophin antisense probe contains two RNA polymerase promoters flanking the neurotrophin sequence, it is possible to use this construct to synthesize the cRNA. Cut the plasmid DNA with a restriction enzyme that does not cut within the neurotrophin sequence, but cuts the plasmid downstream of the insert (*see* **Fig. 1**). Purify this template and use it with the RNA polymerase that recognizes the upstream promoter to synthesize RNA. When preparing cRNA, substitute the labeled nucleotide with 1 µL of 4 m*M* UTP and purify the cRNA by precipitating it as described in **Subheading 3.2.** Serial dilutions of this RNA should be analyzed to find out suitable amounts to yield linear signal. It is useful to always include this RNA as a positive control in assays containing unknown samples.
14. Use as little probe as possible, as this will result in less background. The probe should always be in excess over the mRNA species being measured. This is best tested by analyzing varying amounts of the same RNA sample. A linear response should be obtained. This is usually not a problem for rare mRNAs as neurotrophin transcripts, but can become an issue for internal control probes that detect much more abundant transcripts; hence, the need for synthesizing probes of lower specific activity that can be used at higher molar concentrations. If you are using 18S ribosomal RNA as control with total RNA, then this probe must be prepared using nonlimiting amounts of all ribonucleotides and a small amount (0.05–1 µCi) of labeled UTP. This is because about 20% of total RNA is 18S RNA and 500–1000 ng of probe (100–200 bp long) are needed for each 10 µg of total RNA in the sample

to ensure that the probe is in excess of target. The use of special labeling kits such as the Maxiscript™ from Ambion (Austin, TX, USA) is recommended for this purpose.

15. The hybridization temperature can be varied. Temperatures up to 60°C can be used and a higher hybridization temperature may give cleaner results. If pressed for time, hybridization of 3–4 h may give satisfactory results.
16. This step is necessary to remove the RNAse before RNA–RNA hybrids are denatured prior to gel electrophoresis.
17. Clean glass plates guarantee a bubble-free gel. Make sure that the paper towels do not leave lint.
18. Gel mix can either poured between the plates while holding them at an angle, or a 50 mL syringe can be used. If air bubbles get trapped between the glass plates, hold the gel vertically and gently tap to remove them. Try to work fast as the gel may set in a few minutes. Always prepare a fresh gel for best results.
19. The use of Kodak X-ar 5 or any equally sensitive film is recommended. A signal should be visible after 1–2 d exposure to film. If precise quantification of signals is required, a phosphorimager, such as the ones manufactured by Bio-Rad or Molecular Dynamics, may be used.
20. Any other plasmid/enzyme combination may be used, provided that the digest results in a known pattern of fragments that are 50–400 bp long and can be labeled with a fill-in reaction with Klenow DNA-polymerase. This requires a 5′ overhang after digestion.
21. The use of ^{35}S–dATP is recommended, as it results in a marker that is usable for several months. ^{32}P nucleotides may also be used, but because of their shorter half-life, a fresh marker needs to prepared more frequently.

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7

Quantification of Neurotrophin mRNA by RT-PCR

Edward Chie, Dennis Liu, Xin-Fu Zhou, and Robert A. Rush

1. Introduction

Neurotrophin mRNAs are expressed at a wide variety of levels in many types of neurons and nonneuronal cells both within the central and peripheral nervous systems as well as in cells unrelated to neuronal function ***(1–6)***. However, in mature animals, most tissues synthesize neurotrophin mRNAs in low abundance. Low recoveries of RNA isolated from limited amounts of tissues adds to the problem of quantification of individual mRNA species. The required sensitivity is often beyond the detection of conventional quantification methods, such as Northern blot (*see* Chapter 5), S1 nuclease, and RNase protection (*see* Chapter 6) assays, particularly if several neurotrophin species are to be measured. *In situ* hybridization allows detection within individual cells but lacks quantification strength. For these reasons, a reverse transcriptase–polymerase chain reaction (RT-PCR) method has been developed for its high sensitivity and versatility, where total RNA is first reverse transcribed and then amplified by PCR reaction ***(7,8)***. The technique can be used to detect the presence or absence of gene transcripts, to measure expression levels semiquantitatively for comparison of mRNA levels in different tissues, or to determine the absolute amount of mRNA in a tissue if the competitive form of the assay is used.

The power of RT-PCR is based on the exponential amplification of the PCR reaction by a thermal DNA polymerase, even after many reaction cycles. The amount of PCR product formed is proportional to the initial amount of mRNA being investigated ***(9)***. The efficiency of the PCR amplification is determined by the properties of all components in the reaction including amplification cycle number, reaction temperatures, reagent concentrations, the nature of the primers, and the specific sequence to be amplified. A small change in any param-

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eter affects the amplification efficiency that results in a greater change in the amount of PCR products. It is essential that, in different PCR reactions, efficiencies remain constant during the exponential amplification phase to allow quantitative comparison between samples.

Relative quantification of neurotrophin mRNAs by RT-PCR methods have been used previously. In this chapter, we describe a quick, simple, and reliable quantitative solution to accurate measurement of changes of neurotrophin mRNA levels in normal and experimental tissues. The assay involves a first-strand complementary DNA (cDNA) synthesis, followed by PCR amplification and image analysis of fluorescent-stained gels. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is used as an internal control for monitoring any variation between tissue samples ***(10)***. The relative level of neurotrophin mRNA is calculated from the ratio of PCR product of neurotrophin mRNA to that of the GAPDH control. The GAPDH control is amplified in parallel in separate tubes. This avoids any interference between amplifications of neurotrophin cDNA and the GAPDH control in the same tube that would occur as a result of the expressed GAPDH consuming the majority of reagents ***(11)***.

2. Materials

1. Dissection forceps, scissors, and microscope.
2. Water bath or dry block heater.
3. Plastic disposable pestles and matching round-bottom 1.5-mL reaction tube.
4. Polytron homogenizer.
5. Total RNA isolation reagent (Advanced Biotechnologies, UK, or equivalent).
6. Chloroform.
7. Desktop centrifuge (approx 21,000*g*).
8. Isopropanol.
9. 75% ethanol, ice cold.
10. DEPC-treated H_2O (Sigma): In a fume hood, add diethylpyrocarbonate (DEPC) by 0.1% (v/v) to deionized water. The solution is then shaken and autoclaved.
11. RQ1 RNase-free DNase (5 U/µL, Promega, USA).
12. 0.5-mL PCR tube.
13. Oligo-$(dT)_{15}$: 15-mers of oligodeoxythymidine.
14. RNasin ribonuclease inhibitor (20–40 U/µL, Promega, USA).
15. AMV reverse transcriptase (5 U/µL, Promega, USA).
16. 5X reaction buffer for AMV transcriptase: 250 m*M* Tris-HCl (pH 8.3), 250 m*M* KCl, 50 m*M* $MgCl_2$, 2.5 m*M* spermidine, and 50 m*M* DTT.
17. 10 µ*M* PCR primer pair stocks (Crude purity, Bresatec, Australia).
18. 100 m*M* dNTP (Promega, USA).
19. RT Master Mix for 10 reactions: 5 µL of RNA inhibitor, 60 µL of 5X AMV reaction buffer, 20 µL of 10 m*M* dNTP, 5 µL of AMV transcriptase (5 U/µL).

20. 25 m*M* $MgCl_2$ (Advanced Biotechnologies, UK).
21. DNA thermal cycler (Perkin Elmer, USA, or equivalent).
22. Red Hot DNA polymerase (5 U/µL, Advanced Biotechnologies, UK, or equivalent).
23. 10X reaction buffer DNA polymerase: 200 m*M* $(NH_4)_2SO_4$, 750 m*M* Tris-HCl (pH 8.8), 01% (v/v) Tween-20.
24. PCR Master Mix for 10 reactions: 25 µL of 10X reaction buffer, 50 µL of 10 µ*M* primer pair, 20 µL of 25 m*M* $MgCl_2$, 10 µL of 10 m*M* dNTP.
25. Dilution of Red Hot DNA polymerase for 20 reactions: 87.5 µL of H_2O, 10 µL of 10X PCR reaction buffer, 2.5 µL of Red Hot DNA polymerase.
26. Nuclease-free mineral oil (Sigma, USA); not autoclaved.
27. Vacuum.
28. 6X DNA loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400) in water.
29. 5X TBE buffer: 0.45*M* Tris-borate, 0.01 *M* EDTA. Working solution is made by a 10-fold dilution.
30. 0.25 µg/mL ethidium bromide in 0.5X TBE buffer.
31. Electrophoresis apparatus and power pack (Biorad): Use double comb with (20 wells) in 1 gel for loading 40 samples.
32. Rotating shaker.
33. FluorImager 595 and ImageQuaNT software (Version 4.2a, Molecular Dynamics).

3. Methods

3.1. RNA Isolation

Total RNA is prepared from a variety of tissues according to Chomczynski and Sacchi ***(12)*** using a commercial RNA isolation reagent. The homogenization is a key step to the successful preparation of high-quality total RNA in terms of yield and DNA contamination. The procedure is as follows:

1. Dissect tissues from animals and freeze their aliquots (10–50 mg) in liquid nitrogen quickly. A deep freezer (–70°C) is used for long-term storage of tissue samples.
2. For minipreparations, transfer 10–50 mg fresh or frozen tissues to round-bottom 1.5-mL reaction tubes containing 400–800 µL of RNA isolation reagent and homogenize with plastic pestles (*see* **Note 1**). Go to **step 3** if the starting material is cultured cells ($[5–10] \times 10^6$).
3. Pass the homogenate through a 23-gage needle (0.63 mm × 25 mm) at least three times using a 1- or 2-mL syringe.
4. Store the samples for 5 min at room temperature to dissociate the nucleoprotein complex.
5. Add 0.2 volume of chloroform to the homogenates and hand-shake for 15 s.
6. Centrifuge (21,000*g*) for 15 min at 4°C. Transfer the upper aqueous phase carefully to a fresh tube on site and avoid touching the white middle layer. A second round of chloroform extraction is necessary if the supernatant is turbid because of a high protein content.

7. Precipitate RNA by mixing with an equal volume of isopropanol and store on ice for 10 min or longer for tissues with low recovery.
8. Recover the RNA sample by centrifugation for 15 min at 4°C.
9. Wash with an equal volume of cold 75% ethanol twice by pipeting and spin briefly.
10. Remove residual liquid from the RNA sample by pipet and dry briefly under the cover of a facial tissue to avoid aerosol contamination.
11. Resuspend the RNA pellet in 20–50 µL of DEPC-treated H_2O and incubate for 10 min at 70°C. Vortex the RNA sample vigorously until the solution becomes sticky.
12. The quantity and purity are assessed by the ratio between the optical density (OD) readings at 260 nm and 280 nm, or by electrophoresis on a 2.0% agarose gel. High-quality RNA preparations have OD_{260}/OD_{280} values of 2.0 and show both a 28S and 18S band as well as mRNA smears between them following ethidium bromide staining.
13. Remove possible DNA contamination from RNA samples by treatment with RNase-free DNase (l U/20 µL RNA sample) for 60 min at 37°C. The RNA samples can be used directly for cDNA synthesis without further extraction and purification (*see* **Note 2**).

3.2. cDNA Synthesis

First-strand cDNA is synthesized from the isolated total RNA in a 30-µL reaction ***(13)***. Oligo-$(dT)_{15}$ is used as a primer that allows cDNA to be amplified for multiple mRNA species. To ensure the accuracy of solution transfer, a master mix is made for multiple samples in this step and also in the PCR amplification step (*see* **Notes 3** and **4**). Detailed steps are given as follows:

1. Place 1–18.5 µL of the total RNA and oligo-$(dT)_{15}$ primer into a 0.5 mL PCR tube. Denature the mixture at 95°C for 5 min and anneal at 65°C for 5 min on a programmed DNA thermal cycler. Take the tube out and allow to cool to room temperature.
2. Add 9.5 µL of freshly made AMV master mix to the tube. All components for the cDNA synthesis are as follows:

Reagent	Volume	Final concentration
Template RNA	1–18.5 µL	
Primer	2.0 µL	20 pmol
AMV Master Mix	9.5 µL	
5X buffer	6.0 µL	1X
RNasin RNA inhibitor	0.5 µL	10–20 U
dNTP (10 m*M* each)	2.0 µL	0.3 m*M* each
AMV transcriptase (5 units/µL)	1.0 µL	5 U
Double-distilled, sterile H_2O to	30.0 µL	

3. On a programmed PCR DNA thermal cycler, incubate the reaction mixture at 42°C for 30 min and 48°C for 30 min. Then, heat to 95°C for 5 min to dissociate and inactivate the transcriptase and also to denature the RNA and cDNA hybrid.

4. Dilute the cDNA into 100 µL and store the aliquots at –20°C. These are stable for at least a year. The cDNA is usually enough for the analysis of a minimum of 20 individual mRNA species.

3.3. PCR Reaction

Neurotrophin mRNA from many tissues can be detected using 27–35 amplification cycles in optimized PCR conditions (*see* **Notes 5** and **6**). The starting amount of cDNA and optimal cycle number for amplification of each mRNA species are determined by preliminary PCR reactions. To monitor contamination from any source and validation of any PCR reactions, both positive and negative controls should be included. The positive control also serves as a quantification control for analysis of any interassay variability.

1. Combine 10.5 µL of the PCR master mix, cDNA sample, and water in a DNase/RNase-free 0.2-mL or 0.5-mL thin-wall reaction tube.
2. Denature the mixture at 95°C for 5 min and store on ice throughout the following steps. Spin briefly to ensure all liquids are collected at the bottom. This step improves the performance of cDNA amplification by overcoming nonspecific primer binding to the cDNA template.
3. Add 5.0 µL of the diluted thermal DNA polymerase to each tube. All components for the PCR reaction are as follows:

Reagent	Volume	Final concentration
PCR master mix	10.5 µL	
10X buffer (without Mg^{2+})	2.5 µL	1X
Primer mix (10 µ*M* each)	5.0 µL	1.6 µ*M* each
dNTP (10 m*M* each)	1.0 µL	0.3 m*M* each
$MgCl_2$	2.0 µL	1.6 m*M*
Template DNA	1–14.5 µL	
DNA polymerase diluted	5.0 µL	0.625 U
Double-distilled, sterile H_2O to	30.0 µL	

4. If a PCR thermal cycler without heating lid is used, overlay the top of reaction with two drops (30 µL) of nuclease-free mineral oil to prevent condensation and evaporation.
5. Heat the tubes at 95°C in the thermal cycler for 2 min and proceed with the amplification for 23–35 cycles at a programmed profile of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s (*see* **Note 6**).
6. After the last amplification cycle, transfer the reaction to a water bath prewarmed at 72°C and incubate for a further 10 min to complete the extension of both DNA strands and cool the reaction mix to 4°C. The samples can be analyzed immediately or stored at –20°C.

3.4. Data Analysis

The traditional procedure for DNA quantification is analysis of radioactive counts of DNA after Southern transfer and DNA hybridization, which is a

multiple-step and time-consuming technique. The laser scanning instrument, FluorImager 595, provides a significant advantage for fluorescent analysis of dye stained gels because it allows the direct use of ethidium bromide staining for quantification of the PCR products ***(14,15)***. In a saturated solution of ethidium bromide, there is a good linear correlation between fluorescence intensities and DNA bands at the low range of DNA concentration, thus a lower PCR cycle number is preferred to reduce the fluorescent value of DNA band. The following is a step-by-step guideline (*see* **Note 7**):

1. Prepare a 1.8 % agarose gel and solidify at least 0.5 h before use.
2. Add 5 µL of 6X DNA loading buffer to the PCR products (30 µL) after removing mineral oil by vacuum.
3. Run the DNA samples onto the agarose gel until the first dye marker has migrated about 2 cm.
4. Immerse the gel into ethidium bromide staining solution and shake gently for 15 min.
5. Wash briefly with water and place the stained gel onto a plate screen for laser scanning using the FluorImager 595.
6. In the scan program, set "Pixel" at 100 Micron, "Detection" at Normal, and "Digital resolution" at 16 bits in the scan program and capture digital images.
7. Open image files with ImageQuaNT software and select bands within same-sized rectangles. Quantify fluorescence value by volume report based on local average background.
8. Calculate the ratio of fluorescence value of the DNA amplified from neurotrophin cDNA to that of the GAPDH control.

3.5. Quantitative Assay

The primary reason for developing the relative quantitative RT-PCR assay was to measure the changes of gene expression of neurotrophin at the mRNA level during development and under different experimental conditions. We have found it essential to determine appropriate amplification cycle numbers for each individual neurotrophin mRNA species in each specific tissue. The cycle number should be located in a range where the cDNA is amplified linearly. Data from the plateau phase should not be considered for quantification. Standard curves are constructed by using the same amount of cDNA and different cycle numbers. The linear ranges for the control and all mRNA species to be assayed must be found by preliminary testing.

Figure 1 shows a standard curve for NT-3 and GAPDH amplification from spinal cord RNA of young rat. The cDNA was amplified for NT-3 at 27, 29, 31, and 33 cycles and for the GAPDH control at 19, 21, 23, and 25 cycles because of its higher abundance in total RNA. The linear range of the PCR reaction occurs across 8 cycles before reaching the plateau phase. This permits a consistent ratio of values obtained from neurotrophin cDNA to control, and a

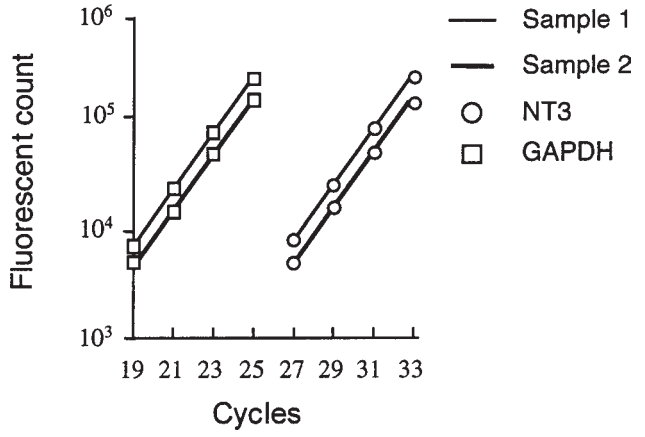

Fig. 1. Linear relationship of PCR amplification of neurotrophin 3 (NT-3) and GAPDH cDNAs isolated from young rat spinal cords. PCR amplifications were performed at 19, 21, 23, and 25 cycles for GAPDH and 27, 29, 31, and 33 cycles for NT-3. The log of fluorescence values of each sample are plotted against the amplification cycle numbers.

minimum 30-fold change measurable in the linear range when adjusted for a lower cycle number for the GAPDH control and a higher cycle number for the neurotrophin mRNA. There are two groups of parallel lines present in the linear range of a standard curve (**Fig. 1**), indicating that the amplification efficiency for individual mRNA species (NT-3) or control (GAPDH) are the same from two tissue samples. This is important for achieving accuracy for a relative quantification analysis.

Reproducibility is another important issue for this assay. The use of a ratio of specific mRNA to the GAPDH internal control eliminates sample-to-sample variations. In addition, an assay control is used to evaluate any day-to-day variations. Our results show quite small standard errors, even when only three animals are used to collect experimental data (**Fig. 2**). In our experience, the quality of the total RNA isolation is most important in the determination of sample-to-sample variations. Total RNA isolation from tissues of experimental animals are always run together with those from normal and sham control animals.

Inhibition is encountered occasionally in PCR amplification of neurotrophin mRNA samples prepared from some tissues, such as the mesenteric artery. An additional round of phenol extraction and ethanol precipitation is performed to remove possible factors that are inhibitory to AMV reverse transcriptase or DNA polymerase. A high concentration of glycerol in the sample may reduce

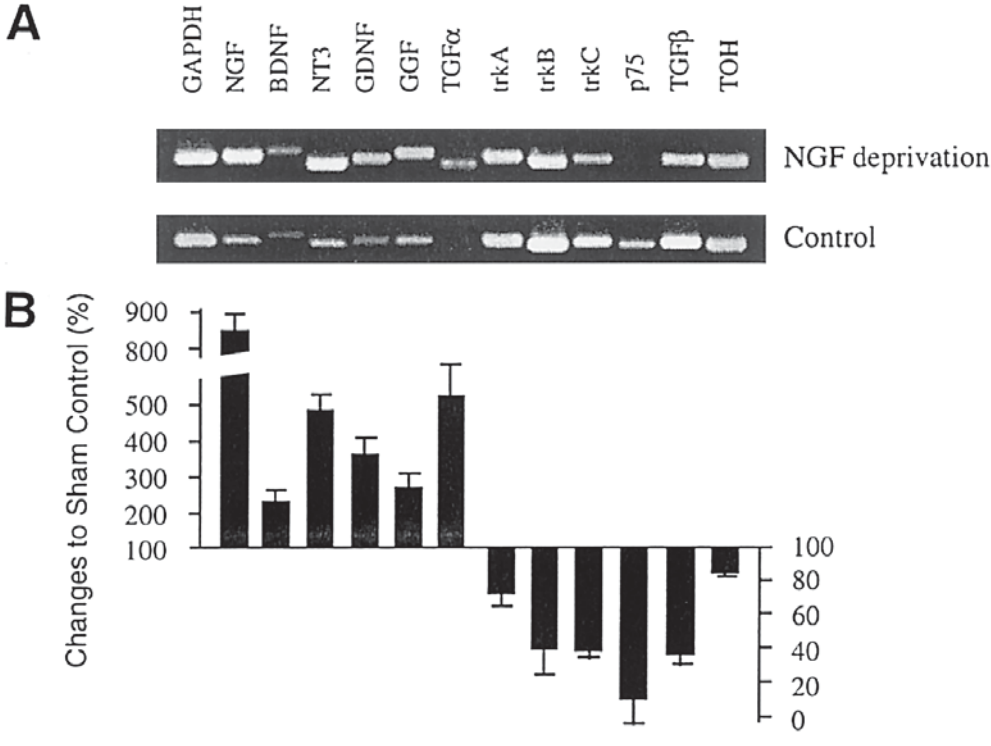

Fig. 2. RT-PCR quantification assay of effects of nerve growth factor (NGF) deprivation for 7 d on mRNA levels of growth factors and their receptors in superior cervical ganglia of young adult rats. The PCR cycle number for amplification are 33 for NGF, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, transforming growth factor (TGF)-α, and glial growth factor; 30 for high-affinity tyrosine kinase receptor (trk) B, trkC, low-affinity neurotrophin receptor p75, and TGF-β; 27 for trkA and tyrosine hydroxylase; and 23 for GAPDH. The PCR products were electrophoresed on a 1.5% agarose gel and were stained in 0.25 μg/mL ethidium bromide solution. Changes of mRNA levels are expressed as the percentage of the value of sham rats, which are set at 100% **(B)**. Values shown here are the means of three rats; error bars indicate ± standard errors of the means.

the activity of AMV reverse transcriptase or DNA polymerase. This could be avoided by reducing the volume of the sample for the cDNA synthesis or PCR reaction and the use of more DNA polymerase or more amplification cycles.

4. Notes

1. We have measured neurotrophin mRNA levels using the RT-PCR assays in an extensive range of tissues. It is convenient to use a plastic pestle as a homogeni-

zation tool when working with multiple tissue samples. The Polytron homogenizer is only used for some tissues, such as mesenteric artery and muscle.

2. The treatment with RNase-free DNase is required only for the RNA sample with possible DNA contamination. After the inactivation of DNase, the RNA sample is used for cDNA synthesis directly with no need of re-extraction with phenol and chloroform. This saves time for the assay and is especially good for analysis of limited samples. The efficiency of the cDNA synthesis may be affected slightly.
3. Small tips for handling samples or buffers are helpful. Always spin tubes with liquids sticking on the tube lid or wall. Always ensure tube lids are capped after transfer of sample or reagent. Aliquot buffers and cDNA samples, and avoid frequent freezing and thawing. Thaw frozen buffer quickly and thoroughly by vortexing, particularly for Mg^{2+} used for the PCR reaction.
4. Contamination may become a serious problem in this assay, mostly in the PCR amplification step. It is therefore necessary to include both positive and negative controls at all times when the assay is performed. The negative controls should be both an amplification of the total RNA sample using an amount equivalent to the cDNA sample and the PCR amplification using water instead of a cDNA template.
5. Primers for the PCR amplification are designed as 21–24 mers and are not required purified. The G + C content is kept at 50–60% to make the annealing temperature at 55°C for all mRNA species. Longer primers ensure higher specific binding to DNA template. The 3′ ends of the primers should not be self-complementary or complementary to each other to reduce the formation of primer–dimers. Multiple, nonspecific amplification products may result from poor PCR primer design and high concentrations of primers. It is preferred that one primer pair generate one single band.
6. Optimize Mg^{2+} concentration, annealing temperature, extension time, and cycle number to minimize nonspecific priming. An extra step of denaturation for cDNA sample in the preparation of PCR reaction, and a hot start for PCR amplifications are also helpful.
7. No difficulties should occur in the electrophoresis step, but extra care should be taken in handling PCR products. Improper operations in removal of mineral oil and loading DNA onto agarose gel can generate large errors in the assay.

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8

Sensitive and Nonradioactive *In Situ* Detection of Neurotrophin mRNAs in the Nervous System

Cory J. Xian and Xin-Fu Zhou

1. Introduction

In situ hybridization, a unique method for evaluating gene expression, allows nucleic acid sequences to be detected in morphologically preserved chromosomes, intact cells, and tissue sections. The localized sequences within the cells can be viewed in relation to the morphological features of tissue sections, thus permitting anatomically meaningful interpretations of gene activity. In neurobiology, *in situ* hybridization is a commonly used sensitive technique to identify, localize, and study regulation of mRNA expression for neurotrophins or neural growth factors in tissue sections. Neurotrophins, such as nerve growth factor (NGF), neurotrophin-3 (NT-3), and brain-derived neurotrophic factor (BDNF), are neurotrophic factors produced by effector tissues, neurons, and glial cells, and regulate differentiation, survival, and functions of neurons in the central and peripheral nervous systems. Functional roles of neurotrophins are often closely related to their gene expression during development. Detection of their mRNA expression in developing and in experimental animals has accumulated considerable data revealing many biological functions of these molecules in the normal development and in pathological conditions of the nervous system ***(1,2)***.

We have developed a protocol for a nonradioactive, quick, and sensitive *in situ* detection of neurotrophin or growth factor mRNAs in nervous tissue sections. This protocol, using nonradioactive cRNA probes labeled with a stable hapten, digoxigenin (DIG), and a sensitive immunoenzymatic colorimetric detection ***(3)***, eliminates the problems associated with radioactive probes of the conventional *in situ* hybridization technique, including safety requirements,

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limitation of probe shelf life, and extensive time required for autoradiography. In this chapter, materials and methods are described for the generation of riboprobes, preparation of slides and tissue sections, pretreatment of sections on slides, hybridization, posthybridization washes, and immunohistochemical detection of target sequences.

2. Materials

2.1. DIG-Labeled Riboprobe Generation and Quantitation

1. DNA templates: Plasmid DNAs containing 800 bp rat NGF [pGEM-3Zf(+), 800 bp mouse BDNF, and NT-3 (both pGEM-7Zf) [generous gifts from Dr. Takashi Ueyama and Prof. Emiko Senba, Wakayama Medical College, Japan]) for making RNA probes.
2. Restriction enzymes: Enzymes (stored at –20°C) used to linearize the plasmid DNA templates are obtained from Bresatec (Adelaide, Australia).
3. DIG RNA labeling kit: Stored at –20°C (Boehringer Mannheim, Mannheim, Germany).
4. DIG-labeled RNA probes: Stored at –70°C in aliquots, they are stable for years. Prevent repeated freezing and thawing.
5. Positively charged nylon membrane (Boehringer Mannheim).

2.2. Slide Preparation

3-Aminopropyltriethoxysilane (Sigma, St. Louis, MO, USA).

2.3. Pretreatments of Tissue Sections

1. Proteinase K (Sigma): Make a stock solution of 1 mg/mL in water and store at –20°C in aliquots.
2. Proteinase K buffer: 20 m*M* Tris-HCl, 2 m*M* $CaCl_2$, pH 7.4.

2.4. Hybridization

1. Basic hybridization solution ***(4)***: 50% Formamide, 10% dextran sulfate, 0.05% Triton X-100, 500 µg/mL herring sperm DNA (Boehringer Mannheim), 0.05% polyvinyl pyrrolidone, and 5X SSC (750 m*M* NaCl and 75 m*M* Na citrate, pH 7.0). Stored at –20°C in aliquots.
2. Hybridization solution with probe: Prior to hybridization, dilute probes in basic hybridization solution to 0.5 µg/mL, and heat the mixture at 85°C for 10 min to denature the probe.
3. Ribonuclease (RNase A) (Boehringer Mannheim): Used in negative controls to destroy target mRNA prior to hybridization.

2.5. Posthybridization Washes

20X SSC stock solution: 3 *M* NaCl, 300 m*M* Na citrate, pH 7.0. Make working solutions of 2X, 1X, and, 0.1X SSC and warm up at 45°C over night prior to use.

2.6. Immunoenzymatic Colorimetric Detection

1. Anti-DIG sheep IgG conjugated with alkaline phosphatase: Stored at 4°C (Boehringer Mannheim).
2. Blocking reagent (Boehringer Mannheim): Heat (at approx 70°C) and stir to make a 10% (10X) stock solution in maleic acid buffer (1 *M* maleic acid, 1.5 *M* NaCl, pH 7.5). Autoclave and store at – 20°C in aliquots. Dilute in water to make a 1% working solution.
3. NBT/BCIP alkaline phosphatase substrate (Boehringer Mannheim): Store at 4°C. Dilute 1 : 200 in 100 m*M* Tris-HCl, 100 m*M* NaCl, 50 m*M* $MgCl_2$ (pH 9.5) prior to use.
4. Glycerol gel-mounting medium (Dako, Carpinteria, CA, USA).

3. Methods

3.1. In Vitro Transcription for Riboprobe Generation

In vitro transcription labeling of cRNA probes represents the best strategy to generate sensitive probes ***(5)***. In our study, we make riboprobes from a linearized DNA template that is cloned into a transcription vector containing a promoter for SP6, T7, or T3 RNA polymerase.

1. Linearize the template plasmid DNA using an appropriate restriction enzyme to create a 5′ overhang and purify linearized DNA by standard phenol extraction and ethanol precipitation (*see* **Note 1**).
2. Prepare DIG-labeled RNA probes by in vitro transcription using a DIG RNA labeling kit according to the kit's instruction. The probes are purified by ethanol precipitation and stored in 10-µL aliquots at –70°C.

   For NGF, antisense and sense riboprobes were made using a DIG RNA labeling kit according to instructions from a ribovector pGEM-3Zf(+) containing 800 bp rat NGF after being linearized by *Eco*RI and *Hind* III and using SP6 and T7 RNA polymerase, respectively. For both BDNF and NT-3, antisense and sense probes were made from a pGEM-7Zf vector containing 800 bp mouse BDNF or 800 bp mouse NT-3 after being linearized with *Eco*RI and *Bam*HI, and using SP6 and T7 polymerase, respectively.
3. To ascertain an optimal probe concentration for obtaining optimal and reproducible hybridization, the yields of the DIG labeling are estimated by dot blots. Serial dilutions of labeled probes and serial dilutions of a control labeled RNA (included in the DIG–RNA labeling kit) are spotted on a nylon membrane and detected according to the supplier's instructions (Boehringer Mannheim). The concentrations of the labeled probes are estimated by comparing the detection signals of the serially diluted probes with those of the control labeled RNA.

3.2. Tissue and Slide Preparation

To ensure that glass slides adhere tissue sections strongly and free of RNase, slides are subjected to the following treatments prior to use:

1. Slides are placed in metal slide racks, washed with ethanol in glass solvent tanks for 1 h, and dried at 70°C.
2. Slides in racks are submerged for 1 min in 2% 3-aminopropyltriethoxysilane in acetone and rinsed in acetone.
3. Slides in rack are baked at 180°C for 2 h and stored dust-free after cooling.
4. Onto these pretreated slides, paraffin sections 4 μm thick are cut from tissue specimens dissected from rats perfuse-fixed with 10% formalin.
5. The sections are dewaxed first by heating slides in a slide rack at 60°C, followed by twice incubation at room temperature in xylene of 10 min each, and one incubation for 10 min in xylene : ethanol (1 : 1).
6. Wash in ethanol for 10 min. The sections are then hydrated through 70% ethanol for 5 min, then in water for 10 min.

3.3. Pretreatments of Tissue Sections

Pretreatments of sections are important steps, as they increase target accessibility for the probe and improve the signal-to-noise ratio (*see* **Note 3**).

1. After the rehydration step, treat the sections with freshly prepared 0.2 *N* HCl for 20 min at room temperature.
2. After washing for 10 min in water, sections are permeabilized with 1–10 μg/mL proteinase K for 20 min at 37°C (the optimal concentration must be determined by an individual researcher for different tissues) in 20 m*M* Tris-HCl and 2 m*M* $CaCl_2$, pH 7.4.
3. Sections are then fixed for 5 min in freshly prepared 4°C, 2% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4).
4. Sections are neutralized for 10 min with 0.2 % glycine in PBS.
5. Following washing in water for 10 min and dehydration in 70% and 100% ethanol 4 min each; sections are ready for hybridization.

3.4. Hybridization

The aims of hybridization are to maximize the reaction of the probe with the target sequence while minimizing the extent of potential nonspecific interaction. These aims can be met by controlling the balance of the various components of the hybridization solution and the hybridization temperature. The following protocol works generally well for the neurotrophin RNA probes on the above-prepared tissue sections. We find it unnecessary to have a prehybridization step to prevent background staining, because this protocol generates good signal-to-noise ratios with neurotrophin RNA probes on paraffin sections. Proper controls are essential to judge whether the hybridization signal is sprecific (*see* **Note 4**).

1. Prior to use, the hybridization mix is made up by combining the basic hybridization solution (*see* **Subheading 2.**) with the probe and vortexing to mix. For

neurotrophin probes, we routinely dilute probes of 1–5 µL label with 100 µL solution (with the probe concentration approx 0.5 µg/mL). For an optimal signal and a minimal background, the optimal probe concentration needs to be predetermined by using serial dilutions of the probe.
2. Denature the diluted probe by heating for 5 min at 85°C.
3. Sections are then applied with sufficient hybridization mix and covered with glass cover slips. Place cover slips carefully to prevent air bubbles. Before use, the cover slips are baked at 180°C to destroy any RNase and stored dust-free, as for glass slides (*see* **Note 3**).
4. Incubate slides in a humidified chamber for 18 h at 52°C. Be sure that the chamber is well sealed to prevent drying out of sections.

3.5. Posthybridization Washes

The aims of posthybridization washes are to dissociate the less stable nonspecific binding or hybridization of the labeled probe to partially homologous sequences or other cellular components. The stringency of the washes to dissociate the partial hybrids can be manipulated by varying the salt concentration and temperature of the washing solutions. The following washing condition generates a clean background in our hand; we do not find it necessary to include a RNase treatment step to degrade the unhybridized probe (it is best to avoid using RNase in the protocol just in case of contamination). Posthydridization washes are undertaken with gentle shaking.

1. Soak slides in 2X SSC solution at 45°C for 5 min to remove cover slips. Most cover slips will drop off easily in the warm washing solution; for those not dropping off, gently remove by sliding the cover slips.
2. Wash twice in 2X SSC at 45°C for 5 min each.
3. Wash twice in 1X SSC at 45°C for 5 min each.
4. Wash once in 0.1X SSC at 45°C for 5 min, and, finally, in 0.1X SSC at room temperature for 5 min.

3.6. Immunoenzymatic, Colorimetric Detection of Hybridized Probes

1. After the posthybridization washes, sections are subjected to blocking of nonspecific binding sites with 1% blocking reagent for 40 min at room temperature.
2. The sections are then incubated for 30 min at 37°C with an alkaline phosphatase-coupled sheep anti-DIG IgG diluted at 1:450 in 1% blocking agent in a humidified chamber. Prevent section from drying out.
3. Wash slides twice for 15 min each in Tris-buffered saline (TBS, pH 7.4) with 0.01% Tween-20, then once in TBS for 5 min.
4. Incubate in diluted NBT/BCIP alkaline phosphatase substrate solution in the dark at room temperature. Dilute substrate stock solution at 1:200 in 100 m*M* Tris-HCl, 100 m*M* NaCl, 50 m*M* $MgCl_2$ (pH 9.5) prior to use. Incubation time can

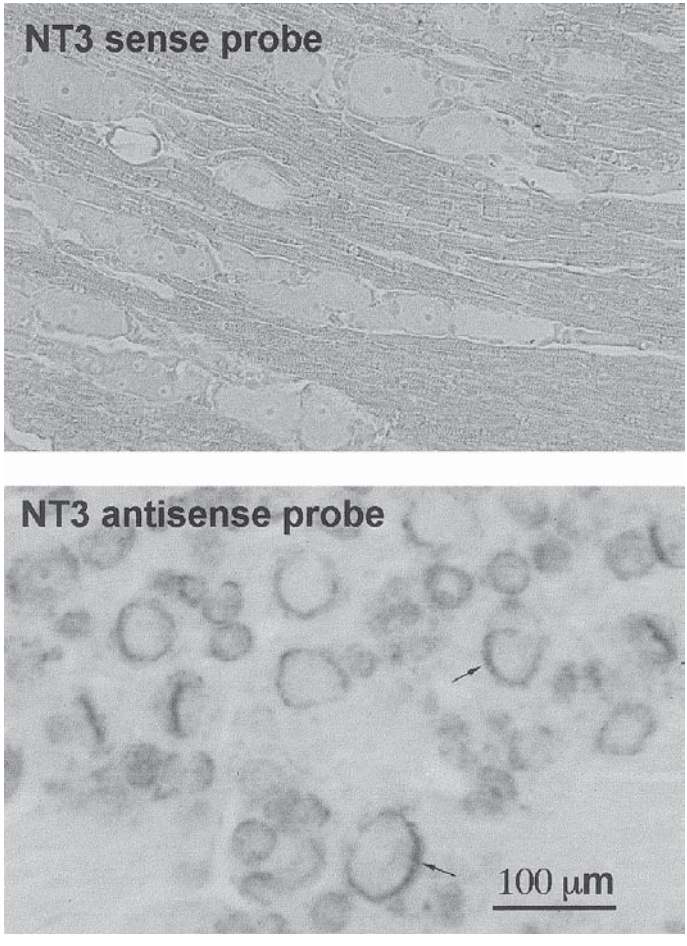

Fig. 1. Micrographs of sections from a lumbar 5 dorsal root ganglion of a rat with sciatic nerve transection for 7 d. NT-3 mRNA was localized in satellite cells around large sensory neurons (bottom panel, arrows). No staining was seen in sections hybridized with sense NT-3 probe (top panel).

vary (from 2 to 18 h) with the abundance of mRNA targets, probe concentration, and antibody concentration, and needs to be predetermined for optimal signal.

5. Sections are gently rinsed with water, counterstained lightly with 0.05% methyl green for 5 min, and mounted with glycerol gel. The sections are observed under a light microscope and images should be captured as soon as possible to avoid fading of the color (**Fig. 1**).

4. Notes

1. Ribonuclease (RNase) contamination and thus RNA degradation in probe preparation and in tissue sections can be a great problem up to and including the hybridization step. Therefore, extreme care should be taken to ensure buffers, water, reagents, slide holders (or racks), tubes, tips, and slide handling are RNase-free. We routinely use 0.1% diethylpyrocarbonate (DEPC) to treat water or buffers overnight, followed by autoclaving and 180°C heat treatment for 2 h for glassware, slides, and cover slips, slide holding racks, and glass solvent tanks. It is important to wear clean gloves at all times.
2. A clean linearized DNA template is imperative for good in vitro transcription labeling of DIG–RNA probes. Use phenol/chloroform extraction and ethanol precipitation to purify linearized plasmid DNA after the restriction digestion.
3. This protocol works well in paraffin sections; however, the tissue specimens need to be fixed appropriately for preservation of mRNA and morphology, and sections need to be cut in RNase-free conditions. For neurotrophin studies, we routinely perform perfusion fixation with 10% buffered formalin, followed by further immersion-fixing for 4 h, and routine tissue processing and paraffin embedding. To obtain RNase-free sections, cut sections with a clean knife and float them on DEPC-treated water and place them on pretreated slides.
4. A number of positive and negative controls can be used to establish the validity of *in situ* hybridization results. The best controls are the positive or negative specimen controls. Tissues or cells known to lack the sequence of interest should produce negative results. It is known that male rat submaxillary gland has a much higher NGF level than female submaxillary gland ***(6)***; thus, hybridization signal with a NGF probe should produce a much stronger signal in the male submaxillary gland than the female one. For negative technical controls, predigestion of target mRNA with RNase A prior to hybridization, hybridization with a sense probe, or without a probe should all give negative results.

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9

Expression of Neurotrophic Factors such as GDNF in the *E. coli* System

Bing-Ren Huang and Xue-Mei Ma

1. Introduction

Neurotrophic factors play an essential role in the growth, survival, and differentiation of neurons in the nervous system. Several well-characterized neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, glial-cell-line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), insulin-like growth factor-1 (IGF-I), are currently in clinical trials and have potential for the treatment of neurological diseases.

Because of their low concentrations in animal tissues, isolation of neurotrophic factors from natural sources is technically difficult, expensive, and impractical. Considering the many clinical and research applications of these factors, a ready supply of these neurotrophic factors from fermentation of organisms expressing these as recombinant proteins would be of great value to medicine and biotechnology. Recently, efforts have centered on the development of efficient recombinant methods for the production of neurotrophic factors. Of the hosts widely used for the production of heterologous proteins, *Escherichia coli*, *Saccharomyces cerevisiae* (baker's yeast), and mammalian cells are best understood ***(1,2)***. *E. coli* expression system is efficient and inexpensive, but it does not possess the ability to produce disulfide bonds in proteins ***(3)***; thus, recombinant proteins are often not stable and tend to aggregate into inactive complexes in the presence of endogenous bacterial proteases. As a result, recombinant proteins produced in *E. coli* require further extraction and oxidation so that disulfide bonds can be formed ***(4)***. Furthermore, in order to produce neurotrophic factor molecules with an authentic N-terminal amino

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acid but not the initiating methionine in the primary translation product, it is necessary to express neurotrophic factors as a fusion protein in *E. coli*. Thus, an additional step is required to obtain mature neurotrophic factors by cleavage of initial fusion proteins.

Yeast can offer clear advantages over bacteria for its ability to secrete heterologous proteins into culture medium. The production of protein from culture medium is generally superior to that from the cytoplasm ***(5)***. A high degree of initial purity can be obtained directly from secreted products, and it is much easier to further purify the products because of the absence of cellular debris. In the case of sulfhydryl-rich proteins, there is another compelling reason for the development of eukaryotic hosts capable of secreting such proteins into the culture medium in which their correct tertiary structure is produced and maintained via disulfide bonds ***(6–8)***. This is because the secretory pathway from the cell and the extracellular medium are oxidizing environments that can support disulfide bond formation. In contrast, in the case of *E. coli*, the cytoplasm is a reducing environment in which disulfide bonds cannot be produced. Upon cell breakage, rapid formation of the disulfide linkages can result in random disulfide bond formation. Therefore, production of sulfhydryl-rich proteins with appropriately formed disulfide bonds can be achieved best through the slow process of a secretory pathway. However, the yields of the expressed protein in *S. cerevisiae* are quite low and may require the use of high-cell-density fermentation methods or other yeast hosts such as *Pichia pastoris* strains.

Expression of cloned genes in cultured mammalian cells is another way to produce recombinant proteins ***(9–11)***. The neurotrophic factors coding the sequence of interest are inserted into an appropriate expression vector and then the resulting recombinant plasmid is transfected into the cells to form a transient or stable system. The yield of expressed neurotrophic factors is usually low, but the expression can be used for a variety of different purposes.

In order to produce a large quantity of neurotrophic factors for in vivo studies, we have used the *E. coli* expression system for the production of recombinant human GDNF. GDNF is a potent survival factor for embryonic dopaminergic, spinal motor, cranial sensory, sympathetic, and hindbrain nonadrenergic neurons and may repay further evaluation with treatments for amyotrophic lateral sclerosis (ALS) and Parkinson's disease. High yields in the production of this polypeptide will greatly promote the process of basic research and pave the way for clinical trials. We have now successfully cloned the genes for GDNF and purified active rhGDNF. The gene for GDNF mature peptide was cloned by the polymerase chain reaction (PCR) method and then transferred into an *E. coli* expression vector, pET. The recombinant protein was expressed in the *E. coli* host strain BL21(DE3)pLysS in the form of inclu-

sion bodies, and the expression level of GDNF reached more than 21% of the cell lysate total proteins. Metal chelate chromatography was used to purify rhGDNF with high purity, and biological activity was achieved by in vitro refolding ***(12)***.

2. Materials

There are many different *E. coli* expression systems, but the pET system is the most powerful system for the cloning and expression of recombinant proteins. This system has strong bacteriophage T7 transcription and translation signals and contains a chromosomal copy of the T7 RNA polymerase gene under LacUV5 control ***(13,14)***. Thus, the expression is induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) and is facilitated by abundant T7 polymerase synthesized in the host cell. A recombinant protein can comprise more than 50% of the total cell protein.

2.1. pET Vectors and Strains

These are shown in **Table 1** and are all available from Novagen (Madison, WI, USA).

2.2. Media

1. Growth Medium (LB) Per Liter: Tryptone (Difco Laboratories, Detroit, MI, USA) (10 g), yeast extract (Difco Laboratories) (5 g), NaCl (10 g), pH to 7.5 with 1 *N* NaOH. Autoclave.
2. M9ZB (per Liter): N-Z-Amine A (Sigma, ST. Louis, MO, USA) (10 g), NaCl (5 g). Autoclave, cool, and then add 100 mL of 10X M9 salts, 1 mL of 1 *M* $MgSO_4$, 10 mL of 40% glucose (from autoclaved stocks).
3. TB (per Liter): Tryptone (12 g), yeast extract (same as in **Subheading 2.2.1.**) (24 g), glycerol (4 mL). Autoclave, cool to 60°C, and add 100 mL sterile potassium phosphate.
4. Potassium Phosphate (per Liter): KH_2PO_4 (23.1 g), K_2HPO_4 (125.4 g). Autoclave.
5. 10X M9 salts (per Liter): NH_4Cl (10 g), KH_2PO_4 (30 g), $Na_2PO_4{\cdot}7H_2O$ (60 g). Autoclave.

2.3. Stock Solutions

1. 100 m*M* IPTG (isopropyl β-D-thiogalactopyranoside; Sigma) 2.38 g IPTG in 100 mL deionized water and stored at –20°C.
2. Carbenicillin (disodium salt; Sigma): 50 mg/mL in deionized water. Store at –20°C. Use at 50 µg/mL.
3. Ampicillin (disodium salt; Sigma): 25 mg/mL in deionized water. Store at –20°C. Use at 50 µg/mL.

Table 1
Brief of pET Vector Composition

Vector	Selection marker and protease site	Promoter	N-Terminal leader	5′ insert site for natural protein fusion protein	Host strain
pET16b	Amp/carb, factor Xa	T7lac	His.Tag	*Nde*I, *Nco*I	BL21(DE3)pLyS
pET27	Kan	T7lac	C-His.Tag, pelB	*Nde*I, *Nco*I	BL21(DE3)
pET28	Kan, thrombin	T7lac	N- and C-His.Tag	*Nco*I, *Nde*I	BL21(DE3)
pET30	Kan, enterokinase	T7lac	N- and C-His.Tag	*Nde*I, *Nco*I	BL21(DE3)

Note: Host system:
For cloning: *E. coli* K12 strains HB101, JM109, DH5a
For expression: BL21(DE3), BL21(DE3)plysS, BL21(DE3)plysE.

4. Chloramphenicol (Sigma): 34 mg/mL in ethanol. Store at –20°C. Use at 34 µg/mL.
5. Kanamycin (sulfate; Sigma): 30 mg/mL in deionized water. Store at –20°C. Use at 30 µg/mL.

2.4. Enzymes

Restriction enzymes, ligase, and other enzymes available from many supplies of recombinant DNA technology.

2.5. Solutions for Purification of Recombinant Proteins with His.Tag System

1. His-Bind ∗ Resin (Novagen, Madison, WI, USA).
2. 8X binding buffer: 40 m*M* imidazole, 4*M* NaCl, 160 m*M* Tris-HCl, pH 7.9.
3. 8X wash buffer: 480 m*M* imidazole, 4*M* NaCl, 160 m*M* Tris-HCl, pH 7.9.
4. 4X elution buffer: 4*M* imidazole, 2*M* NaCl, 80 m*M* Tris-HCl, pH 7.9.
5. 4X Strip buffer: 400 m*M* EDTA, 2*M* NaCl, 80 m*M* Tris-HCl, pH 7.9.
6. 8X Charge buffer: 400 m*M* $NiSO_4$.

3. Methods

3.1. Vector Preparation

1. Mix the following: 2 µL restriction enzyme buffer, 1 µg pET vector, 10 µL appropriate restriction enzyme, such as *Nco*I or *Nde*I, add water up to 20 µL. Incubate at 37°C for 2 h. If a gene is cloned into a single site, the vector should be dephosphorylated following the digestion to reduce the vector self-ligation.
2. In addition to alkaline phosphatase, sometimes a modification enzyme reaction may be needed. For example, polymerase I Large Fragment can be used to fill in the cohesive end that results from the digestion with a endorestriction enzyme such as *Nco*I or *Nde*I. To do so, just add 1 µL of 1.25 m*M*/L dNTP and 1–5 units of Large Fragment to the mixture and incubate at room temperature for 15 min.

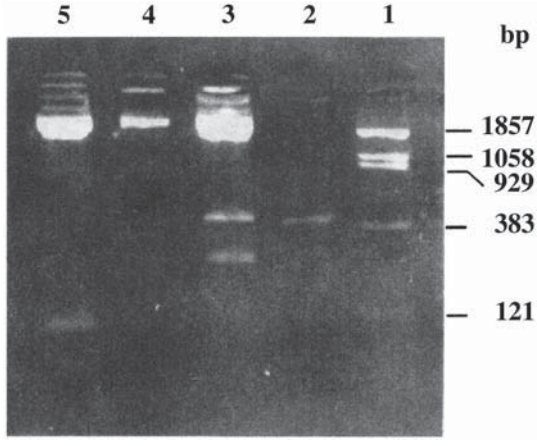

Fig. 1. Restriction enzyme analysis of pUC18-GDNF. Lane 1: pBR322/BstN I DNA size standard; lane 2: PCR fragment of GDNF; lane 3: pUC18–GDNF/*Nde*I, *Bam*HI; lane 4: pUC18/*Sma*I; lane 5: pUC18–GDNF/*Eco*RI.

3. Extract the digested and modified DNA with phenol : chloroform : isoamylol (25 : 24 : 1) and precipitate the DNA with ethanol and dry.
4. The plasmid may need to be digested with a second restriction enzyme and re-extraction, such as *Bam*HI. In this experiment, if the plasmid were to be digested with *Nde*I and *Bam*HI, now the prepared plasmid would be ready for ligation with a coding GDNF mature peptide cDNA fragment.

3.2. Preparation of the Neurotrophic Factor Coding Sequence

The PCR or reverse transcriptase (RT)-PCR methods can be used to isolate and/or modify a target neurotrophic factor gene (*see* **Note 1**). It is necessary to design a set of primers that will help you to get the translated portion of a cDNA sequence with convenient restriction enzyme sites and place the coding sequence in the proper reading frame ***(15,16)***. In order to get the mature GDNF cDNA sequence, the upstream and downstream primers are synthesized as follows:

Upstream:	5′CATATGTCACCAGATAAACAAATG 3′
	(underlined is *Nde*I site)
Downstream:	5′ TCAGATACATCCACACCT 3′

Amplify the GDNF cDNA and clone the PCR fragment into the pUC18 *Sma*I site. Sequence the recombinant plasmid to determine if the cDNA has a correct sequence (*see* **Note 2**). Digest the recombinant plasmid with *Nde*I and *Bam*HI and purify the GDNF fragment from low-melting-point agarose. The fragment, which has a size of 411 bp, will be ligated to the vector; *see* **Figs. 1** and **2**.

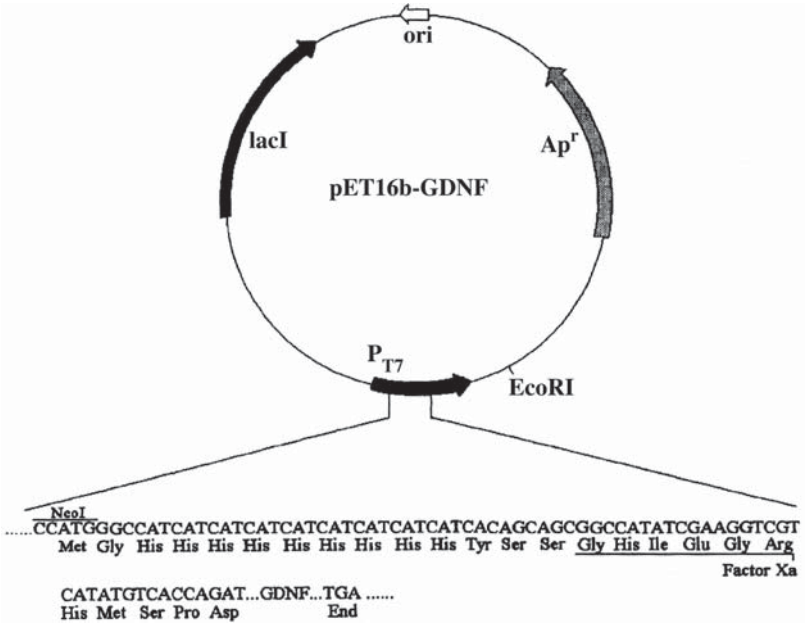

Fig. 2. pET16b–GDNF.

3.3. Ligation and Transformation

1. Add 0.01–0.03 pmol pET vector (33–100 ng) and 0.2 pmol insert fragment (50 ng of a 500-bp fragment) into the ligation mixture with a total volume of 10 µL: 1 µL of 10X ligase buffer, 1 µL of 100 mmol/L dithiothreitol (DTT), 1 µL of 10 mmol/L or 1 mmol/L ATP (for sticky or blunt end, respectively), 1 µL T4 DNA ligase. Incubation at 16°C for 16 h.
2. Preparation of the competent cells JM109. The transformation procedure is as described in any molecular cloning manual.

3.4. Identification of the Recombinant Clones

1. Pick up several clones (at least 1 mm in diameter) with sterile toothpicks from the agar plate and transfer each of them to a separate 1.5-mL tube containing 50 µL H_2O, then disperse the pellet. Place the tubes in boiling water for 5 min to lyse the cells. Centrifuge at 12,000*g* for 1 min to remove cell debris. Take 10 µL of the supernatant as a template and use T7 promoter and T7 terminator primers, dNTP and Taq polymerase to carry out a PCR—35 cycles for 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C, with a final extension at 72°C for 10 min. Run a 1% agarose gel containing 0.5 µg/mL ethidium bromide to analyze the PCR products to determine which clones are correct recombinant clones.
2. The other method is to make a restriction map to identify the recombinant clones; *see* **Fig. 3**.

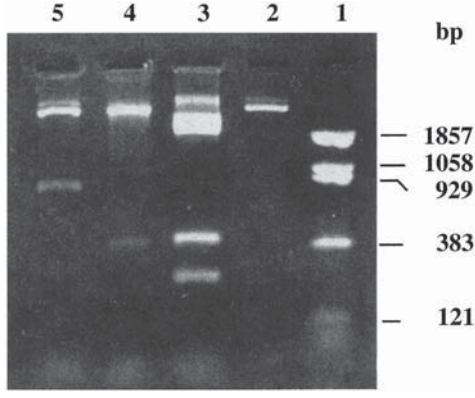

Fig. 3. Restriction enzyme analysis of pET16b–GDNF. Lane 1: pBR322/BstNI DNA size standard; lane 2: pET16b/*Nde*I, *Bam*H; lane 3: pUC18–GDNF/*Nde*I, *Bam*HI; lane 4: pET16b–GDNF/*Nde*I, *Bam*HI; lane 5: pET16b–GDNF/*Eco*RI.

3.5. Recombinant Plasmid Preparation

After positive clones are identified, the pET-derived plasmid is prepared by the alkaline lysis method or the boiling method and used for transformation into an expression host. Restriction mapping and sequence analysis are used to determine if the neurotrophic factor can be expressed, restrict enzyme sites are correct, and the fragment is inserted in a correct reading frame.

3.6. Gene Expression

1. Transformation of expression host and induction. After a pET-derived plasmid is transformed into *E. coli* strain BL21 (DE3) or into one of these strains containing pLysS or pLysE, the transformed strain is incubated and shaken at 37°C until the optical density (OD_{600}) is 0.4–1 (*see* **Note 3**). Take a sample for an uninduced control; then, the expression of neurotrophic factor is induced by the addition of IPTG to the culture at a final concentration of 0.4 m*M* (T7 promoter) or 1 m*M* (T7lac promoter). The incubation continues for 2–3 h.
2. Harvest the cells by centrifugation at 5000*g* for 5 min at 4°C. Resuspend the cells in 0.25 culture volume of cold 50 m*M* Tris-HCl (pH 8.0), 2 m*M* EDTA, and centrifuge again as above. Store the cells at –70°C. The expression can be assessed by analysis of total cell protein on an SDS-PAGE followed by Coomassie staining. *See* **Fig. 4**.

3.7. Identification and Purification of Target Proteins

3.7.1. Soluble and Insoluble Fractions

Collect induced cells by centrifugation at 5000*g* for 5 min. Discard the supernatant and resuspend the cell pellet in 1/10 culture volume of 50 m*M* Tris-

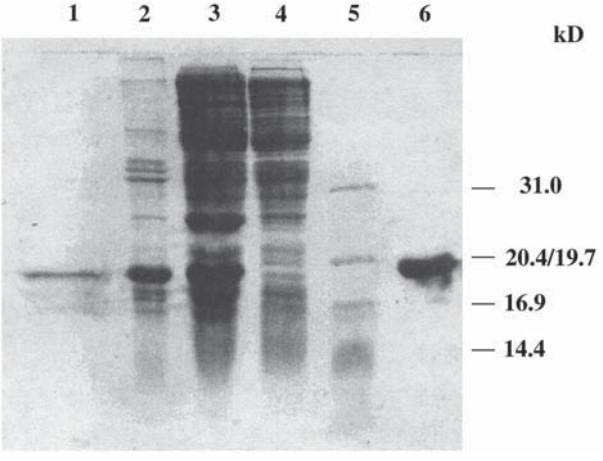

Fig. 4. 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the expression and purification of rhGDNF. Samples were heated to 100°C without a reducing agent. Lane 1: refolding supernantent; lane 2: inclusion body; lane 3: total cell lysate of *E. coli* expressing GDNF; lane 4: Total cell lysate of controled *E. coli* strain; lane 5: molecular-weight standards; lane 6: His-Bind metal chelation resin purified rhGDNF.

HCl (pH 8.0), 2 m*M* EDTA. Add lysozyme to a concentration of 100 mg/mL and 1/10 volume 1% Triton X-100. Incubate at 30°C for 15 min. Place the tube in an ice bath and sonicate the solution with a microtip to shear the DNA. The solution should lose its viscosity after one or two 10-s pulses at a high output setting. Centrifuge at 12,000*g* for 15 min at 4°C. The supernatant contains soluble proteins, add an equal volume of 2X SDS sample buffer to a sample for gel analysis. The insoluble pellet is resuspended in 1X SDS sample buffer for gel analysis also.

3.7.2. Periplasmic Fraction

When vectors with ompT or petB leaders, such as pET27, are used, the expressed neurotrophic factor can be directed to the periplasmic space (*see* **Note 4**). Centrifuge a 1.5-mL sample of induced culture in a microcentrifuge for 1 min. Decant the supernatant, resuspend the cells in residual supernatant, and add 15 µL chloroform at room temperature for 15 min. Add 75 µL of 10 m*M* Tris-HCl (pH 8) and centrifuge the sample at 12,000*g* for 15 min. Transfer the supernatant to a fresh tube and add an equal volume of 2X sample buffer. Heat samples at 70°C for 3 min for the gel analysis.

3.7.3. Rapid Purification With pET His.Tag® System (see **Note 5***).*

*His-Bind * Resin (Novagen) preparation*: Mix the bottle containing His-Bind resin and transfer the desired amount of slurry to a column of 2.5 mL volume. Allow the resin to pack under gravity flow and then wash the column with 3 volumes of sterile deionized water, 5 volumes of 1X charge buffer, and 3 volumes of 1X binding buffer.

Preparation of the cell extract: Harvest the cells from 100 mL culture medium and resuspend the cells in 4 mL cold binding buffer. NP-40 or another nonionic detergent is added to 0.1% to reduce nonspecific binding. Sonicate the sample until it is no longer viscous. Centrifuge the lysate at 40,000*g* for 30 min. If the expressed protein is soluble, filter the supernatant and run through the affinity column (*see* **Note 6**).

Column chromatography (*see* **Note 7**): Allow the binding buffer to run though the column (such as 2.5 mL resin for 20 mg protein purification) and load the column with the prepared extract. A flow rate of about 10 column volumes per hour is optimal for efficient purification. Wash the column with 10 column volumes of 1X binding buffer (*see* **Note 8**). Wash the column with 6 column volumes of 1X wash buffer. Elute the bound protein with 6 column volumes of 1X elute buffer. Run 6 column volumes of strip buffer through the column to strip the column of the protein.

Purification under denaturing conditions: The resin preparation, the cell harvest, and sonication are all the same as described above. Centrifuge the extract at 20,000*g* for 15 min to collect inclusion bodies and cellular debris. Remove the supernatant and suspend the pellet in 20 mL binding buffer. Centrifuge again. Remove the supernatant and resuspend the pellet in 5 mL binding buffer containing 6*M* guanidine or 6*M* urea. Incubate on ice for 1 h to completely dissolve the protein. Remove any remaining insoluble material by centrifugation at 39,000*g* for 20 min. Filter the supernatant through a 0.45 µm micromembrane before loading it on the column. In order to purify protein under denaturing conditions, guanidine (6*M*) or urea (6*M*) is present in all buffers and the protein tends to be eluted at low imidazole concentration. Run the binding buffer through the column and then load the column with the prepared extract. Wash the column with 10 column volumes of 1X binding buffer followed by 6 column volumes of 1X binding buffer containing 20 m*M* imidazole. Elute the protein with 1X elution buffer containing 300 m*M* imidazole.

Salts and the denaturing agent in the purified sample must be removed gradually by dialysis or by a microconcentrator or gel filtration, followed by a protease cleavage. The recombinant protein (1 mg) is digested at 20–37°C for

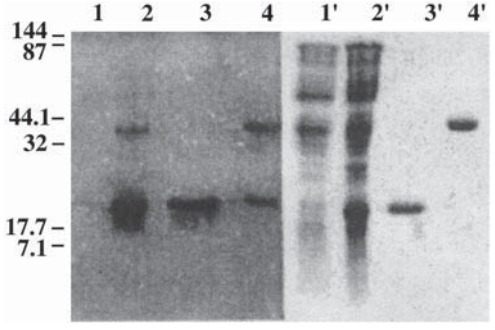

Fig. 5. Removal of oligohistidine by treatment with proteases, Factor Xa. Samples were heated to 100°C for 10 min with or without a reducing agent (100 m*M* DTT; *see* **Note 9**). Lane 1: molecular-weight standards; lane 2: GDNF with His.Tag leader; lane 3: GDNF with His.Tag leader removed; lane 4: Native SDS-PAGE of GDNF with His.Tag leader; lane 5: Native SDS-PAGE of GDNF with His.Tag leader removed.

8–16 h with 20–50 µg Xa in Xa buffer containing 100 m*M* NaCl, 50 m*M* Tris-HCl, and 1 m*M* $CaCl_2$ (pH 8.0). Alternatively, 1 mg recombinant protein is digested by 100 µg enterokinase at 37°C for 8–24 h in the buffer containing 10 m*M* Tris-HCl (pH 8.0), and 10 m*M* $CaCl_2$. Human thrombin is one of the most active site-specific protease that has a very low mass ratio of enzyme to target protein, required for efficient cleavage. For example, a 1:2000 (w/w) ratio is sufficient in buffer containing 20 m*M* Tris-HCl, 150 m*M* NaCl, and 2.5 m*M* $CaCl_2$ (pH 8.4) at 4–37°C for 2–16 h. After cleavage reaction, the pure native protein can be obtained by using the His-Bind resin column again to separate the fragment containing His.Tag from the target protein. *See* **Fig. 5**.

3.8. Western Blot

Western blot is designed for the rapid, sensitive detection of proteins immobilized on nitrocellulose membranes. Thus, the expressed neurotrophic factor is transferred from gels after electrophoresis or bound directly from solution (dot blots) and detected by a method based on the enzyme-linked immunodetection of antigen-specific antibody. Following sequential incubations with the primary antibody and appropriate secondary antibodies conjugated with alkaline phosphatase (AP) or horseradish peroxidase (HRP), the color of bands (dots) is developed in a solution containing appropriate substrates (*see* **Note 10**). **Figure 6** is the result achieved using the method.

3.9. Bioassay

To determine the biological activity of recombinant neurotrophic factors, a cell line or a neuronal tissue expressing the neurotrophic factor receptor is used

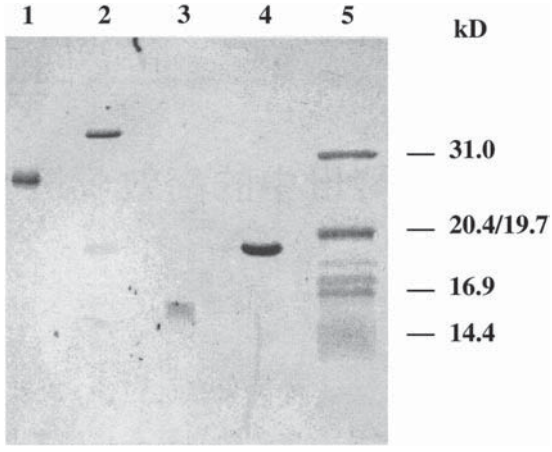

Fig. 6. Western-blot analysis of rhGDNF. Samples were heated to 100°C for 10 min with or without reducing agent (100 m*M* DTT). Lanes 1′–4′ and 1 were detected by Coomassie brilliant blue staining; lanes 2–4 were detected in an immunoblot with antibodies to rhGDNF; lanes 1 and 1′: total cell lysate of control *E. coli* strain; lanes 2 and 2′: total cell lysate of *E. coli* expressing GDNF; lanes 3 and 3′: His-Bind metal chelation resin-purified rhGDNF; lanes 4 and 4′: purified rhGDNF in its native state.

for assessing the effects of the neurotrophic factor on survival, proliferation, or differentiation. **Figure 7** illustrates a biological assay of rhGDNF using cultured E8 chicken embryonic dorsal root ganglia (DRG).

If needed, the p*I* of the protein is determined or N-terminal amino acid sequences is examined by using the Edman degradation method. Pharmacological tests in vivo can be used to further characterize the recombinant protein.

4. Notes

1. If a coding sequence of interest is amplified by using the RT-PCR method, it is very important to digest contaminant DNA in the RNA sample with RNase-free DNase. DNA can be degraded by brief treatment of samples with DNase. Add $MgCl_2$ to 10 m*M* and DNase to 12 µg/mL and then incubate at room temperature for 20 min.
2. It is possible to obain a single-strain DNA template from pET (numbered 20 or greater) recombinants with a vector that carries the phage f1 origin of replication and to use the template for sequencing.
3. Because the expressed proteins are unique, the optimal scheme and time-course can vary. Growth at 37°C causes some proteins to accumulate in inclusion bodies, whereas incubation at 30°C leads to a soluble active protein. If export of the target protein is desirable, the signal sequence leaders present in pET27 are used, and the induction at 25°C or 30°C may be optimal. Inclusion bodies are easily isolated by centrifugation to yield highly concentrated and relatively pure pro-

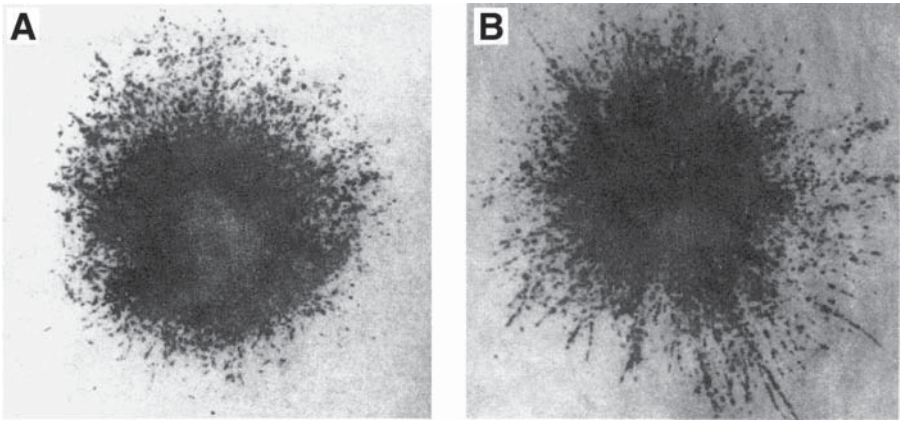

Fig. 7. Biological activity of rhGDNF in promotion of neurite outgrowth of cultured chicken embryonic DRG: **(A)** control; **(B)** rhGDNF.

tein. With a large quantity of proteins, it is possible to solubilize the protein in the inclusion body with urea or guanidine–HCl and then refold it by slow dilution or by dialysis of the denaturant at a low protein concentration.

4. Translocation across the cell membrane of *E. coli* is not completely understood. However, it is clear that translocation can also depend on the mature domain of the target protein, which is recognized by Sec B, the major chaperone of export.
5. Many pET vectors have a property of carrying the His.Tag sequence, a stretch of ether 6 or 10 consecutive histidine residues can be expressed at the N- or C-terminal ends of the target protein. The His.Tag sequence binds to divalent cations (e.g., Ni^{2+}) immobilized on the His-Bind metal chelation resin. First, unbound proteins are washed away and then the target protein is recovered by elution with imidazole. Columns can be regenerated and reused many times. This versatile system provides a convenient, economical method of purification without the need to develop new protocols for each proteins. It also allows the purification of target proteins under gentle, native conditions for maintaining activity of soluble proteins, as well as under denaturing conditions necessary for solubilization of inclusion bodies. A volume of a 2.5-mL column can be used to purify 20 mg target protein, and for the pET system, a yield of target protein per 100 mL culture is about 20 mg.
6. To protect against different degradation enzymes, protease inhibitors may be added to the buffer. For example, add 1/200 volume of 0.2 *M* phenylmethylsulfonyl fluoride (PMSF), or 12 µg/mL pepstatin, 12 µg/mL leupeptin, and 1% aprotinin.
7. It is important to avoid 2-mercaptoethanol, DDT, and EDTA in solutions that will be run through the column. The reducing regents react to form brown precipitates. EDTA will chelate the Ni^{2+} and strip the column of the active-affinity group.
8. If the recombinant protein contains a six histidine stretch, the wash buffer should contain 60 m*M* imidazole. On the other hand, if a few minor contaminants remain

bound to the column, the wash buffer should contain 100 m*M* imidazole. The elution buffer can contain 100–400 m*M* or 1*M* imidazole.

9. When urea is used, the sample may be mixed with the sample buffer and loaded directly on an SDS-PAGE, whereas a sample in 6*M* guanidine must be diluted 1 : 5 in water or dialyzed before running on an SDS-PAGE gel.
10. The secondary structure in the mRNA transcript can interfere with the AUG translation initiation codon and/or the ribosome binding site. All pET vectors will generate the following transcripts:

    5′..... AAGAAGGAGAUAUACAUAUG ...3′

    5′.. AAGAAGGAGAUAUACCUGGA*..3′

    If the expression is poor, the coding strand of your insert should be examined for stretches of complementarity with the above sequences to reveal a potential problem in the secondary structure. Excessive rare codon usage in the target protein has also been implicated as a cause for low-level expression. If so, it is necessary to make mutations to change the codon into a normal one.

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10

Construction of Cells Expressing Neurotrophins

Jasodhara Ray and Fred H. Gage

1. Introduction

The ability to introduce genes of neurotrophic factors into eukaryotic cells provides a novel way to study neurobiological processes in vitro and in vivo ***(1–6)***. To study the functional role of the growth factors in vivo, genetically modified cells expressing neurotrophin have been grafted in the central nervous system (CNS) ***(2,3,6)***. In different animal models of CNS degenerative diseases, neurotrophic factors secreted from grafted cells can prevent neuronal degeneration and promote functional recovery after injury ***(1–11)***. The neurotrophin family is comprised of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) ***(12–14)***. Although structurally similar, they differ in their regional and temporal distribution, receptor specificity, and biological activity. NGF, the first prototypic neurotrophin, is synthesized as part of a large precursor protein within which mature, bioactive NGF occupies the C-terminal half, and the N-terminal half contains a signal peptide (18 amino acid) preceding a large pro region. The pro sequence in combination with the signal (pre) sequence is necessary for the secretion of correctly processed, biologically active NGF ***(15)***. All neurotrophins show considerable structural homology to NGF and contain similar signal peptide and pro sequences.

The basic steps required for gene transfer into cells are construction of plasmid vectors, harvest and propagation of target cells, and transfection. Successful gene transfer depends on a number of factors, including type of cell, methods of gene transfer, and the regulatory elements that control the long-term expression of transgenes. The cells used for gene transfer should be readily available, divide rapidly in culture, and be maintainable for a time in vitro. For use in gene therapy purposes, cells should not grow in vivo. Although immor-

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talized cells meet these criteria, some of them form tumors upon grafting ***(1,2)***. Although no single cell type is suitable for all cell-based gene therapy, the accessibility and the replicative nature of skin fibroblasts, endothelial cells, muscle cells, and keratinocytes have made them the cells of choice for this purpose. Primary skin fibroblasts are the most commonly used cells, based in part on knowledge gained from in vitro studies over decades. Fibroblasts can be readily obtained from skin biopsy and cultured free from keratinocytes, the other major cell type present in skin.

Three components are generally considered in the construction of an expression system: the DNA template, the promoter/enhancers, and the vectors. Neurotrophin cDNAs encompassing the coding sequence for the protein, 5′ untranslated regions containing the signal, and the pro sequences are used as DNA template. cDNA is placed downstream of the promoter/enhancer in the expression vectors. To drive the mRNA expression, housekeeping or viral promoters are often used. Housekeeping promoters encode proteins necessary for the every day survival of cells. Although these promoters are not strong transcriptional initiators, they are expressed in a variety of cell types at a constant level and are little affected by external factors. Another approach is to use tissue-specific promoter/enhancers to produce high levels of transcripts in a particular cell type. However, strong viral promoter/enhancers are the elements of choice ***(16–18)***. Vectors for gene transfer generally contain a bacterial origin of replication (Ori) and antibiotic-resistant gene. The presence of Ori would facilitate their assembly in a competent bacterial carrier and allow for large-scale preparation of the plasmid DNA to be used for the analysis of the construct by restriction mapping and direct transfection of this DNA into mammalian cells. Antibiotic-resistant genes would allow for the selection of vector-carrying colonies. Usually strong viral promoter-driven selectable markers (e.g., aminoglycoside phosphotransferase, hygromycin B phosphotransferase) are introduced into the constructs to select for stable populations of transgene-expressing mammalian cells. Cells expressing these proteins are resistant to the antibiotics neomycin or hygromycin.

A number of physicochemical (calcium phosphate, cationic lipids, or electroporation) and biological methods (viral vectors) are available for transducing cells ***(16–25)***. Presently, retrovirus-mediated gene transfer to mammalian cells is the method of choice ***(16–19,25)***. Most retroviral vectors are derived from Moloney murine leukemia virus (MoMLV) ***(4,18,25)*** in which the native viral protein sequences are replaced with recombinant sequences like the gene of interest and selectable marker genes (**Fig. 1A**). The long terminal repeats (LTRs) in the proviral DNA are organized as direct repeats and contain *cis*-acting promoter/enhancer signals as well as signals for viral integration and reverse transcription of RNA genome. LTRs also contain the

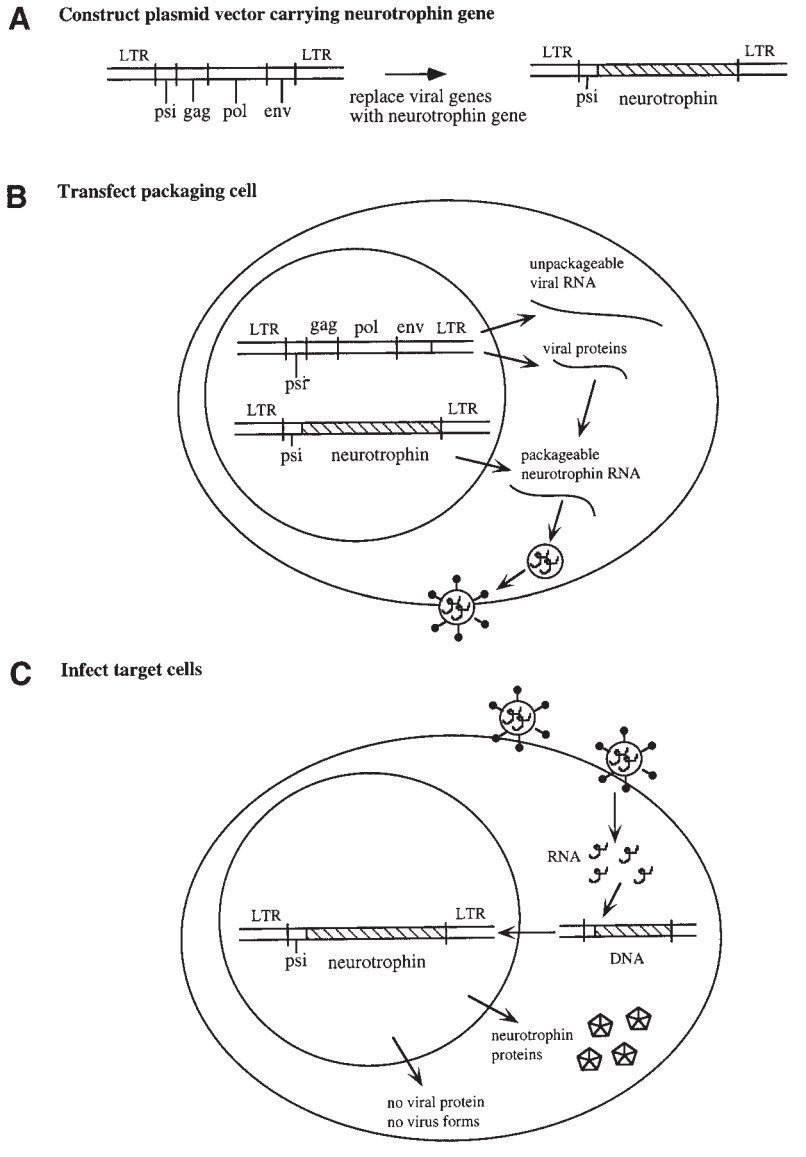

Fig. 1. Schematic of generation of target cells expressing neurotrophins. **(A)** The viral genes in a provirus are replaced with neurotrophin gene of interest and are transfected into packaging cells **(B)**. The viral DNA directs the synthesis of viral RNA, but lacking the viral proteins, it cannot package the RNA into infectious viral particles. The viral proteins necessary for packaging are provided in trans from a helper virus that lacks the psi region crucial for inclusion of RNA in viral particles. The virus produced by the packaging cells contains neurotrophin RNA but no viral genes. **(C)** The virus can infect target cells and splice the gene into cellular DNA, but because of the absence of viral proteins, it cannot reproduce.

polyadenylation signal for termination of viral transcription. Encapsidation of genomic-length RNA into virions is dependent on the presence of an untranslated psi packaging sequence that lies downstream of the 5′ LTR and 5′ portion of the *gag* gene. Viral structural proteins are encoded within the *gag* gene, reverse transcriptase and integrase within the *pol* gene, and the virion envelope gene within the *env* gene. For safety reasons the *gag*, *pol*, and *env* genes are deleted from the retroviral genome to make them replication-defective, as the virions generated in the initial packaging step can go through only a single round of infection ***(16–19,25)***. Deletion of the viral *gag*, *pol*, and *env* genes necessitates that these functions should be provided in *trans*. The producer cell lines contain a provirus expressing only the viral structural gene (*gag–pol–env*) required for the production of transmissible viral particles (**Fig. 1B**). However, the provirus lacks the packaging signals; hence, only empty viral particles are generated by producer cells. When recombinant retroviral vectors are introduced into producer lines, the RNA transcripts of the vectors are recognized by the packaging mechanism and the RNA transcripts are encapsidated into replication-defective infectious virus particles (**Fig. 1B**), which are then used to infect the target cells (**Fig. 1C**). Removal of viral structural genes frees up approx 8 kb of the genome, which can be replaced with a transgene. One disadvantage of retroviral vectors is the rare possibility of their random insertion, causing disruption of a cellular gene, or insertion of regulatory sequences (promoter or enhancers) near a cellular gene. This may cause inappropriate or uncontrollable expression of this gene.

In a typical retrovirus vector, the transgene is expressed from either viral LTR or from an internal promoter that can be a housekeeping gene promoter, tissue-specific gene promoter, or viral promoter (**Fig. 2A,B**). The selectable marker gene is expressed from either internal promoter or LTR (**Fig. 2A,B**). In these vectors, the transgenes are constitutively expressed. However, when it is necessary to regulate transgene expression, retroviral vectors containing tetracycline (*tet*)-responsive reporter systems can be used (**Fig. 2C.**; and **refs. *18, 26,27***). In these vectors, the prokaryotic *tet* repressor is converted to a eukaryotic transactivator by fusion of the repressor with the activating domain (C-terminal) of VP16 of herpes simplex virus. The *tet*-controlled transactivator (tTA or rtTA) strongly influences transcription from the human cytomegalovirus (hCMV) minimal promoter P_{hCMV} fused to the *tet* operator (tetO) sequence (**Fig. 3**). Gene expression in the tTA-containing system is inhibited by the addition of *tet*, whereas the effector stimulates transgene expression in vectors containing the mutant transactivator (rtTA). Thus, both vector systems allow for the differential control of specific genes in mammalian cells and are suitable for turning genes “on/off” in a reversible way.

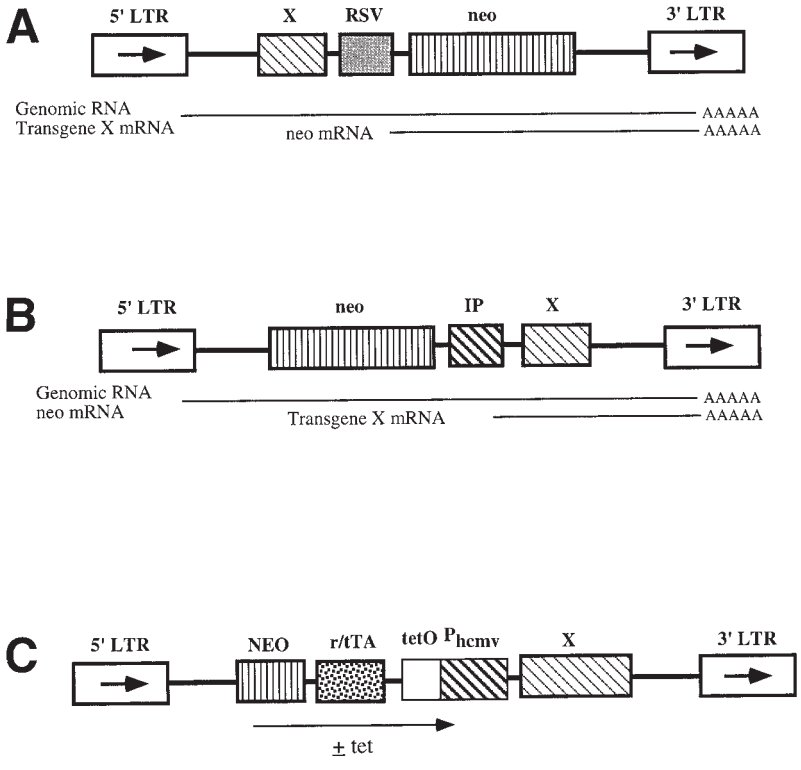

Fig. 2. Replication-defective retroviral vectors. Transgene X and the drug-resistant marker gene *neo* can be expressed from viral LTR **(A)** or an internal promoter (IP;) **(B)**. **(C)** tetracycline (*tet*)-regulatable retroviral systems in which the transcription of transgene can be downregulated (using transactivator tTA) or upregulated (using mutant transactivator rtTA) in the absence or presence of the antibiotic or its derivatives.

In this chapter, we detail the methods for production of retroviral packaging cell lines, generation of primary fibroblast cultures, retroviral-mediated transfer of neurotrophin genes into these cells, and characterization of transduced cells. Although the methods described here use fibroblasts as the target cells, the same general methods are applicable to other cell types.

2. Materials

2.1. Plasmid Vectors

1. Nonregulatable retroviral vectors pLRNL (**Fig. 2A**, and **refs. *16,17,25***) or any equivalent retroviral vectors like pLNSX or pLNCX (**Fig. 2B**, and **refs. *16,17,25***).

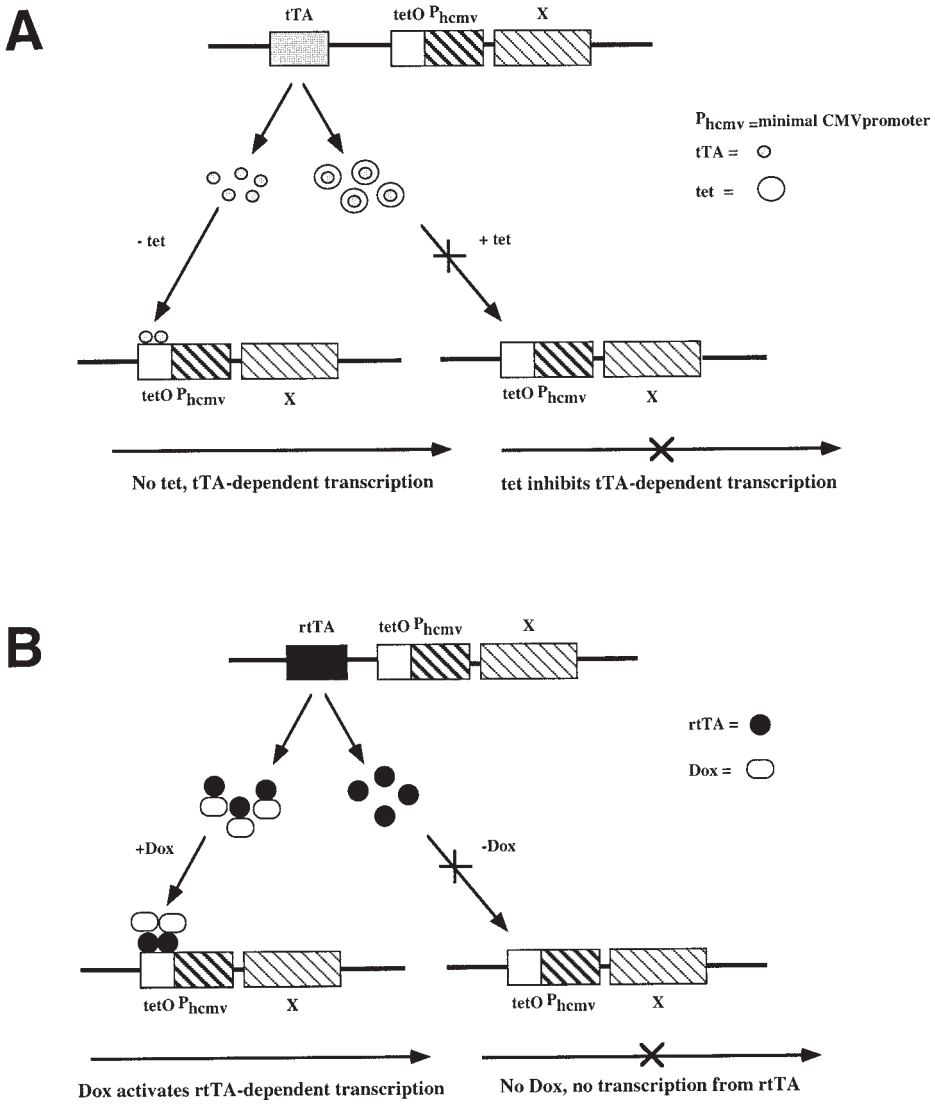

Fig. 3. Schematic representation of tetracycline-controlled transactivation systems. **(A)** The regulator unit (tTA), which is a fusion protein of *Escherichia coli* transposon (Tn) 10-derived repressor (tetR) and VP 16, strongly activates transcription from hCMV minimal promoter (lacking enhancers) fused to the *tet* operator (tetO) sequences in the absence of tetracycline (*tet*). The presence of *tet* almost completely abolishes transcription activation by tTA and downregulates transgene expression. **(B)** When the regulator unit contains a mutant of the Tn 10-tetR gene (rtTA), the transactivator does not activate transcription in the absence of Dox (a derivative of *tet*). In the presence of Dox, rtTA activates transcription from hybrid promoter and transgene expression is upregulated.

2. Tetracycline-regulatable retroviral vectors (**Fig. 2C**, and **refs. *26*,*27***). For a comprehensive review, *see* **ref. *18***.

2.2. Neurotrophin cDNAs

Neurotrophin cDNA containing the complete coding sequence, the signal sequence, and the pro sequence are used for vector construction.

2.3. Animal Biopsies and Generation of Primary Fibroblasts

Anesthesia mixture: Ketaset (Ketamin, Veterinary Corp America), PromAce (Acepromazine, Western Medical Supply), and Rompan or Anased (Xylazine, Western Medical Supply).

2.4. Tissue Culture

1. Ecotropic psi-2 cell line ***(28)***; amphotropic PA317 cell line (ATCC #CRL9078); immortalized fibroblasts NIH 3T3 (ATCC #CRL 1658); human 293T cells ***(29)***.
2. Phosphate-buffered saline (PBS; Irvine Scientific) and Isotonic saline solution (Fisher Scientific).
5. Dulbecco's modified essential medium (DMEM) containing 4.5 g/L glucose.
3. Fetal bovine serum (FBS; Sigma, Irvine Scientific, Gemini).
4. Complete medium: mix DMEM with 2 m*M* L-glutamine (Irvine Scientific), 2.5 µg/mL fungizone (Irvine Scientific), 50 µg/mL gentamycin (Gemini Bioproducts) or 1X penicillin/streptomycin (Gibco), and 10% FBS. Store at 4°C for 1–2 mo. Before use, prewarm medium in a 37°C water bath for 15–20 min.
5. ATV trypsin (trypsin/EDTA solution; Irvine Scientific).
6. Superfect transfection reagent (Qiagen, Inc, Valencia, CA).
7. Polybrene (Hexadimethrine bromide, Sigma). Dissolve polybrene in sterile water to make a solution of 10 mg/mL and filter through a 0.22-µm filter. Aliquot in small volumes and store the stock solutions at –20°C and the working solution at 4°C.
8. Geneticin (G418 sulfate, Gibco): Note the active concentration of G418. Dissolve G418 in HEPES buffer (pH 7.4) to make a solution of 40 mg/mL (active concentration). Filter through a 0.22-µm filter and store at 4°C for 1–2 mo. G418 is added to medium at a concentration of 400 µg/mL.
9. Agarose (3%): Add 3 g agarose to 100 mL PBS and autoclave. Store in capped bottle at room temperature. Just prior to use, melt agarose in a microwave oven and cool to 45–50°C.
10. N2 medium: Add N2 supplement (1:100, Gibco) into DMEM:F12 medium (Irvine Scientific) containing 1 m*M* L-glutamine and 3.1 g/L glucose.
11. Laminar flow hood; CO_2 incubator (Forma Scientific).
12. 37°C water bath; clinical centrifuge (Fisher Scientific).
13. Coulter counter, model ZF (Coulter Electronics, Inc.).
14. Tissue culture dishes (T75 flasks and 35-, 60-, or 100-mm dishes and six-well plates).
15. 0.45-µm and 0.22-µm filters (Nalgene).

16. Dimethylsulfoxide (DMSO, Sigma); Coomassie brilliant blue-G (Sigma) in 75% methanol, 5% acetic acid.
17. Cryovials (Nalgene), freezing chambers, and liquid nitrogen tank.
18. Enzyme-linked immunosorbent assay (ELISA) kits for NGF and NT-3 assays (Boehringer Mannheim, Chemicon, and Promega).
19. Antibodies to NGF, BDNF, NT-3, and NT-4 (Boehringer Mannheim, Chemicon, and Promega).
20. Recombinant human (rh) NGF, BDNF, NT-3, and NT-4 (Promega or other supplier).
21. Rat PC12 cells.
22. Poly-L-ornithine, mol. wt. 30–70,000 (Sigma); Laminin (Gibco), collagen (Sigma).

3. Methods

The production of genetically modified cells is done in three stages. First, plasmid vectors containing neurotrophins are designed and constructed according to standard plasmid subcloning techniques ***(30)***. Second, a clonal virus-producing cell line is generated by transfection of vectors and the viral titer is determined. Finally, target cells (e.g., primary fibroblasts) are stably infected with the recombinant virus collected from producer lines. Cells are further propagated and characterized for the expression of transgene products. The purity of plasmid DNA is important for obtaining high transfection efficiency in producer cells (*see* **Note 1**). Here, we will briefly outline the standard protocol for plasmid DNA preparation that has been described in detail elsewhere ***(30)***. The production and characterization of producer cell lines expressing recombinant virus and the generation of genetically modified fibroblasts will be discussed in detail.

3.1. Preparation of Plasmid DNA

Closed circular plasmid DNA can be prepared by the alkaline lysis method followed by precipitation with polyethylene glycol ***(30)***, by banding the cleared bacterial lysate on a CsCl gradient ***(30)***, or by using any of the commercially available plasmid DNA preparation kits (Qiagen, Inc.; In Vitrogen, Inc.). Dissolve DNA in 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0. Measure the optical density (OD_{260}) and calculate the concentration (1 OD_{260} = 50 µg DNA/mL). The purity of DNA can be determined from the ratio of OD_{260}/OD_{280}. Pure preparation of DNA has a value of 1.8. A contamination with protein or phenol will exhibit a significantly lower value.

3.2. Generation of Retrovirus-Producing Cell Lines

All the steps in the following protocol should be done in a tissue culture hood unless otherwise specified. Cells are incubated in a 10% CO_2 incubator at 37°C (*see* **Note 2**).

Day 1: Plate log phase psi-2 cells (5×10^5 cells/60-mm plate) in complete medium (*see* **Note 3**). Make one plate/plasmid vector and one for negative control.

Day 2: Cultures should be at least 40% confluent. Transfect cells with plasmid DNA using Superfect transfection reagent (*see* **Note 4**). Dilute 5 µg plasmid DNA to 150 µL in DMEM (without any serum or antibiotic). The concentration of DNA should not be less than 0.1 µg/µL. Mix and spin down briefly. Add 30 µL Superfect transfection reagent to DNA solution and mix gently by pipeting up and down four or five times. Allow the complex to form by incubating the mixture at room temperature for 10 min. During this incubation period, wash psi-2 cells once with 4 mL PBS. Add 1 mL complete medium to the tube containing the Superfect/DNA complex, mix, and immediately transfer the whole volume to psi-2 cells. Incubate for 2–3 h. Gently aspirate out the incubation mixture, wash two or three times with 4 mL PBS; add fresh complete medium. Leave control plate untransfected.

Day 3: Replace medium from psi-2 plates with 4 mL fresh complete medium. Plate 5×10^5 PA317 cells/60-mm dishes. Use one plate each for psi-2 infection and one plate for control (no infection).

Day 4: Collect medium from psi-2 cells and remove floating cells by filtration through a 0.45-µm filter or by centrifugation at 5000*g* for 5 min. Replace medium on PA317 cells with 4 mL complete medium containing 4 µg/mL polybrene. Add 0.1 or 1 mL of virus-containing medium from psi-2 cells to PA317 cells; mix by swirling the plates.

To assess the transfection efficiency, treat psi-2 cells with ATV trypsin and replate 10% of the cells in 60-mm plates in medium containing 400 µg/mL G418. To ensure that drug selection conditions are working, plate untransfected cells and treat with G418

Day 5: Trypsinize PA317 cells, plate 10% cells in a 100-mm dish in 10 mL complete medium containing 400 µg/mL G418 and select for drug resistance as described for psi-2 cells (*see* **Note 5**).

Day 6 and onward: Monitor the cell survival and formation of colonies. Plates should be checked under light microscope to visualize the small colonies. Replace medium with fresh medium as necessary to remove the dead floating cells. Determine the number of individual colonies in psi-2 and PA317 plates. The number of colonies in psi-2 cells will determine the transfection efficiency (*see* **Note 6**).

Tremendous variation (three to four orders of magnitude) has been noted in viral titer in concurrently generated colonies from a single transfection/infection. To obtain one or more high-titered virus-producing producer cell lines, it is necessary to isolate clones and determine viral titers. Pick clones from PA317 plates that have relatively large (approx 50–100 cells) and well-

separated colonies. Mark a clone by circling it on the back of the dish. Melt 3% agarose solution in a microwave oven and cool it to approx 45–50°C. Mix 1 mL of agarose with 2 mL ATV trypsin (warmed to 37°C), add immediately to the dish containing the colonies, gently spread over the cells by swirling the plate, allow to solidify, and let sit for 2–3 min for trypsin to work. Gently cut around the circles with a sterile Pasteur pipet and lift the agarose plugs (with cells attached to it) and transfer them to individual wells of a 24-well plate containing complete medium and 400 µg/mL G418. Gently wash the area of the plug twice with the medium (approx 100 µL) and transfer to the well containing the cells. Allow the cells to grow to approx 70–80% confluency. Determine viral titer (**Subheading 3.4.**) (*see* **Note 7**).

3.3. Production of Helper-Free High-Titer Retrovirus

Recombinant virus can be concentrated to obtain a high-titer virus (*see* **Note 8**). Transiently transfect the highly transfectable 293T cell line that stably expresses retroviral packaging functions ***(30)*** with the plasmid vector using Superfect transfection reagent (*see* **Subheading 3.2.**). Remove the medium after 48–72 h and either centrifuge at 5000*g* for 5 min or filter through a 0.45-µm filter. The packaging cell line produces infectious retrovirus at $>10^6$ colony-forming units (CFU)/mL within 72 h after transfection ***(30)***. For further concentration, centrifuge supernatant at 50,000*g* for 90 min in an ultracentrifuge. Suspend pellets in Tris-buffered saline (TBS), pool, and recentrifuge at the same speed for 90 min ***(31)***. Resuspend pellet in sterile saline containing polybrene. The resulting virus stock usually has a titer of 10^7 CFU/mL. The concentrated virus can be frozen at –70°C and thawed before use.

3.4. Determination of Viral Titer

Day 1: Plate PA317 cells at 5×10^5 cells/60-mm plate in medium containing G418.

Day 2: Plate 5×10^5 target cells (NIH 3T3)/60-mm dishes. Make two plates for each clone to be titered, one plate for a positive control (to be infected with a virus of known titer) and another for negative control (receives no virus). Replace medium on PA317 cells with fresh medium containing no drug.

Day 3: Collect medium from PA317 cells and filter through a 0.45-µm filter. The virus-containing medium can be kept frozen at –70°C and thawed once without the loss of virus titer. Replace the medium of target cells with fresh medium containing 4 µg/mL polybrene. Add 0.1–10 µL virus-containing medium to each dish and mix gently by swirling the dish.

Day 4: Trypsinize and plate 10% of the target cells in 60-mm plates (two plates/infection) and 90% in 100-mm plates in G418-containing medium. Split the control dish (uninfected) the same way and select for drug resistance.

Days 5–10: Replace medium with fresh G418-containing medium as needed and continue selection until all cells in control cultures are dead. Monitor cells for survival and colony formation in experimental plates. A dish containing < 50 colonies is suitable for determining the titer.

After colonies appear, remove the medium from the 60-mm plates (two plates/infection) and wash cells with PBS. Add enough Coomassie solution (1 mg/mL) to cover cells completely and let stand for 5–10 min. Wash plates gently in tap water until the background is clear and the blue colonies are visible. Count number of colonies per plate. Number of colonies in duplicate plates should be similar. Virus titer CFU/mL) = 10 (split ratio) × Number of colonies × Dilution of virus.

A guideline for dilution of virus stock is as follows:

High-titer virus (10^6 CFU/mL) = 0.1–1 µL/dish
Medium-titer virus (10^5 CFU/mL) = 1–10 µL/mL
Low-titer virus (10^4 CFU/mL) = 10–100 µL/mL

Virus stocks with a titer lower that 10^4 CFU/mL are generally not useful and can be discarded. Many vector constructs will produce virus in the range of 10^5 CFU/mL, although titers of 10^6 CFU/mL or higher can be generated with some virus constructs by concentration.

3.5. Detection of Replication-Competent Helper Retrovirus (neo Marker Rescue Assay)

Recombination between packaging sequences and the retroviral vector will occasionally generate replication-competent helper virus. The assay is based on the ability of the helper virus to package a replication-defective vector RNA and transfer it to naive target cells.

Day 1: Plate 1×10^5 marker cells in a 60-mm plate. Marker cells can be any 3T3 cells transduced with a helper-free virus containing a selectable marker like pLNCX. Plate one dish of cloned PA317 cells to be assayed.

Day 2: Replace the medium of marker cells with fresh medium containing 4 µg/mL polybrene. Add 1 mL of cell-free supernatant from PA317 cells (prepared as for titering the virus). Often the virus titering and detection of the helper virus are done at the same time using the same virus-containing supernatant from PA317 cells.

Days 3–14: Do not select the marker cells, as they are already G418 resistant (*see* **Note 9**). Passage cells as needed and maintain them at high cell

density but in the log phase of growth. This would help the spread of replicating helper virus throughout the culture (*see* **Note 10**).

Day 14 and onward: Treat the infected marker cells as if they are virus-producing cells and determine the viral titer on naive NIH 3T3 cells as described in **Subheading 3.4.** Generation of G418-resistant colonies would indicate the presence of helper virus.

3.6. Generation of Fibroblast Cultures from Skin Explants (*see* Note 11)

Day 1: Anesthetize a rat by intramuscular or intraperitoneal injection of the anesthetic solution. Wash a portion of the abdomen with 70% ethanol to sterilize the region and shave the area. Wash the area with an iodine solution followed by a wash with 70% ethanol; air-dry. Cut out a section of the full-thickness skin (approx 1–2 cm^2) with a scalpel blade. Dip the skin in 70% ethanol wash and then place in a sterile tube (50 mL) containing complete medium. Close the wound and allow the animal to recover. All these procedures are done on the laboratory bench.

In a tissue culture hood, wash biopsied skin twice with complete medium. Place the skin in a 60-mm dish containing a small amount of medium. Remove the fatty connective tissue from underneath the skin and dice the skin into smaller pieces (approx 1–2 mm^2) by holding the skin against the dish with a scalpel and then cutting it with a second scalpel or sterile blades. Place 6–10 pieces of explants in a well of a six-well plate and put a sterile cover slip (22 mm^2) whose corners are dipped in sterile silicone grease for anchoring. Slowly add 3 mL complete medium per well and incubate. Alternatively, place the diced tissues under the cover slip and slowly add enough medium under the cover slip just to wet the bottom of the plate. If the top of the cover slip is left dry and the depth of the medium is slightly less than the top of the cover slip, then surface tension will hold it down to the plate.

Day 2 and onward: Incubate explants for 1–2 wk and monitor cell growth. Replace medium with fresh complete medium every 4–6 d. When the tissues are placed under the cover slips without silicone grease, the medium should be changed every 2–4 d until the cover slip adheres well to the tissue. Then, the larger volume of medium can be used with less frequent changing. Fibroblasts will grow out of the explants and form monolayers. When a small to medium area of cell monolayer is established on the cover slip or on the underlying tissue culture dish, passage cells to a new plate. Consider this as passage 1.

3.7. Passaging and Expansion of Cells

Remove the culture medium by gentle aspiration from the side of the well with a Pasteur pipet and a vacuum pump. For a well in a six-well plate wash

with 2–3 mL PBS twice. Add 1 mL ATV trypsin to cover the cells. Incubate for approx 1 min and then gently hit the side of the dish to dislodge cells. Examine the culture periodically under light microscope to make sure that all cells have lifted from the plate. Add 2–3 mL of complete medium and resuspend the cells with a pipet. Inoculate a T75 flask containing 8–10 mL of complete medium with this culture (equivalent of three to six wells) and allow the culture to reach confluency.

Passage the culture from a near-confluent (90%) T75 flask by trypsinization, as just described. Split the culture 1 : 4 (i.e., if the cells are taken up in 10 mL total volume of the medium and trypsin, then transfer 2.5 mL cell suspension per T75 flask). Feed the cultures only once or twice a week depending on the confluency.

3.8. Generation of Genetically Modified Fibroblasts

Day 1: Plate (5–10) × 10^5 fibroblasts in complete medium/100-mm dish. Prepare one dish per infection and one as the negative control.

Day 2: Replace the medium with fresh complete medium containing 4 μg/mL polybrene. Add 10% volume of thawed cell-free virus-containing medium collected from transduced PA317 cell lines as described for titering of the virus.

Day 3: Passage fibroblasts to four 100-mm dishes and allow the cells to grow in complete medium containing 400 μg/mL G418. Place the cells in the control (untransfected) plate under drug selection (*see* **Notes 12** and **13**).

Day 4 and onwards: Monitor for cell survival and passage cells as necessary. Cells should be under the log phase of growth during selection, as at high cell density, stationary nontransduced cells do not die (*see* **Note 14**). The efficiency of the selection process can be determined by the death of all cells in the negative control plate. Expand cells, characterize for the expression of neurotrophins, and freeze cells for long-term storage. Clonal cultures expressing high amounts of neurotrophins can be generated by cloning cells from bulk infected cultures as described in **Subheading 3.2.** Clonality of the cultures can be determined by Southern blot analysis ***(30)***.

3.9. Storage and Reculturing of cells

Fibroblasts can be stored frozen in liquid nitrogen for a long period of time. To store fibroblasts, trypsinize cells from a near-confluent T75 flask (*see* **Subheading 3.7.**) and pellet them by centrifugation at 5000*g* for 3 min. Resuspend cells in 3 mL complete medium containing 10% DMSO. Place 1 mL per freezing vial (cryovial), put the tubes in a Nalgene freezing chamber, and freeze at –70°C. After 16–24 h, place cells in a liquid-nitrogen tank.

Reculture frozen cells by thawing them quickly in a 37°C water bath and then plating in a 60-mm or 100-mm dish, depending on the cell density of the

frozen culture. Change the medium 5–6 h after plating to remove DMSO from the medium. Feed cultures once or twice a week, depending on the confluency.

3.10. Cell Count

3.10.1. Coulter Counter

Trypsinize and resuspend cells in complete medium. The total volume will depend on the confluency of the culture. For example, cells from a near-confluent (90%) T75 flask can be suspended in a total volume of 10 mL. Dilute 100 µL of cell suspension in 10 mL Isotone solution and count twice in a Coulter counter. The counter counts cells present in the 0.5-mL solution; counting twice gives the number of cells per milliliter. Total number of cells = Number of cells/mL × dilution × total volume.

3.10.2. Hemocytometer

Dilute a portion of the cell suspension 1:4. Add enough cell suspension to the hemocytometer to cover all of the chamber but not overflow. Count the number of cells in four external squares. With a chamber depth of 0.1 mm, the sample volume will be 4×0.1 mm or 0.4 mm^2. Total number of cells = Number of cells counted $\times 10^4 \times$ Dilution × Total volume.

3.11. Characterization of Genetically Modified Fibroblasts

Expression of neurotrophins by genetically modified cells is examined at the RNA and protein levels.

3.11.1. Detection of mRNA

Isolate mRNA from control and modified fibroblasts and analyze by Northern blot using standard methods ***(30)***. The transgene-specific mRNA expression can also be analyzed by reverse transcriptase–polymerase chain reaction ***(32)***.

3.11.2. Detection of Protein

The production of neurotrophin proteins can be monitored by Western blot analysis and ELISA. Because neurotrophins are secreted from cells, the culture medium (CM) collected from modified cells after 24–48 h of incubation or cell lysates can be used for this purpose. Western blot analysis is done with concentrated CM using commercially available antibodies against neurotrophins ***(30)***. ELISA provides a sensitive method for quantitative measurement of neurotrophin production. ELISA kits to assay NGF and NT-3 are commercially available (Boehringer Mannheim, Chemicon, and Promega, respectively). These kits can detect 5–10 pg/mL mouse NGF, 30 pg/mL of human NGF, and 4.7–300 pg/mL human NT-3. Antibodies against BDNF and

NT-4 are also commercially available and ELISA assays can be developed for the detection of these proteins. However, ELISA methods do not determine whether the proteins are biologically active.

3.11.3. Detection of Biologically Active Transgene Products

The biological activity of NGF is determined by the ability of CM from NGF-expressing cells (CM-NGF) to elicit neurite outgrowth for rat PC12 cells, an assay sensitive to 100 pg/mL ***(33)***. Grow 5×10^4 to 2×10^5 cells in collagen-coated 35-mm dishes. Filter CM through 0.45-µm filters. Add filtered CM-NGF, CM-lacZ, CM from untransfected fibroblasts (CM-control), and defined medium containing known amounts of rhNGF to PC12 cultures and incubate for 16–24 h. Photograph cells at different fields and measure neurite outgrowth. BDNF supports the survival and neurite elongation from embryonic chick dorsal root ganglion (DRG) neurons ***(34)***. Dissect DRG from E9 chick embryos, suspend in 0.8% trypsin, incubate for 20 min at 37°C, add complete medium, and gently titurate. Plate cells for 2–4 h for preferential adhesion of non-neuronal cells. After gentle washing, plate unattached cells (15,000 cell/cm^2) in 24-well plates coated with poly-ornithine (5 µg/mL) and laminin (10 µg/mL). Incubate cells in CM-LacZ, CM-BDNF, CM-control, and defined medium containing known amounts of rhBDNF (100 ng/mL) for 48 h, fix with 4% paraformaldehyde, and photograph ***(10)***. Measure cell survival and neurite outgrowth. Biological activity of NT-3 is assayed using cultured embryonic (E9) chick sympathetic (SG) or DRG ganglia neurons ***(34)***. Condition fibroblasts (control or expressing NT-3) in N2 medium for 48–72 h, add filtered medium to cells ***(11)***, and assess cell survival and neurite elongation after 48 h incubation.

4. Notes

1. To obtain optimal transfection, a number of parameters should be optimized. The best transfection efficiency is achieved with plasmid DNA of highest purity. For each cell line and new plasmid construct, it is necessary to optimize the ratio of DNA and Superfect reagent. The cells should be in the log phase of growth and the cell density should not be too high or low. A 40–80% confluent plate is desirable for this purpose.
2. DMEM and DMEM:F12 media require 10 and 5% CO_2, respectively. If a different medium is used, check for the CO_2 requirement described in the catalog.
3. The quality of the serum is important for cell growth. Samples of serum from different batches and from different suppliers should be tested for optimal growth of a particular cell type before purchasing the batch. To avoid repeated freeze–thawing, aliquot serum in small volumes and keep at –70°C.
4. The protocol described here uses Superfect transfection reagent (cationic liposomes) for transduction of producer cells. However, other methods like calcium

phosphate-mediated gene transfer ***(21)***, lipofection ***(22)***, electroporation ***(23)***, and microinjection ***(24)*** can be used successfully.

5. The presence of numerous G418-resistant psi-2 colonies indicates a good transfection efficiency, whereas drug-resistant PA317 colonies indicate successful virus packaging and integration. Although producer lines can be generated by transfecting packaging cell lines directly and isolating virus-producing clones, the plasmid DNA often integrates as multiple rearranged copies. As a result, clones often produce a mixture of viruses containing the expected vector genome as well as rearranged genomes. Infection of a second packaging line with the limiting dilution of the rescued virus generates clones with a single integrated proviral genome whose unrearranged nature can be confirmed. Transduction of packaging cells with vectors of the same tropism is more than 1000 times less efficient than the transduction of vectors with a different tropism. For this reason, two cell lines of different tropism are used for the production of recombinant virus.
6. Genomic DNA prepared from clones of packaging cell line with acceptable titers should be analyzed by Southern blot analysis ***(30)*** to determine if the integrated virus contains large deletions or insertions. The blot when probed for the *neo* sequence should show a single band of the size of the plasmid.
7. Viruses with a titer of 10^4 CFU/mL are not useful because the transduction efficiency in primary cells will be quite low. Recombinant viruses with a titer of 10^5 CFU/mL are routinely used in the authors' laboratory. This concentration should transduce approx 10–50% of a fibroblast culture. A high-titer helper-free retrovirus (*see* Subheading **3.3.**) can also be used.
8. It is important to determine if transduced cells are free of the helper virus. If these cells are used for grafting, the helper virus can cause the spread of the transgene sequence to the surrounding tissue and complicate the evaluation of the results.
9. If the virus generates G418 resistant colonies in target cells but they fail to express the transgene products, confirm that the integrated proviral sequence is not rearranged, transgene mRNAs are present in target cells, and they are of expected size. If mRNA is detected, then analyze for protein production by transient transfection of cDNA carried in a simple plasmid expression vector. If both of these experiments give positive results, then a minor defect may be present in the vector or there may be a mutation in transgene-coding sequences. Test an alternate bacterial clone of the vector plasmid.
10. Sometimes, over the course of time, a high-titer packaging cell line may generate a reduced titer virus because of the selection of cells that have lost vector expression or packaging function. Reselect cells for drug resistance or subclone to obtain a functional clone.
11. For allogenic grafts, primary fibroblasts can be cultured from the biopsy of a single animal and subsequently used in other animals of the same strain. For autologous grafts, a small skin biopsy sample can be used for this purpose. Cell cultures are more readily established from a young animal than from older animals. Cells from aged animals usually go through a crisis period from which few

cells survive. Cells from young animals can be expanded for up to 10–15 passages with a twofold to fourfold dilution at each passage. Stocks of cells from earlier passages should be frozen to ensure supply later. Extensive passaging may select for a rare, spontaneously mutated cell whose survival and transgene expression characteristics may not reflect the properties of early passage deploid fibroblasts.

12. Continuous maintenance of transduced cells in G418 can reduce the clonal diversity of a population, as the cells expressing a high level of *neo* gene product will grow more rapidly than the ones with a lower level of *neo* expression. Maintain cells in medium without G418 and periodically select to remove cells that are no longer drug resistant.
13. Occasionally transfected psi-2 cells fail to produce virus resulting from poor transfection efficiency. Either optimize the transfection protocols or use a different physical transfection method. A less likely reason for lack of viral production may be because the producer cell lines have lost the packaging function. During passaging over long periods, cells not expressing viral protein encoding genes may have been selected. Use an earlier passage of the packaging cell line or obtain new cells from ATCC.
14. One common problem with the retroviral vector system is the downregulation of the transgene expression with passage and time. To a large extent, this is the result of the selection of cells with a low expression level (cells expressing a low level of transgene product grow faster) or gradual transcriptional downregulation particularly in quiescent cells. It is difficult to distinguish between these two processes. If the transgene is trancriptionally downregulated, then either work with cells growing in the log phase or develop vectors that remain transcriptionally active in specific cell populations. If differential growth selects for low expressors, then use only low passage cells.

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11

Efficient Generation of Stable Pheochromocytoma (PC12) Cell Lines Using a Recombinant Retrovirus (LNC)

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1. Introduction

Investigation into the nature and mechanism of action of neurotrophins dates back to the description of a "nerve growth factor" activity by Levi-Montalcini (described in **refs.** ***1*** and ***2***) and has progressed to include a family of factors, several of which have been assessed in Federal Drug Administration (FDA) trials to treat neurodegenerative diseases. Study of neurotrophins and their receptors has employed many paradigms, including knockout animals, primary neuronal cultures, and a variety of cell lines. Perhaps the most popular of the cell lines used in these investigations is the rat pheochromocytoma cell line PC12 ***(3)***. PC12 cells resemble immature adrenal chromaffin cells until stimulated to differentiate into a sympathetic neuronlike state by nerve growth factor (NGF) treatment. The documented similarities between neuronally differentiated PC12 cells and cultured neurons, at both the morphological and biochemical levels, are striking and have underscored the utility of the PC12 cell system in neurotrophin research (reviewed in **refs. 4–6**).

PC12 cells have been indispensable in acquiring much of the understanding we have about the NGF mechanism of action. Many experimental designs utilizing the advantages and exploiting the PC12 cell system to this end have been detailed elsewhere (reviewed in **refs. 7** and **8**). Here, in contrast, we describe protocols to overcome a shortcoming of PC12 cells, namely, the difficulty they show for efficient transfection and transgene expression. The property of PC12 cells that most contributes to this shortcoming is their slow transit through the cell cycle, with cell doubling occurring only every 3–4 d. This chapter

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describes methods for the efficient generation and use of the retrovirus pLNC-X ***(9)*** to generate PC12 cells that express foreign genes. We have recently used these methods to study mutant TRK receptor function in PC12-derived cells ***(10)***. The pLNC-X vector is especially suited for PC12 cell work because the transgene is under the control of the cytomegalovirus promoter, which has been found to promote high gene expression levels in PC12 cells ***(11)***. However, the procedures can likely be used for other viruses of this series (e.g., pLNS-X or pL-X-SN, which use the SV40 promoter or MLV-long terminal repeat to drive transgene expression, respectively) ***(9)*** or of another design (e.g., pCRIP or pGD) ***(12)***.

The retroviral transgene delivery system has several advantages that make it an attractive alternative to previously described transfection techniques for PC12 cells, such as electroporation ***(8)***. First, the retrovirus system does not require the use/purchase of the electroporation apparatus or electroporation cuvets. Additionally, transduction efficiency with viruses can be better controlled (by assessing viral titer and inoculating appropriately) than in the case of transfection. Moreover, viral inoculum can be raised (discussed later) to levels that allow PC12 cell infection at rates that transfection cannot presently achieve. In addition, we have noted that retroviral infection yields a substantially higher percentage of selected lines that express the transgene and that the levels of transgene expression are generally higher in the infected cells. Finally, using the coculturing technique described in this chapter, clonal colonies of G418-selected PC12 cells are ready for expansion at a substantially earlier time (presumably the result of a "feeder layer" effect).

Approaches described in this chapter are presented as defined steps and can be summarized as follows. The production of recombinant retrovirus involves transiently transfecting a viral packaging cell line with a plasmid containing the recombinant LNC virus, and culturing these cells to generate a virus-containing supernatant. This packaging cell supernatant is filter sterilized and is the viral stock used for infection of the PC12 cells. By infecting several subcultures of PC12 cells with different dilutions of this viral stock, one can generate G418-resistant colonies sparse enough for cloning of cells or dense enough for use as mixed (polyclonal) cultures. At the end of the chapter, we describe an alternate method using a coculture of the transfected viral packaging cells with PC12 cells. This coculture technique can be used to boost LNC titers/infection to levels higher than is possible by the inoculation method. As mentioned earlier, this coculturing technique substantially decreases the time required to generate transfected clones for further culture and analysis and is especially useful in the generation of polyclonal PC12 cultures. Using these protocols, we believe that one can overcome the resistance that PC12 cells have shown to genetic manipulation.

2. Materials

2.1. Cells and Cell Culture

1. The rat pheochromocytoma cell line (PC12) can be obtained from a variety of sources, including our laboratory.
2. The retroviral packaging cell line BOSC23 can be obtained from the American Type Culture Collection (Rockville, MD) and requires a material transfer agreement from Rockefeller University (New York, NY).
3. Medium for culture of PC12 cells is RPMI 1640 (Gibco-BRL), supplemented with 10% heat-inactivated horse serum (56°C for 45 min) and 5% fetal calf serum (both from JRH). PC12 cells are grown on tissue culture plates (Falcon) coated with rat-tail collagen prepared and applied as described in detail elsewhere ***(8)***. BOSC23 cells are cultured in DMEM high glucose (Gibco-BRL) supplemented with 10% fetal calf serum (JRH) on tissue culture plastic (Falcon). BOSC23 cells are passaged with trypsin/EDTA (Sigma).

2.2. Reagents

1. A 1000X (25 m*M*) aqueous stock of chloroquine (Sigma) is used for BOSC23 cell transfection.
2. The two other transfection solutions are calcium chloride and HEPES-buffered saline (2X HBS). Stock calcium chloride is 250 m*M* and is filter sterilized. The 2X HBS is 50 m*M* HEPES, 1.5 m*M* Na_2HPO_4 and 280 m*M* NaCl, adjusted to pH 7.1 ± 0.05, and is filter sterilized.
3. pLNC-X plasmids can be obtained from A. D. Miller ***(9)*** (Fred Hutchinson Cancer Research Center, Seattle, WA) or have recently become commercially available through Clontech (Retro-X™, cat. no. K1060-1). The pLNC-X plasmid is easily manipulated by standard molecular-biological approaches ***(13,14)***. A brief description of our cloning strategies and methods will be described in **Subheading 3.** Recombinant pLNC plasmids prepared for virus production should be of a purity sufficient for transfection (we use a resin-binding-based system from Qiagen), and 20 µg is required per transfection.
4. Mitomycin C (mutamycin) used in the coculturing technique is from Sigma and is made as a 100X (1 mg/mL) stock using sterile phosphate-buffered saline (PBS) ***(14)***.
5. Geneticin (G418) is from Sigma, is prepared in water as a 100X stock and is filter sterilized. The final concentration in culture of pure (active) G418 should be 500 µg/mL and the concentration of the stock adjusted according to the purity of the starting material.
6. Standard laboratory supplies, such as cryotubes for cell storage, 0.22-µm bottle-top and syringe filters for sterilizing reagents, 10-mL syringes, tissue culture plates of various sizes, and the like.
7. Molecular-biology apparatus and supplies, including but not limited to: agarose gel electrophoresis apparatus and power supply, various restriction endonucleases, DNA ligase, DH5-α *Escherichia coli*, and components for LB and SOB media (recipes in **ref. *14***).

3. Methods

Subheadings 3.1. and **3.2.** describe the preparation of recombinant pLNC-X plasmids and general comments about retroviral life cycle and viral packaging cell lines, respectively. **Subheadings 3.3.–3.6.** describe transfection of the packaging cell line with recombinant pLNC constructs, target field preparation, viral harvesting, and PC12 cell infection, respectively. **Subheading 3.7.** describes a technique for coculturing virus-producing packaging cells with PC12 cells.

3.1. Preparation of Recombinant pLNC-X Constructs

LNC-X is a recombinant form of the Moloney murine leukemia virus (MLV) and gets its name from the organization of its subdomains (i.e., long terminal repeat [LTR], neomycin resistance, cytomegalovirus [CMV] promoter, gene of interest, LTR). This structure recapitulates the natural state of MLV with the sequences encoding viral proteins (gag, pol, and env) replaced by sequences for expression of the neomycin resistance and the transgene. The pLNC-X plasmid has the proviral LNC DNA incorporated into the cloning vector pBR322, which makes pLNC-X quite easy to handle and use as an expression vector. Genes to be driven off the CMV promoter need to have their own translation start sequence (ATG) and need to be directionally cloned into the multiple cloning site (MCS) that immediately follows the promoter. In addition, genes to be expressed cannot exceed about 4.5 kb, because of size constraints on virion packaging. Several versions of pLNC-X exist that are characterized by having different restriction enzyme recognition sequences in their respective MCS. We have been utilizing pLNC-X.5, which has *Hind*III, *Xho*I and *Cla*I in its MCS (5′ to 3′, respectively). The version (pLNC-X.4) available from Clontech has somewhat different sites in its MCS. A full description for generating a recombinant pLNC-(gene) is beyond the scope of this chapter; however, the following guidelines may assist efforts to this end:

1. To express any gene of interest from the CMV promoter, it must be inserted into the MCS in the proper 5′ to 3′ orientation. This is most easily accomplished by directionally subcloning the gene from its cloning plasmid using restriction enzymes with noncompatible cohesive overhangs (e.g., *Hind*III and *Xho*I from the example of pLNC-X.5). Alternately, nondirectional cloning may be the only option. If this is the case, insert orientation must be evaluated and a subclone with the proper directionality chosen for propagation. To assess insert orientation, there is a unique *Bam*HI site 800 bp 5′ of the MCS (preceding the CMV promoter) and a unique *Xba*I site 350 bp 3′ to the MCS (in the 3′ LTR). Further information about restriction sites of pLNC-X can be obtained from the Internet

at http://www.medkem.gu.se/cutter/ using "pLNCX" as a search term, and then selecting the information required. Note that the form described there is pLNC-X.4.

2. Subcloning involves digesting the plasmids with restriction endonucleases, purifying the DNA fragments by agarose gel electrophoresis, and ligating the fragment(s) into an ampicillin-resistance-carrying pLNC-X plasmid. These steps are well described elsewhere ***(13,14)***.
3. Ligated plasmids are then introduced into *Escherichia coli* (transformation) for amplification and assessment (miniprepping). Standard protocols ***(13,14)*** are sufficient for performing these steps; however, to maximize the probability of obtaining a subclone of interest, better protocols exist. Transformation is affected by bacterial competence and transformation technique, both of which have been recently improved/optimized ***(15,16)***. Additionally, miniprepping is tedious and is limited by the number of samples that can be processed at one time; a much more efficient method has been described ***(17)***, which can increase both the rate and number of samples processed in one batch.
4. pLNC-(gene) plasmids determined to be properly constructed by restriction enzyme analysis (and/or sequencing) need to be amplified for virus production. pLNC is not a high-copy plasmid (likely a result of its 6.6 kb size without insert), so about 2 mL of bacterial culture is needed for each microgram of DNA required. We typically grow 250-mL cultures in LB medium to get 100 µg of pLNC-(gene) and have enough plasmid to perform several transfections. We purify scaled-up bacterial cultures with a resin-binding based kit (Qiagen, cat. no. 12143) that delivers DNA suitable for immediate transfection.

3.2. Retroviral Life Cycle and Recombinant Virus Packaging

To aid in understanding the protocols that will be provided in the following sections, a brief description of the mechanics of the system is in order. Although a broad description of the subject is beyond the scope of this chapter, readers who desire more detail are encouraged to consult the review by Brown and Dougherty ***(18)*** and appropriate references therein. Ultimately, the LNC-(transgene) delivery system mimics portions of the MLV life cycle, which merits introduction. The characteristic parts of any retroviral life cycle are that after the virus adheres to the target cell and delivers (transduces) its RNA genome into the cell, the genome is reverse transcribed (by an enzyme delivered with the genome) and the viral DNA incorporates into the genome of the host cell, forming a provirus. In the case of a wild-type virus, the incorporated provirus has all of the structural genes required for completing the life cycle (i.e., *gag*, *pol*, and *env*), and the proviral DNA is transcribed into RNA viral genomes that are packaged and released. Nonlytic spread of viral infection then occurs *horizontally* (described directly above) in replication-competent viruses and *vertically* (from mother to daughter cells by host cell division) in

replication-competent or nonreplicative viruses. LNC-X is restricted to vertical transmission because of its lack of viral structural genes.

The question then arises as to how LNC can be harnessed to deliver its genome in the initial infection if the provirus is incapable of packaging and initiating a horizontal (free-virus) propagation. The answer lies in supplying the viral structural genes in *trans*, through use of a viral packaging cell line. There are many packaging lines to choose among, all of which are a bit different, but they all accomplish the same end: they have been transfected to provide the structural genes required for packaging and release of infectious virus. Some of these cell lines, such as Psi-2 ***(19)***, require stable transfection of the recombinant LNC plasmid to obtain virus (which requires up to a month of cell culture), whereas others such as BOSC23 ***(12)*** and PHOENIX ***(20)*** produce a virus with transient transfection of pLNC (requiring only 3 days of culture). The latter two cell lines differ in that BOSC23 tends to lose packaging ability when kept in culture without selection, whereas PHOENIX is more stable. All three of these lines deliver MLV gene products in *trans*. Other lines, such as 293GPG ***(21)***, deliver the VSV-G (vesicular stomatitis virus) gene (instead of MLV-*env*) which provides for production of pseudotyped viruses that have broader host range and are much more stable to concentration and freeze–thaw than those obtained with MLV-*env* ***(22)***. We routinely use the BOSC23 cell line, and the protocols that follow describe its use; however, these methods are applicable for use with other packaging lines such as noted earlier, with minor modification.

3.3. Transient Transfection of BOSC23 Cells with pLNC-X

Detailed descriptions for routine culturing of the cell lines used in these methods is beyond the scope of this chapter and is described elsewhere ***(7,8,12)***. However, all of the practical information for required subculturing is described in the following sections or in the notes section (*see* **Note 1**). As a cautionary note, although LNC is a murine retrovirus ***(9)*** that has been demonstrated to be unstable in nonoptimal conditions ***(22)***, proper care should be taken to avoid self-contamination (wear gloves). To facilitate their use, the following protocols are written in a day-by-day format.

Transfection of BOSC23 cells is completed by modification of previously reported methods ***(12,23)***. Factors contributing to optimal transfection efficiency are freshly seeding BOSC23 cells in culture dishes (appropriate cell number in log phase growth), the presence of chloroquine (optimizes plasmid entry), accurate pH of the HBS, and timing of the DNA mixing (both ensure proper precipitation of plasmid DNA). Transfection efficiency can be monitored by using a pLNC-lacZ construct in the protocol, followed by X-Gal staining of the BOSC23 cells the next day (*see* **Note 2**). Transfection requires

subcloning BOSC23 cells the day prior to DNA addition, takes approx 7 h on the day of DNA addition, and follows these steps:

1. Day 1: In a tissue culture hood, subculture a confluent 10-cm plate of BOSC23 cells 1:3. Adjust the final culture volume to 9 mL per plate. One subcultured plate is required for each transfection.
2. Day 2 (16–24 h later): Add 1 mL of chloroquine-containing medium (10 µL of the chloroquine stock/mL) to each of the dishes prepared on d 1. This will result in a final concentration of 25 µ*M* in the 10 mL of medium in the plates (*see* **Note 3**).
3. Next, for each transfection, dispense 500 µL of 250 m*M* calcium chloride into a sterile 15-mL conical tube.
4. To this, add 20 µg of the recombinant pLNC-X plasmid and swirl tube to mix.
5. Using a sterile 1-mL pipet and pipet-aid, blow bubbles through the mixture to keep the tube contents continually mixing.
6. While continuing the bubbling started in **step 5**, slowly add 500 µL of the 2X HBS.
7. When all of the 2X HBS has been added, continue to bubble the medium for 10 s, then stop bubbles, and allow tube to stand undisturbed for 2 min at room temperature while the DNA forms a precipitate (2 min is optimal, but 2–5 min works).
8. Next, take the cells prepared in **step 2** and add the precipitated DNA drop by drop over the entire area of the plate and then rock the plate to swirl the medium and mix.
9. Put the cells back into a tissue culture incubator for 6 h.
10. After the 6-h incubation, replace the 10 mL of transfection media with 5–7 mL of growth media (without chloroquine) and culture for 2 d. Do not feed the cultures again before virus collection.

3.4. Preparation of Target (PC12) Cells

As discussed previously, the life cycle of all retroviruses includes a step wherein the reverse-transcribed genome of the virus incorporates into the genome of the host. This is the feature of the vector that allows the production of stable transgene-expressing cell lines with recombinant LNC-X(s). A point to be stressed is that in order for the two DNAs to combine, they must be in the same cellular location. For the Moloney murine leukemia virus (LNC's parent), this requires nuclear membrane breakdown; in other words, the cell needs to divide. Thus, in order to get viral incorporation and expression of the transgene as well as the neomycin resistance, the cells must divide at least once. To guarantee that this division can occur, we routinely infect PC12 cells that are between 30 and 50% confluent, and wait a week before commencing selection with G418. Subculturing of PC12 cells (on d 3) is conducted as follows:

1. Day 3: Using a sterile Pasteur pipet, mechanically detach a confluent 10-cm dish of cells by briskly, and repeatedly, squirting the growth medium at the monolayer (trituration).

2. Seed approximately one-third of the suspended cells into each of three collagen-coated 10-cm plates.
3. Unless you plan to titer the viral supernatant(s) (*see* **Note 4**), three to five plates of target cells will be needed for different dilutions of each retrovirus to be used.

3.5. Collection of LNC Supernatants and Production of Frozen Stocks

On the second day posttransfection (d 4 of the protocol), the BOSC23 cells will have accumulated recombinant LNC retrovirus in their growth medium. It is this supernatant medium (5–7 mL) that will become the viral stock used for the infection of the target PC12 cells prepared on d 3. The supernatant is prepared for use as a viral stock by clearing any nonadherent packaging cells by passing it through a 0.22-µm syringe filter. It should be noted that once filter sterilized, aliquots of the viral stock can be flash-frozen on liquid nitrogen and stored in liquid nitrogen for future use. We have found that stock viral titers drop approximately 50% when freeze–thawed; however, owing to the instability of the virus when stored in any other fashion, this is an acceptable loss (*see* **Note 5**). Preparation of stocks proceeds in the following steps:

1. Day 4: In a tissue culture hood, pass the medium from the transfected BOSC23 cells through a 0.22-µm syringe filter and into a sterile 15-mL tube.
2. The filtered viral stock can be used for direct infection of target cells (*see* **Subheading 3.6.**) or for flash-freezing described as follows.
3. Dispense 0.5–1 mL of the viral stock into labeled tissue culture cryotubes.
4. Flash-freeze the aliquots of virus by immersion of the tubes in liquid nitrogen.
5. Transfer into a liquid-nitrogen cryopreservation unit; liquid-phase storage is preferable, but as long as temperature fluctuations are minimized, it is not necessary.

3.6. Infection of Target Cells with Prepared Retroviral Stock

Filtered fresh (or previously frozen) virus is inoculated into the target PC12 cell cultures (prepared on d 3) by delivering an appropriate amount of the stock. As mentioned previously, the "appropriate amount" of stock to add depends on its titer and the purpose of the infection. Unless the stock is titered (which is not necessary), the most effective approach is to infect separate cultures with different amounts of inoculum. This will ensure that the desired transfection density is achieved. This is to say that if the purpose is to obtain single-cell clones, then the inoculum must be low enough so that colonies are sufficiently sparse enough to be isolated from one another; however, if the transfected cells are to be used to generate polyclonal cultures, then the highest inoculum is probably appropriate. Infection proceeds as follows:

1. Day 4: Label plates of target cells with recombinant virus to be used and volume that will be delivered.

2. In a tissue culture hood and using a sterile technique, deliver the predetermined volume of viral stock to the supernatant medium (usually 5–6 mL) of each PC12 cell target dish. Disperse the inoculum by rocking the plate to swirl the medium. We typically deliver 20 µL to 1 mL of the viral stock in fivefold dilutions (i.e., 20, 100, 500, and 1000 µL). If no titering is done, assume that it is about 1000 colony-forming units (CFU)/mL.
3. Return the target cells to the tissue culture incubator. Do not feed the target cells for at least 4 h. The virions need a period of time to adhere to target cells and transduce their genomes.
4. The PC12 cells are then cultured as per usual for 1 wk. Do not passage the infected target cells (as this would preclude any effort for subcloning transfected populations).
5. Day 11: One week after infection, G418 selection is applied. After medium exchange, deliver G418 from the 100X stock solution to a final active concentration of 500 µg/mL. Maintain G418 selection for 2 wk. Change medium three times the first week and then once the second week. PC12 cell cultures tend to grow better when not overly sparse (presumably a result of a released factor); consequently, we like to culture G418-selected PC12 cell cultures with 50% new and 50% conditioned medium (*see* **Note 6**) to aid in propagation, and we feed once or twice weekly as culture density dictates.
6. Approximately 1 mo: If subcloning of transfectant PC12 colonies is the desired end point, then when colonies in the G418-selected target plates become sufficiently large (i.e., approx 150–300 cells), mechanically subclone the cells (described in **ref. *8***). Briefly, this is done using a sterile technique in a tissue culture hood under a dissecting microscope. First, colonies are inspected under high-magnification light microscopy to assess morphology and are marked for cloning. Then, under observation with a dissecting microscope, the colonies are individually dislodged from their position, recovered, and transferred to collagen-coated 24-well plates using sterile pipets. We find that yellow micropipet tips attached to a 200-µL micropipettor work well for this purpose. When colonies expand to fill the 24-well surface area, the cells are transferred to collagen-coated 35-mm plates, and then to 10-cm plates. Aliquots should be frozen as early as possible to avoid loss of clones.
7. At culture confluence: If generating a polyclonal population of cells is the desired end point, then the passage 1 plate has been established. We routinely passage the cells purely for expansion and freezing at early stages, because of the changing nature of polyclonal cultures. It is best to have several aliquots of early passage cells to forestall problems with variations in cultures after multiple passages.

3.7. Target Cell Infection Through Coculture with BOSC23 Cells

We have noted that coculturing of BOSC23 cells with target PC12 cells seems to provide a "feeder layer" that allows PC12 colonies to grow faster. In addition, coculturing can help reverse problems with infection efficiency (by delivering more virus than is possible by inoculation), which is crucial if polyclonal cultures are desired. Coculturing is done by treating the transfected

BOSC23 cells with a compound (mitomycin C) that permanently prevents them from proliferating. Be careful when using mitomycin C; it is carcinogenic. The treated cells are then cocultured with the target PC12 cells so that they can shed recombinant retrovirus continually until they die from the mitomycin C treatment (which can take up to several weeks). Differences between this technique and that described earlier include that the target cells must be prepared on d 2 instead of d 3, that infection begins on d 3 instead of d 4, and that care must be taken at each feeding of the cocultures to avoid cross-contamination, because live virus will be present in the supernatant until all of the BOSC23 cells have died off. The technique proceeds as follows:

1. Days 1 and 2: Transfect BOSC23 cells as described above in **Subheading 3.3.**
2. Day 2: Subculture the PC12 cells for infection as described in **Subheading 3.4.**
3. Day 3: Put on gloves, and using a sterile technique, feed the transfected BOSC23 cells with growth medium containing 10 µg/mL (from the 100X stock) of mitomycin C (*see* **Note 7**).
4. Return the treated cells to the tissue culture incubator for 5 h.
5. After 5 h, the mitomycin C needs to be washed away from the BOSC23 cells so that when the coculture is initiated, the target PC12 cells are not exposed to the drug. Three exchanges with warmed, sterile PBS or with growth medium is sufficient.
6. Suspend the washed BOSC23 cells by adding 1 mL of trypsin (to a plate drained of medium) and rapping the plate for 1 min to dislodge the cells. Neutralize the trypsin by adding 4–7 mL of growth medium to the trypsinized cells.
7. Transfer the suspended cells to a 15-mL conical tube and pellet the cells at 150 to 250*g* for 3 to 5 min.
8. Remove the supernatant and suspend the cell pellet in 3 mL of growth medium.
9. Deliver the suspension to the PC12 target cells and return the plate(s) to the tissue culture incubator. Feed the cultures on the usual schedule, but be *very careful* with the discarded supernatant medium (it contains live virus as described earlier).
10. Monitor the coculture(s) for their morphology and proliferative behavior. BOSC23 cells grown on collagen-coated plates tend to spread out and be flatter than PC12 cells and appear stippled and more phase dark. Additionally, BOSC23 cells, when proliferating, divide every 18–24 h (in comparison to every 3–4 d for PC12 cells). Thus, if the mitomycin C treatment of the BOSC23 cells was insufficient, relative proliferation rates should illustrate the problem, and BOSC23 cell overgrowth will be apparent.
11. Day 10: At 1 wk of coculture, if there is no evidence of BOSC23 cell proliferation, the PC12 target cells can be G418 selected, as described in **Subheading 3.6.** As a cautionary note, do not confuse *presence* of BOSC23 cells with *proliferation*, and recall that the BOSC23 cells delivered were from a semiconfluent plate (there will be many of them). Until you are comfortable making assessments of

proliferation in coculture, it may be helpful to culture an aliquot of the mitomycin-treated cells in an empty dish (in parallel) to simplify the process.

12. Do not be alarmed by the persistence of the cocultured BOSC23 cells. Although most of the cells die off within 1–2 wk, BOSC23 cocultured cells may be carried along in the G418-selected PC12 cell cultures for several passages (recall that they were transfected with pLNC-X and are G418 resistant). As described earlier, these cells tend to be much flatter, larger, and phase dark than the phase-bright PC12 cells. The PC12 cells tend to grow on top of and around the BOSC23 cells.
13. PC12 cell colonies can be expected to appear in 1–3 wk (and sometimes during G418 selection) and can be isolated for generating single-cell colonies if they are sparse enough and well spread (which can be adjusted by the proportion of the BOSC23 cell suspension used for coculturing).

4. Notes

1. Standard subculturing of BOSC23 cells is done by trypsinizing cultures and splitting 1:5 at confluence. This typically means splitting cells every 3–4 d without need for medium exchange between subculturings.
2. If target PC12 cell infection is absent, or unacceptably low, and using lower-passage BOSC23 cells does not relieve the problem, then you may need to dissect the system. This is most easily done using a recombinant LNC-lacZ virus and a standard X-Gal staining protocol ***(13)***; however, any reporter will do (e.g., LNC-CAT). After checking the proper construction of the recombinant LNC, simply perform the above protocols using the reporter construct(s) and stain the cell populations used. If no staining of the BOSC23 cells is found, then there is a problem with the initial transfection. You should check the pH of the HBS stock and be sure that the DNA precipitation is done for 2 min (and not longer). Also, ascertain that the pLNC-X DNA is of good quality (not fragmented or contaminated with bacterial proteins).

   The other possible outcome will be that the BOSC23 cells are positive for the reporter, but that the targets are not. If you do not wish to coculture as described earlier, then the addition of polybrene (10 mg/mL is a 2000X stock) to the target PC12 cell infection step may help raise apparent titers. Additionally, keeping infection dilutions to a minimum may help increase infectivity. This can be done by aspirating PC12 cell medium completely and replacing with 2–5 mL of viral stock (with no consequent dilution), and after a 4-h incubation for adherence and transduction, bringing up the total culture volume to 5–6 mL with growth medium.
3. Chloroquine is quite toxic to the BOSC23 cells, and for this reason, it is best to keep the time that the cells are exposed to the drug to a minimum. It is suggested that chloroquine addition to the cultures be done immediately prior to the DNA precipitation/transfection and that the time be monitored. Transfections conducted over 7–11 h (and beyond) have worked, although the cultures were quite impaired.

4. When we have titered the virus on NIH-3T3 cells (by inoculating 1 mL of stock into a confluent 35-mm plate, 6 h later splitting this 1:5 into a 10-cm plate, and 16–24 h later, G418 selecting for 1–2 wk), we typically get (0.5–3) × 10^3 G418-resistant colonies per milliliter of BOSC23 cell supernatant.
5. LNC titers present in BOSC23 cell supernatants tend to decrease the longer that the cells are kept in culture. For this reason, it is advisable to obtain low-passage cells initially and to freeze down several aliquots of low-passage cells (by suspending in growth medium supplemented with 10% DMSO, storing at –80°C overnight, and then transferring to liquid-nitrogen storage). We try to thaw new aliquots every 2 mo, or as falling titers dictate.
6. Conditioned PC12 cell medium is prepared by collecting the supernatant PC12 cell medium normally discarded during feeding. Avoid collecting medium from PC12 cultures that are in poor condition, contaminated, near confluence, or have been previously transfected. Conditioned medium should be sterile filtered before being used to avoid cross-contamination (with nonadherent PC12 cells or microbes).
7. Mitomycin C is stable in aqueous solution for approx 2 wk at 4°C.

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12

Construction and Analysis of Transgenic Animals Overexpressing Neurotrophins

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1. Introduction

The ability to manipulate the mouse genome has provided extremely useful model systems for studying the underlying molecular mechanisms of neurotrophin peptide action in mammalian development and adult systems. One way to use transgenics is to overexpress neurotrophins in neuronal target tissues to investigate how target expression of neurotrophic factors affects neuronal development and function in the adult. This review presents the basic experimental design considerations of this approach and describes selected techniques used to evaluate transgenic phenotypes.

Overexpression of neurotrophins offers a means to study the temporal and biochemical controls regulating target tissue–neuronal interactions. Neurotrophins are normally synthesized by numerous non-neuronal tissues during development and in the adult ***(1)***. This production facilitates neuronal targeting and survival during development and appears to nurture and maintain phenotypic specialization of adult neurons. To identify mechanisms whereby target production regulates neuron development, tissue-specific promoters have been used in transgenesis to drive neurotrophin overproduction. For example, non-neuronal promoters have been used to target nerve growth factor (NGF) expression to the pancreas [insulin promoter ***(2)***], pituitary [prolactin promoter ***(3)***], skin [keratin 14 promoter ***(4)***], oligodendrocytes [myelin protein promoter ***(5)***], heart [cardiac-specific α-myosin ***(6)***], GFAP-expressing cells [GFAP promoter ***(7)***], and lung [Clara-cell secretory protein promoter ***(8)***].

Mice are most frequently used for mammalian transgenic applications because of the relative ease of fertilized egg isolation and microinjection, and

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their well-characterized genetics and short reproductive cycle. Pronuclear microinjection of transgene DNA constructs is now a rapid, reliable means to manipulate gene expression in vivo when done by experienced personnel. Given the time and expense to acquire the technical expertise and equipment for embryo retrieval and microinjection, it is preferable to have the transgene construct injected by either an academic or commercial facility.

In designing a transgenesis experiment, the strain of mouse is an important consideration. It has become increasingly clear that the genetic background of a mouse affects developmental, behavioral, and physiological parameters ***(9,10)***. Inbred strains widely used for microinjection studies are C57BL/6 or FVB/N. If strict genetic homogeneity is not required, F2 hybrid zygotes generated from matings between F1 hybrid male and female mice (e.g., B6 × C3H) can be used to improve egg production, transgenic generation, and reproductive performance ***(11)***. Although a mixed genetic background is generated, littermates provide appropriate controls. Toward providing a common genetic background for better comparative studies between laboratories, a proposal was recently made that transgenic mice isolated by microinjection be derived in either the C57BL/6 or 129/J inbred strains ***(12)***. Counter to this proposal was the limitations imposed in restricting studies to one strain and possible missed opportunities in understanding neurodiversity in structure and function ***(10,13)***. Although the choice of strain remains a debated issue, what is important is that the background be simple and described in detail.

1.1. Design and Isolation of a Transgene DNA Construct

Design of a transgene construct is dependent on the aims of the project and, optimally, should use promoter and enhancer sequences that have been characterized by transfection expression studies in culture cells. This allows testing of promoter efficiency and tissue specificity prior to investing in the more technically and time-intensive microinjection and screening of isolated mice. Transgene constructs typically range in size from 1.5 to 15 kbp and must include a promoter and enhancer region, a start codon, a cDNA or genomic DNA sequence encoding the protein of interest, a stop codon, and a poly(A) signal. Intron sequences should also be included either within the coding region or in the 3′ noncoding sequence to enhance mRNA stability and processing ***(14)***. Because the purified transgene insert should be free of bacterial plasmid sequences (or less than 100 bp), restriction sites should be included that flank the 5′ and 3′ ends of the transgene insert in order to purify insert DNA from vector sequences.

1.2. Genetic Screening of Founder Offspring

Screening of founder mice and their offspring can be done either by Southern, polymerase chain reaction (PCR), or slot blot analysis of genomic DNA

isolated from tail biopsies. DNA isolation takes about 1 d and produces DNA pure enough for restriction digestion or PCR amplification. Tail biopsies should be done on mice 3 wk or younger, at which time they should be tagged for identification by ear punch. Tail samples are collected by pushing the end of the tail forward to remove only skin and not tail vertebrae. Southern blotting should be used for initial identification of transgenic founder animals because this analysis provides information about transgene copy number and possible transgene rearrangements or deletions. Genomic slot blotting is preferable for rapid screening of subsequent generations of transgenics assuming hybridization signal from endogenous gene sequences can be minimized or avoided. Transgene copy number can also be determined using slot blots by comparing the density of band hybridization with those of known insert standards.

1.3. Analysis of mRNA Expression Using RT-PCR

Determining the pattern and level of transgene mRNA expression is essential for characterizing transgenic overexpression lines and is typically done using northern hybridization analysis. Based on these data, two to three lines should be established to (1) verify linkage between the transgene and possible phenotype (i.e., to rule out a positional effect), (2) to determine tissue specificity of transgene expression, and (3) to study gene dosage effects. Commercially available RNA extraction reagents containing phenol and guanidine isothiocyanate are available that allow fast RNA isolation from many tissue samples within 1 d.

Reverse transcriptase (RT)–PCR assays are helpful because they allow detection of gene expression in small amounts of tissue (ganglia, embryonic samples) that do not allow sufficient RNA recovery for either Northern or RNase protection assays. The degree and specificity of RT-PCR depends on magnesium ion concentration, enzyme concentration, primer specificity, and annealing temperature. As PCR generates millions of copies of a sequence, meticulous care must be taken to avoid carryover of DNA from one tube to another and one reaction to another to prevent false positives. Using ^{32}P-dCTP as a tracer in the reaction greatly improves assay sensitivity and allows semiquantitation of expression level when measured by a phosphorimager.

1.4. Analysis of Protein Expression Using Enzyme-Linked Immunosorbent Assays

Determining the level of transgene protein expression is required to verify translation of the transgene mRNA and correlate the protein level to phenotype. This can be done using Western blot analysis or the more sensitive enzyme-linked immunosorbent assay (ELISA). In ELISAs, antigens in a tissue sample are bound to a fixed antibody substrate coated on a 96-well culture

plate that is then treated to produce a colorimetric reaction product proportional to the amount of ligand bound. Sensitive ELISAs to measure NGF (Boehringer Mannheim, Chemicon, and Promega), neurotrophin (NT)-3 (Chemicon and Promega), and brain-derived neurotrophic factor (BDNF) (Chemicon and Promega) peptide levels are now commercially available. These assays are particularly useful for measures of neurotrophins in small amounts of tissues. For example, NGF, NT-3, and BDNF measures can be made by combining trigeminal ganglion from one animal or from 8–10 lumbar dorsal root ganglion. The ability to measure neurotrophin levels in a single animal allows an investigator to obtain a large "*n*" and assess the variability in a population.

Although the level of neurotrophin can be expressed relative to the wet weight of tissue processed or on a per milligram protein basis, values relative to total protein can be difficult because many sample buffers in ELISA protocols contain high levels of bovine serum albumin, which complicates protein measure. If quantification of protein is required, tissue can be homogenized in sterile buffer, an aliquot removed for protein assay, and the remainder quickly combined with sample buffer.

1.5. Neuronal Counting Strategies

There are two general approaches to counting cells in neuronal structures. The first relies on the "profile-counting" method based on the original Abercrombie ***(15)*** formulation and subsequent revisions (e.g., **ref. *16***). In this method, ganglia are serially sectioned and cellular structures (e.g., nuclei or nucleoli) are counted in evenly spaced sections (e.g., every tenth section). The number of neurons is estimated by multiplying the structures observed by the fraction of sections analyzed (in this case by 10). A correction factor is used to account for structures located in more than one section or when cells contain more than one structure (e.g., nucleoli). More recently, "unbiased" stereological methods have been developed [e.g., optical dissector method ***(17–19)***]. These unbiased methods are generally accepted as the best protocols for estimating the number of neurons in a wide range of structures, particularly in the central nervous system (CNS), where they have been used to disprove the theory that significant cell loss occurs during normal aging ***(20,21)***. The success of the optical dissector and related methodologies has been so dramatic that some journals only reluctantly publish estimates of neuronal numbers produced by other protocols [see the editorial by Saper ***(22)***; but also see **ref. *23***)].

Not surprisingly, the call to abandon what many investigators feel are useful techniques has led to much debate and direct comparisons of both approaches. For example, Popken and Farel ***(24)*** compared the profile and optical dissector methods. The estimates obtained by these two methods were normalized to the number of cells determined by three-dimensional reconstruction of sensory

ganglia. Both methods were found to provide accurate estimates of the number of neurons and the correlation coefficient of the two techniques was significant ($r = 0.83$, $p < 0.02$).

There are several considerations when determining which method is best for individual studies. Although protocols exist for using the dissector method without specialized equipment ***(17)***, most investigators rely on either dedicated image analysis systems and/or dedicated microscopes with motorized stages. Analysis using Abercrombie-type methods are usually done on tissue processed for paraffin histology, serially sectioned on a rotary microtome, and examined using a standard microscope equipped with either a drawing tube or video camera system (*see* below). When using a dissector method, it is important that the surface of each section is uniform and that shrinkage after sectioning is minimized (although this can be compensated for). To satisfy these criteria, experts on the dissector method recommend that tissues be embedded in plastic (e.g., cellodin). This requires significantly greater time and resources than paraffin-embedded tissue.

The most important consideration when choosing a counting methodology is the level of accuracy required. Guillery and Herrup ***(23)*** make a balanced argument that all counting methodologies have inherent biases that must be appreciated in the context of an experimental goal. Often, the relative ratio of cell numbers is sufficient, although in other cases "exact" numbers are required. Thus, the method used should be based on the goals of the investigator, with the interpretation of the data limited by the biases imposed by each method.

2. Materials

2.1. Isolation of Transgene DNA Construct

1. TE: 10 m*M* Tris-HCl, pH 8, 1 m*M* EDTA.
2. Tris-equilibrated phenol, pH 8.
3. Chloroform : isoamyl alcohol (24 : 1).
4. 5 *M* NaCl.
5. 50X TAE buffer: 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL of 0.5*M* EDTA, pH 8.
6. Spin Bind columns (FMC Bioproducts, cat. no. 61004).
7. Low-melt agarose (Seaplaque or Seakem GTG; FMC Bioproducts).
8. 10X loading dye: 40% (v/v) glycerol, 10X TBE, 0.1% (w/v) bromphenol blue, 0.1% (w/v) xylene cyanol.
9. Elutip columns (Schleicher & Schuell cat. no. 27370).
10. 1X phosphate-buffered saline (PBS) transgene microinjection buffer: dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 mL of sterile, purified water. Adjust pH to 7.2, bring to 1 L and put through a 0.2-µm filter. Do not use glassware with possible detergent residue, as it is toxic to embryos.

2.2. Genetic Screening of Founder Mice

2.2.1. Isolation of Genomic DNA from Tail

1. Use sharp, sterile, dissecting scissors to cut off approx 0.4 cm of tail from mice 3 wk or younger.
2. Tail digest buffer: 50 m*M* Tris-HCl (pH 8), 100 m*M* EDTA, 0.5% (w/v) sodium dodecyl sulfate (SDS), 25 µL Proteinase K (10 mg/mL, Boehringer Mannheim).
3. Tris-equilibrated phenol, pH 8.
4. Chloroform : isoamyl alcohol (24 : 1).
5. 3*M* sodium acetate, pH 6–7.

2.2.2. Slot Blot Analysis of Genomic DNA

1. Slot blot manifold unit (BRL cat. no. 1055).
2. Nylon hybridization membrane (Nytran, Schleicher & Schuell).
3. 20X SSC stock: 3 *M* NaCl, 0.3 *M* sodium citrate. Dilute accordingly.
4. 3 *M* NaOH.
5. 6X SSC.
6. Gel blot paper (GB002, Schleicher and Schuell, cat. no. 31540).

2.3. Analysis of mRNA Expression

2.3.1. RNA Isolation

1. Polytron homogenizer (Brinkman Instruments, New York).
2. Trizol phenol–guanidine isothiocyanate mixture (BRL cat. no. 15596-026).
3. Chloroform : isoamyl alcohol.
4. Isopropanol.
5. 75% Ethanol.
6. DNase and DNase buffer (BRL Life Technologies).
7. 25 m*M* EDTA made in diethylpyrocarbonate (DEPC) treated water.

2.3.2. Reverse Transcription

1. Random primers.
2. 5X reverse transcriptase buffer (i.e., first-strand buffer, BRL cat. no. Y00146).
3. Superscript II enzyme (BRL cat. no. 18064-014).
4. RNase inhibitor (RNasin, Promega).
5. Nucleotides: Mix equal volumes of 100 m*M* dATP, dCTP, dGTP, and dTTP (Pharmacia) to yield 25 m*M* dNTP stock.

2.3.3. Polymerase Chain Reaction

1. 10X PCR buffer (Promega).
2. *Taq* polymerase (Promega).
3. PCR primers: PCR primer oligonucleotides are typically 20–25 bases long that hybridize to opposite strands of the target DNA and flank the region of interest. Primers should contain 45–55% G+C content and contain no secondary structure or stretches of any one base.

4. Nucleotides: 25 m*M* dNTP stock.
5. Mineral oil.
6. Thermal cycling machine.

2.3.4. Polyacrylamide Gel Analysis of PCR Products

1. Acrylamide: *N′-N′-bis* methylene-acrylamide (30:0.8): mix in water and filter sterilize.
2. 10X TBE buffer: 108 g boric acid, 55 g Tris base, 40 mL of 0.5 *M* EDTA, pH 8, to 1 L. Autoclave to sterilize.
3. Gel blot paper (GB002, Schleicher and Schuell, cat. no. 31540).
4. Gel dryer.

2.4. Analysis of Protein Expression ELISA

2.4.1. Equipment and Reagents

1. NGF ELISA antibodies: Anti-β NGF antibody, 60 µg/mL (Boehringer Mannheim cat. no. 1008 226). Anti-β NGF β-gal antibody (Boehringer Mannheim cat. no. 1008 234).
2. NGF standard: NGF 2.5S (BRL cat. no. 13257-019).
3. ELISA plates: Dynatech immulon 4 96-well plate (Fisher cat. no. 14-245-153).
4. Plate sealer: Dynex Plate Sealers (acetate) (Fisher cat. no. 14-245-18).
5. Bovine serum albumin (BSA) Fraction V (Sigma cat. no. A 8551).
6. Fluorescein di-β-D-galactopyranoside (FDG, Molecular Probes cat. no. F-1179).
7. Optional: Repeat pipettor, multichannel pipettor, plate washer.

2.4.2. NGF ELISA

1. Wash buffer: 0.1 *M* phosphate buffer, 0.4 *M* NaCl, 0.1% Triton X-100, pH 7.4.
2. Carbonate coating buffer: 0.025 *M* sodium bicarbonate, 0.025 *M* sodium carbonate, pH 9.7.
3. Sample buffer: Wash buffer plus 2 m*M* EDTA, 0.1 m*M* benzethonium chloride, 2 m*M* benzamidine, 0.1 m*M* polymethylsulfonyl fluoride (PMSF), 20 TIU/mL Aprotinin, 0.5% BSA.

   To prepare the sample buffer, make the following stocks:
   a. 200 m*M* EDTA in wash buffer (7.44 g/100 mL wash buffer, pH to 8 to dissolve EDTA, then pH to 7.4; store at room temperature [RT]).
   b. 10 m*M* benzethonium chloride in wash buffer (0.448 g/100 mL wash buffer, store at RT).
   c. 200 m*M* benzamidine in wash buffer (3.13 g/100 mL wash buffer, store at RT).
   d. 10 m*M* PMSF (17.5 mg in 10 mL dimethyl sulfoxide (DMSO), store at –20°C in small aliquots).
   e. Aprotinin: Use undiluted (Sigma A-6012, store at 4°C).

   Dilute the EDTA, benzethonium chloride, and benzamidine stocks 1:100 in wash buffer and then add the BSA (500 mg/100 mL). Filter sterilize and store at RT. Just prior to use, add the PMSF stock (1 µL/mL sample buffer) and Aprotinin

(1.64 µL/mL sample buffer). Do not store sample buffer with PMSF and aprotinin added.
4. Substrate buffer: 0.1 *M* phosphate buffer, 1 m*M* $MgCl_2$, pH 7.4.
5. NGF Standard stock solution: Dilute NGF to 10 ng/µL in acetate buffer.

2.5. Neuronal Counting Strategies

1. 4% Paraformaldehyde in 0.1 *M* phosphate buffer.
2. Cresyl violet or hematoxylin and eosin stain.
3. Standard reagents for paraffin embedding.

3. Methods

3.1. Isolation of a Transgene DNA Construct

3.1.1. Gel Separation of Transgene Insert

1. Restriction-enzyme-cut 25–100 µg plasmid (depends on size of insert relative to plasmid) to remove transgene insert.
2. Check DNA digest on 0.8% agarose gel, and if complete, phenol/chloroform (1 : 1) extract, chloroform extract, and precipitate by adding 1/25 volume of 5 *M* NaCl and 100% EtOH (*see* **Note 1**).
3. Microcentrifuge at 4°C for 15 min, wash pellet with 80% EtOH, spin down, and air-dry.
4. Dissolve pellet in 200 µL TE (pH 8) and gel purify the transgene insert from bacterial plasmid sequences by separation on a 125-mL preparative 0.8% Seaplaque or Seakem GTG agarose (FMC Bioproducts) gel made in TAE running buffer.
5. Run gel up to 80 V for 3–6 h, rinse in sterile water, and lightly stain with ethidium bromide to visualize bands. Use a clean razor to cut out the band for injection.

3.1.2. Isolation of DNA from Agarose

1. Cut gel containing insert band into 1–2-cm lengths. Purify DNA from agarose using Spin Bind columns (FMC Bioproducts) following the manufacturer's protocol.
2. Phenol/chlororform (1 : 1) extract DNA from Spin Bind column, chloroform extract, and precipitate with EtOH and 5*M* NaCl overnight at –20°C.
3. Spin down precipitate for 15 min at 4°C, wash pellet with 80% ethanol, spin, remove ethanol, and air-dry the pellet.

3.1.3. Purification of Transgene Insert Using Elutip Minicolumns

1. Final purification is done using Elutip minicolumns. Resuspend DNA pellet in 500 µL low-salt buffer used in Elutip protocol. Load DNA onto column, collect the first flowthrough, and reload column.
2. Wash two or three times with low-salt buffer and then elute DNA with 400 µL of high-salt buffer. A second elution with 400 µL high-salt buffer improves the yield.

3. Precipitate DNA overnight at –20°C with 2 volumes of 100% ethanol (do not add salt).
4. Wash DNA pellet several times with 80% ethanol and air-dry the pellet. DNA washes are important to remove residual salt and ethanol, which are lethal to developing embryos.

3.1.4. Measure of Transgene DNA Concentration

1. Many transgenic facilities prefer transgene DNA be supplied as an alcohol precipitate so they can resuspend the DNA in a quality-controlled injection buffer and do the final concentration measure. If this is not an option, spin samples at 4°C for 15 min, wash with 80% ethanol, air-dry the pellet, and resuspend in 20–100 µL of injection buffer (1X PBS).
2. Run 1–2 µL of DNA solution on agarose gel to verify the size and quality of the DNA fragment.
3. Estimate DNA concentration using either gel electrophoresis with known standards, a spectrophotometer with a microcuvet, or a fluorometer. The DNA solution should be 5–10 µg/mL final concentration.

3.2. Genetic Screening of Founder Offspring

3.2.1. Isolation of Genomic DNA from Tail

1. Cut approx 0.4 cm of the tail off and place in a sterile 1.5-mL microfuge tube on ice. Mincing the tail is not necessary.
2. Add 500 µL of tail digest buffer and incubate at 55°C overnight.
3. Extract at room temperature with 500 µL Tris-buffered phenol. Shake vigorously for 3 min so phases mix completely.
4. Centrifuge for 3 min in a microfuge.
5. Use a large-bore (blue) pipetman tip to remove upper phase and interphase to clean the tube.
6. Add 500 µL of phenol/chloroform (1 : 1) (250 µL each), shake for 2 min, and centrifuge for 2 min. Transfer the top aqueous phase to a 1.5-mL tube. Do a chloroform only extraction (500 µL).
7. Remove the top phase, avoiding interface, and transfer to a clean microfuge tube.
8. Add 60 µL (1/10 volume) of 3 *M* sodium acetate (pH 6–7; a sodium acetate solution with a pH lower than 6 will cause the EDTA to precipitate) and 2 volumes (1 mL) of ethanol at room temperature. Shake gently to mix. DNA will form a stringy, white precipitate. Pellet the precipitate, wash with 1 mL of 70% EtOH to remove traces of SDS and phenol.
9. Resuspend in 300–500 µL of TE and determine the DNA concentration using a spectrophotometer.

3.2.2. Slot Blot Analysis of Genomic DNA

1. Dilute 2 µg isolated tail DNA in TE to a total volume of 50 µl.
2. Add 5 µl of 3*M* NaOH and heat for 1 h at 60°C to denature DNA. Cool and add 1 volume 6X SSC.

3. Apply DNA to Nytran membrane previously equilibrated with 6X SSC using a slot blot unit hooked to a vacuum line.
4. After the DNA solution has passed through the filter, disassemble the slot blot unit, remove the membrane, and place on piece of blotting paper.
5. Covalently crosslink DNA to filter using a Stratalinker (Stratagene).
6. Hybridize membrane with transgene specific probe made by random priming in the presence of either ^{32}P- or digoxigenin-labeled nucleotides.

3.3. Analysis of mRNA Expression

3.3.1. Isolation of RNA

1. Use sterile dissection tools to immediately collect tissues from recently killed mice. Place tissue in the sterile 2059 tube (Falcon) or microfuge tube placed on dry ice. Abundant total RNA is typically obtained from 100 mg of wet weight tissue. For smaller amounts of tissue (e.g., sensory or sympathetic ganglia), collect into microfuge tubes.
2. Add 1 mL Trizol extraction reagent per 100 mg tissue in the 2059 tube and immediately homogenize (in a chemical safety hood) at RT using a Polytron (*see* **Note 2**). For samples with large amounts of connective tissue (e.g., skin), use 2 mL Trizol/100 mg tissue. Homogenize control samples first, then transgenic samples. If sample genotype is unknown, thoroughly wash the homogenizer, tip between samples to avoid contamination that could cause problems in sensitive (e.g., RT-PCR) assays. Small amounts of tissue (pooled ganglia) can be homogenized in microfuge tubes using a small polytron probe and less extraction reagent. RNA from very small amounts of tissue (single ganglia) can be isolated by placing in 100–200 µL extraction reagent and dissociating the tissue by rapid pipeting.
3. Leave homogenized samples at room temperature for at least 5 min, then add 0.2 mL chloroform/mL Trizol, cap tubes tightly, and vigorously shake for 15 s. Let solution sit 2–3 min at room temperature.
4. Centrifuge 15 min at 12,000*g* at 4°C. For larger samples in 2059 tubes, spin at 8000*g* for 15 min.
5. Move the top phase to clean microfuge tube and add 0.5 mL isopropanol/mL Trizol used in homogenization. Mix thoroughly, incubate for 10 min at room temperature, and centrifuge 10 min at 12,000*g* at 4°C (*see* **Note 3**).
6. Discard supernatant and add 1 mL of 75% EtOH/mL Trizol used. Mix sample, let sit 2–3 min, and centrifuge at 7500*g* for 5 min at 4°C.
7. Remove EtOH, air-dry, resuspend in sterile water, and measure RNA concentration using a spectrophotometer or a Genequant calculator (Pharmacia) (*see* **Note 4**).
8. DNase treat 5 µg total RNA by adding 1 µL DNase, 2 µL DNase buffer, and water to 20 µL. Incubate for 15 min at room temperature.
9. Inactivate DNase by adding 1 µL of 25 m*M* EDTA (final concentration = 2 m*M*) and heating for 10 min at 65°C.

3.3.2. RT-PCR Assay

This protocol uses a radioactive nucleotide, therefore, care must be taken to avoid contamination of work surfaces and personnel (*see* **Note 5**).

1. Anneal random hexamer primers to 1 µg DNased RNA by combining in a 0.5-mL flat top tube on ice
   4 µL (1 µg) DNased RNA.
   1 µL random hexamer primers (BRL).
   Sterile, purified water to a volume of 11 µL.
2. Mix by flicking tube and quick spin. Heat for 10 min at 70°C. Chill on ice for 5 min.
3. Prepare on ice RT master mix. Per reaction, add
   4 µL 5X RT buffer (BRL).
   2 µL dithiothreitol (DTT) (0.1*M*).
   0.4 µL dNTP mix (each dNTP at 25 m*M*).
   0.25 µL RNasin (Promega).
   1 µL (0.5 µg) acetylated BSA (BRL cat. no. 15561).
   Sterile water to 8 µL.
4. Add 8 µL of RT master mix to primer annealed RNA, mix, and incubate at 37°C for 2 min.
5. Add 1 µL (200 units) Superscript II reverse transcriptase, flick tube, quick spin, and incubate for 50 min at 42°C.
6. As RT reaction is running, prepare PCR mixtures (for gene of interest and internal standard) on ice. Per reaction, add
   5 µL 10X PCR buffer (containing 1.5 m*M* $MgCl_2$; Promega)
   1 µL amplification primer 1 (20 µ*M*)
   1 µL amplification primer 2 (20 µ*M*)
   0.4 µL dNTP mix (each dNTP at 25 m*M*)
   0.2 µL of ^{32}P-dCTP (3000 Ci/mmole, 10 µCi/µL)
   1.5 units *Taq* polymerase (Promega)
   Water to 49 µL
7. Stop RT reaction by heating at 70°C for 15 min. Transfer to ice.
8. Add 1 µL RT reaction to 49 µL PCR mix, layer two drops (approx 100 µL) light mineral oil over the top of the reaction, place in the thermocycler, and amplify at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. The cycle number, length, and annealing temperature are primer and template dependent and must be determined empirically.

3.3.3. Polyacrylamide Gel Electrophoresis of PCR Products

1. Prepare an 8% polyacrylamide gel (30 mL) by mixing the following in a 50-mL graduated cylinder:
   3 mL 10X TBE buffer
   8 mL 30% acrylamide : 0.8% bis-acrylamide mix

19 mL water
250 µL 10% APS
25 µL *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED)

Cover the cylinder with Parafilm, mix gently, and immediately pour the solution into a 20-cm × 20-cm vertical gel assembled using 0.8-mm-thick spacers. Allow the gel to polymerize for 1 h.

2. Add loading buffer to 5 µL of each RT-PCR reaction and load on the gel using a Hamilton syringe. Run in 1X TBE buffer at 400 V.
3. Run the dye front two-thirds down the gel, carefully separate the glass plates, place a piece of gel blot paper over the gel, and gently lift the gel from the plate. Cover gel with Saran wrap and place on a gel dryer for 1 h at 80°C.
4. Cover the dried gel with Saran wrap and place in film cassette adjacent to X-ray film or to a phosphorimager screen. Measure the band densities and normalize to an internal standard.

3.4. Analysis of Protein Expression Using ELISA

3.4.1. Isolation of Protein for ELISA Measure (See Also Chapter 3)

Collect the tissue immediately from the killed animal and either homogenize immediately or store at –80°C. Obtain the tissue wet weight and homogenize in a sample buffer containing PMSF and aprotinin. The volume used depends on the tissue type. For ganglia (pooled or singular), spinal cord segments, or other nervous tissue, homogenize in a Duall-type ground-glass tissue grinder in a volume of 300 µL. Keep samples on ice. Clean the tissue grinder (a pipe cleaner folded over works well) between each sample to avoid contamination. Store samples at –80°C until assayed (*see* **Note 6**).

For samples with large amounts of connective tissue (e.g., skin), increase the volume to 2 mL and use a Polytron (*see* **Note 7**). Homogenize in Falcon 2059 or similar tubes. Keep on ice before and after homogenization. Thoroughly clean the probe between samples. Homogenized samples are stable for 1–2 mo provided they are not subject to frequent freeze–thaws.

3.4.2. ELISA

To maximize confidence in data collection, run samples in duplicate and assay two to three different dilutions (i.e., for each sample, four to six wells are used) (*see* **Note 8**). Label the top of the plate with a marker to show where the standard curve and the samples will be loaded. Allow the first two columns (1 and 2) on the plate to be used to generate a standard curve. Assuming that an acceptable range has been established, 20 duplicate samples at 2 dilutions can be loaded on one 96-well plate.

For the assay of NGF (adapted from **ref. *25***):

1. Bind primary antibody to plate by first washing wells with 75µl binding buffer. Remove buffer from wells by flicking out contents and then tapping the plate on a stack of paper towels.
2. Add 75 µL of the primary antibody diluted in binding buffer to each well of the plate. To cover one plate, dilute 30 µL of the 60-µg/mL primary anti-β NGF antibody with 7.5 mL of binding buffer. Invert gently to mix without creating bubbles.
3. Seal the plate with a plate sealer and place at 37°C for 3 h (*see* **Note 9**).
4. To block nonspecific binding to plate, add 100 µL of 10 mg/mL BSA solution (made by adding 100 mg BSA/10 mL binding buffer) directly to the primary antibody in each well. Do not empty the primary antibody. Shake the plate on the orbital shaker for 5 min. Reseal the plate and incubate at 37°C for 1.5 h. This step can be continued overnight at 4°C, following the 1.5-h incubation at 37°C.
5. Empty the contents of the plate by flicking and tap the plate on a stack of paper towels to remove as much residual liquid as possible. Add 250 µL of wash buffer to each well, flick out the contents, tap on a stack of paper towels, and repeat five times. Do *not* let the plate dry out. Do not empty last wash until ready to add the next solution.
6. Prepare peptide standards by diluting 3 µL of NGF stock solution in 300 µL sample buffer (final dilution 1 ng/mL). Perform serial dilutions by mixing 150 µL of NGF solution with 150 µL of sample buffer. Repeat seven times, at which point the final standard concentration will equal 0.0156 ng/mL. Thaw the tissue samples and centrifuge at 12,000*g* for 10 min at 4°C. Prepare one to two additional dilutions with sample buffer (e.g., 1 : 10 and 1 : 50) to ensure that sample measurements fall within the linear range of the standard curve (*see* **Note 10**).
7. Pipet in duplicate 50 µL of each standard dilution into wells. In the last two wells of the columns for the standards, add the undiluted sample buffer. Pipet in duplicate 50 µL of each sample dilution into wells.
8. Seal the plate and place at room temperature on an orbital shaker overnight (500 rpm). Incubate for 15 h (*see* **Note 11**).
9. Wash the plate six times. Place on the orbital shaker for 10 min for the last two washes.
10. During the last wash, dissolve 150 mg of BSA in 5 mL of wash buffer (30 mg/mL BSA). Add 12.5 µL of the secondary anti-β NGF β-gal antibody to the BSA solution.
11. Add 50 µL of secondary antibody solution to each well. Reseal plate and incubate 3 h at 37°C. Do not cut this incubation short as it reduces sensitivity of the assay.
12. Wash the plate with wash buffer as in **step 9**.
13. Prepare the FDG solution by adding 62.5 µL of the FDG stock to 5 mL substrate buffer.
14. Rinse the plate twice with 100 µL of substrate buffer.

15. Add 50 µL of the FDG solution per well. Reseal the plate and incubate overnight (12 h) at 37°C.
16. The reaction product is fluorescent with an excitation wavelength of 485 nm and emission wavelength of 530 nm. When reading the plate, use wells at the bottom of the standard curve (i.e., containing only sample buffer) for both the blank and the lowest point on the curve. Before reading, remove the plate sealer and clean the plate bottom with a KimWipe and 70% EtOH to remove residue that can interfere with readout.
17. Plot data to obtain the regression equation for the standard curve. Substitute the optical density (OD) value of the samples into the equation to get neurotrophin concentration. Values obtained will be in nanograms per milliliter. Because sample dilutions were performed, a calculation is done to scale samples to the same concentration as the standard. For example, if 2 µL and 10 µL of sample tissue homogenate in 50 µL of sample buffer were loaded, multiply by 25 and 5, respectively (2 µL × 25 = 50 µL, 10 µL × 5 = 50 µL). No dilution factor is required for the standard curve.

Duplicate wells should be averaged prior to entering them into the regression equation. Multiply the result by the dilution factor. To determine the total amount of NGF in the sample, multiply the nanograms per milliliter by the total volume (e.g., if homogenized in 300 µL, multiply by 0.3). This value is then divided by the tissue wet weight and results expressed as nanograms NGF per gram tissue.

3.5. Neuronal Counting (Based on refs. *15* and *16; See Also Chapter 18)*

1. Perfuse animal with 4% paraformaldehyde in 0.1*M* phosphate buffer.
2. Dissect ganglia and process for paraffin embedding.
3. Embed in paraffin and store at 4°C until sectioned.
4. Serially section the entire ganglia at 5 µm. All sections are collected in order to determine the sampling interval (*see* **step 6**).
5. Stain sections with Nissl or hematoxylin and eosin and cover slip.
6. Determine the interval between counted sections. We have determined empirically that in mouse, analysis of six sections per ganglion is sufficient to obtain an accurate estimate of cell number in ganglia. Therefore, count the total number of sections collected for each ganglia and divide by 6. This is the number of sections to be skipped (interval) between each section counted (e.g., for 66 total sections, every 11th section will be analyzed).
7. Select sections to be counted by randomly selecting a number between 1 and the number calculated for the interval (11 in the above example). This will be the first section counted; that is, if the random number is 4, the sections analyzed are 4, 15, 26, 37, 48, and 59. Use a marking pen to identify these sections on the slide.
8. Count neurons that have one or more nucleoli (*see* **Note 12**) using a drawing tube connected to a microscope (*see* **Note 13**). If a cell has multiple nucleoli, do not count that cell more than once.

9. Count the number of nucleoli on the six sections and multiply this total number by the interval. This is the raw number of cells for the ganglion.
10. Raw counting data must be adjusted to account for the possibility that individual cells have multiple nucleoli and could theoretically be counted more than once. It is important to remember that even though the same cell will never be present in more than one of the six sections analyzed, the raw neuron number is achieved by multiplying the number of cells in the six sections by the interval, thus allowing an estimation of cell number that would be obtained if *every* section was analyzed (*see* **Note 14**).
11. If every section was counted, cells with multiple nucleoli would be scored erroneously more than once, hence the need for a correction factor. To obtain a correction factor, randomly select 50 neurons. Follow each neuron in the serial sections until all portions of the neuron are identified and count the number of nucleoli in each neuron. Determine the total number of nucleoli in the 50 neurons. Divide the number of neurons (50) by the total number of nucleoli. Multiply this correction factor by the raw number of neurons determined in **step 9**. For example, after reconstructing 50 cells, 100 nucleoli are found (*see* **Note 15**). The correction factor would be 0.5 and the corrected estimate of the number of neurons would equal (Raw number of neurons) $\times$ 0.5 (*see* **Note 16**).
12. The correction factor can be refined by determining the number of *sections* that contain nucleoli from a single reconstructed neuron. The reason for this is that during the analysis process, if a neuron contains two nucleoli that are present in the same section, this neuron is only scored once. Thus, the most accurate correction factor accounts for the possibility that a neuron is counted more than once because of multiple nucleoli in *different* sections. In most cases, the two corrections factors will be similar. However, in some transgenic mice exhibiting hypertrophied sensory neurons, we have found significant difference in the two correction factors.

4. Notes

1. Do not vortex genomic DNA, as it will shear the DNA.
2. Use gloves and eye protection, and work in a chemical fume hood when using Trizol. RNA from skin or other tissues with high RNase content should be isolated immediately from tissue as the RNA degrades quickly.
3. To improve RNA yield, extend the room-temperature isopropanol precipitation time (up to 1 h).
4. To avoid contamination from repeated sampling from stock water bottles, aliquot sterile purified water into 2-mL microfuge tubes and reautoclave. Use aliquot and discard unused portion.
5. When setting up RT-PCR reactions, handle everything with care. All solutions and plasticware must be sterile, wash powder off gloves (it can lead to contamination and inhibit enzymatic reactions), keep pipettors and work area clean, and uncap tubes carefully to prevent aerosols. Always have positive and negative controls and an internal control (e.g., actin, cyclophilin).

6. To homogenize large tissue samples, polytrons work best. Small tissue samples (e.g., trigeminal ganglion) may be partially lost using a polytron; thus, it is better to use small (1–3 mL) or micro (200 µL) ground-glass homogenizers.
7. When collecting tissue for an ELISA, some tissues require special handling. For skin, it is best to depilitate prior to collecting tissue. Measures of serum NGF levels can also be difficult ***(26–29)***, although treatment with 1.0*M* guanidine appears to improve neurotrophin recovery ***(30)***.
8. In the initial assay of a tissue, establish the optimal assay volume by measuring several dilutions in duplicate. We have found trigeminal, SCG, and DRG dilutions to be 5–25 µL, whereas 200–400 mg of skin (wet weight) is 2 µL and 10 µL.
9. Do not shorten incubation times, as it will reduce protein measures.
10. Isolate tissue and run assays within 2 wk of harvesting tissues.
11. Always run interassay controls using a '"standard" tissue homogenate.
12. A major concern in cell counting is consistency when identifying neurons with nucleoli. To reduce variability, only one person should count on a given project.
13. When drawing cells with a drawing tube, use a dark fine-tipped pen and illuminate the scoring sheet with a fiber-optic-style light source.
14. Asymmetry in ganglia causes variability in neuron number between animals. To reduce this variability, count pairs of ganglia from each animal and average the numbers obtained.
15. When performing cell reconstruction for the correction factor, make the selection of cells as random as possible. Do not select cells with obvious nucleoli. Choose neurons in which nuclei are *not* present and then scan the slide in either direction to find the rest of the cell. This prevents the analysis from being biased toward neurons with multiple nucleoli.
16. Calculate a correction factor for each animal and each type of ganglia. Correction factors for trigeminal ganglia are not appropriate for SCG.

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13

Construction of Transgenic Animals Overproducing Neurotrophins and Their Receptors

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1. Introduction

The development and analysis of transgenic mice that overexpress or misexpress neurotrophins or their receptors has led to a number of fundamental insights into the mechanisms whereby this family of growth factors exerts its biological effects in vivo ***(1–5)***. These transgenic mice were generated when an appropriate foreign DNA construct, or transgene, expressing a neurotrophin or neurotrophin receptor was stably incorporated in the genome of every cell; the transgene, which was incorporated at the same chromosomal location(s) in germ-line and somatic cells, was transmitted to offspring in a Mendelian manner, allowing for a detailed analysis of the physiological consequences of perturbations in neurotrophin biology. Although conceptually simple, the generation and analysis of transgenic mice is a time-consuming and expensive process that is fraught with potential conceptual and technical problems. In this chapter, we provide (1) an overview of the steps involved in the production and analysis of transgenic mice that overexpress neurotrophins or their receptors, with an emphasis on targetting to neurons, and (2) detailed protocols for the characterization of transgenic mice at the level of DNA, RNA, and protein expression.

1.1. Steps in the Establishment and Characterization of Transgenic Mice

Transgenic mouse experiments are, as stated, long-term projects that should be carefully conceived. In this regard, a typical transgenic mouse experiment should adopt the following sequence of steps:

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1. Choice of promoter (*cis*-sequence elements) to target appropriate transgene expression.
2. Construction of the transgene, including choice of intron(s) and poly-adenylation site.
3. Confirmation that the transgenic construct expresses the protein of interest.
4. Preparation of DNA and microinjection.
5. Identification of transgenic founder animals by genomic DNA analysis.
6. Establishment of transgenic lines from founder animals.
7. Analysis of transgene expression at the level of RNA and protein.
8. Analysis of phenotype in at least two transgenic lines that carry the same transgene. A brief overview of each of these steps will be provided, and detailed protocols are included for **steps 5** and **7**.

1.1.1. Steps 1–3: Design and Construction of an Appropriate Transgene Construct

One of the first, and, ultimately, most important decisions in a transgenic mouse experiment is the choice of promoter that will be used to target overexpression of the protein of interest—in this case, a neurotrophin or neurotrophin receptor. Fortunately, the number of promoters for targeting gene expression to neurons in transgenic mice has significantly increased over the past several years. However, it is essential to consider the precise properties of each of these promoters in light of the particular hypothesis that is being tested. In particular, neuronal promoters (which will be briefly reviewed later) differ in terms of (1) developmental onset [some, like the Tα1 α-tubulin promoter, express as soon as neurons are born ***(6)***, whereas others, like Thy-1, commence expression postnatally ***(7)***], (2) specificity of neuronal subtype [some, like the Tα1 α-tubulin promoter and the neurofilament promoters, are panneuronal ***(6,8,9)***, whereas others, like the dopamine-β-hydroxylase promoter, are limited to specific neuronal populations ***(10,11)***], (3) penetrance (the percentage of transgene-positive lines that express the protein of interest), and (4) level of expression. Thus, if overexpression of a neurotrophin was desired only in a limited subpopulation of mature neurons, this would entail the choice of a very different promoter than if overexpression was desired panneuronally in the embryonic nervous system.

In our studies, we have taken advantage of two very different neuronal promoters. In one study, we utilized the Tα1 α-tubulin promoter, which we had previously isolated and characterized ***(6,12,13)***, to express the intracellular domain of the p75 neurotrophin receptor panneuronally ***(3)***. This study took advantage of the facts that the panneuronal Tα1 α-tubulin promoter is expressed at high levels in developing neurons, is downregulated (but not turned off) in mature neurons, and is subsequently reinduced in injured motor

neurons. In a second set of studies, we utilized a 1.6-kb fragment of the dopamine-β-hydroxylase promoter ***(14)*** to target twofold to fourfold higher expression of brain-derived neurotrophic factor (BDNF) specifically in noradrenergic and adrenergic neurons of the central and peripheral nervous systems ***(1,2)***. This promoter is induced early in noradrenergic and adrenergic neurons, and its expression is maintained in the mature animal. These two different promoters therefore allowed us to ask two very different physiological questions.

A number of other neuron-specific promoters have been used to direct expression of heterologous genes to the nervous system. Examples of such promoters include 5′ regulatory regions of the thy-1, neurofilament light chain (NF-L), and neuron-specific enolase (NSE) genes. The Thy-1 promoter targets strong constitutive expression to neurons, with onset of expression commencing around birth and continuing into adulthood ***(7,15,16)***. The NF-L promoter targets expression either to the embryonic nervous system or to the postnatal neurons, as a function of the particular promoter fragment used ***(17,18)***. Finally, the NSE promoter, like the Tα1 α-tubulin promoter, provides neuron-specific panneuronal gene expression, although, unlike the Tα1 promoter, expression is not induced following neuronal injury ***(19)***. It should be noted that many other neuron-specific promoters are currently available and that these represent only some of the better-characterized examples.

Once a promoter has been chosen, the next step is the construction of an appropriate minigene. A minigene created for transgenesis usually includes the promoter sequences for targeting, the open reading frame of the desired protein product, and additional exogenous sequences that provide an intron and a polyadenylation site. These latter two motifs significantly affect transgene expression ***(20,21)***. In particular, the presence of an intron facilitates the transcription of transgenes in transgenic animals ***(21)*** and a poly(A) tail stabilizes the transcribed transgene for transport from the nucleus to the cell cytoplasm ***(22,23)***. These sequences are usually located downstream of the promoter and cDNA coding sequences. In our previous studies, we have used two different sources of an intron and polyadenylation site; the mouse protamine-1 gene, which contains both an intron and polyadenylation site ***(24)***, and an intron and polyadenylation site from SV40 ***(25)***. Finally, once the construct is made, it is essential (1) to sequence it, at least through the construction sites and (2) to express it in transfected cells such as NIH 3T3 or 293 cells to ensure that the protein product is correct. In this regard, we normally express our constructs in 293 cells and check the protein product by Western blot analysis. Fortunately, most neuron-specific promoters are leaky in transformed cells, although, in many cases, expression will be relatively low.

1.1.2. Steps 4–6: Creation and Establishment of Founder Transgenic Lines Expressing the Construct of Interest

The most common technique for the generation of transgenic mice is via injection of the transgene into the pronucleus of a fertilized egg. Using this method, large pieces of DNA, such as transgenes, can integrate into the genome. Upon microinjection, a single copy or, more often, multiple copies that are arranged in a head-to-tail or head-to-head tandem array randomly integrate into the chromosome ***(26,27)***. The injected eggs are then implanted into psuedopregnant foster mothers, which subsequently give birth to the transgenic offspring ***(28)***. Because expression of a transgene depends on the site of chromosomal incorporation, and not necessarily the copy number, more than one line of transgenic mice should be produced in which the transgene is present at different chromosomal locations in order to rule out position-dependent effects of genomic DNA on the integrated transgene copies. Thus, three lines were generated for the DBH:BDNF mice and six lines were generated for the Tα1:ICD mice. Subsequent analysis was performed on all lines (1) to choose two lines to analyze in detail based on level and pattern of expression and (2) to confirm that the phenotype in question occurred in at least two lines with different chromosomal insertion sites. The latter ensures that the resultant phenotype is truly the result of transgene expression and not a consequence of transgene insertion into a functional gene (i.e., an inadvertant "knockout").

Preparation of DNA for microinjection is a critical component in production of transgenic mice. In particular, the probability of transgene expression is increased when the DNA is linear, free of vector sequences, and free of any impuritites ***(20,29)***. The basic procedure for DNA preparation involves (1) linearizing the DNA with appropriate restriction enzymes, (2) separating the transgene from vector DNA sequences by electrophoresis on a low gelling/melting agarose gel, and (3) isolating and purifying the transgene fragment using a commercially available kit. For microinjection, the final concentration of the DNA should be 1–7 μg/μL. The protocols for microinjections will not be reviewed here, as a detailed review has been described elsewhere ***(28,30)***.

Once potential transgenic founder animals are born, they are genotyped to identify those that have integrated DNA into their genome. The DNA is obtained from a small piece of the tail of each animal and is prepared as described in detail later. Subsequent Southern blot analysis, again as described later, allows identification of transgene-positive animals. These founders are then bred to wild-type mice, and their progeny analyzed for transgene integration using Southern blot analysis of tail DNA. Much of the time, the transgene is transmitted to 50% of the progeny in a Mendelian manner. However, there are a number of exceptions to this generalization. First, if the transgene is

integrated at multiple sites in the genome (resulting in multiple alleles that segregate independently), then more than 50% of the progeny will be transgene positive. In this case, it is essential to segregate the alleles via subsequent breeding, as different alleles may well express the transgene differently. Second, if the founder animal is a mosaic (which occurs when the transgene is integrated after the first round of replication in the fertilized egg cell), then less than 50% of the progeny will carry the transgene. In the extreme case, it will be impossible to transmit the transgene. Finally, expression of biologically potent molecules such as neurotrophins or their receptors can cause embryonic lethality, resulting in few or no transgenic progeny at birth.

Positive progeny of founder animals are termed the F1 generation and will carry the transgene in all somatic and germ cells. There are two approaches to subsequent breeding of these animals. First, heterozygous F1 animals of the same line can be crossed, and their homozygous F2 progeny subsequently bred to generate homozygous animals. In some cases, this is the preferable strategy, because the progeny of homozygous animals no longer need to be routinely genotyped. However, when dealing with biologically potent molecules such as neurotrophins or their receptors, this approach may be infeasible and/or undesirable. For example, in our BDNF overexpression studies, we chose to analyze transgenic animals in which the level of overexpression was relatively modest. Thus, we selected two lines that overexpressed BDNF twofold to fourfold as heterozygotes. In this case, by establishing homozygotes from these animals, we doubled the gene dosage, resulting in higher levels of expression than was desirable and generating animals with a reduced ability to breed.

1.1.3. Step 7: Analysis of Transgene Expression at the Level of the RNA and Protein

Once transgenic lines have been established, it is essential to analyze the spatial and temporal pattern of transgene expression by measuring mRNA and protein levels utilizing Northern blot/*in situ* hybridization analysis and Western blot/immunocytochemical analysis, respectively. Although this analysis is conceptually straightforward, there are a number of technical considerations. First, it is essential to measure both mRNA and protein, as there are documented cases in which increased mRNA from a transgene did not lead to increased protein ***(16,31)***. Second, it is essential to determine which lines actually express the transgene early in the analysis, as neuronal promoters are rarely more than 50% penetrant. Third, the analysis needs to be performed at the single-cell level using *in situ* hybridization or immunocytochemistry, because many neuronal promoters exhibit partial penetrance, a phenomenon where the transgene is expressed in only a subset of cells within the targeted cell popula-

tion ***(11,32)***. This latter phenomenon can actually be advantageous; should the transgene product be detrimental to neurons (such as, for example, expression of a protein that inhibits neurotrophin-mediated survival or growth pathways), then partial expression will allow analysis of the effects of transgene expression while maintaining viability. Finally, it is important to analyze animals as close to the F1 generation as possible. A number of promoters are known to be "silenced" as they are passaged through subsequent generations (i.e., transgene expression is turned down or off) ***(33–35)***, a phenomenon that can make analysis difficult and results variable.

1.2. Protocols for Analysis of Transgene Integration and Expression

As discussed earlier, initial analyses of transgenic animals include identifying those animals that carry the transgene using Southern blot analysis and identifying those that express the gene product at the desired level in the desired cells. In this regard, the following protocals include (1) isolation of genomic DNA from transgenic tails for Southern blot analysis, (2) isolation of RNA from small amounts of tissue for Northern blot analysis, (3) isolation of protein from the brain for Western blot analysis, and (4) tissue preparation for immunocytochemistry.

1.2.1. Identification of Transgenic Animals

To identify those animals that carry the transgene in their genome, DNA is isolated from the tails of these animals and analyzed by using either a specific probe in Southern blot analysis or primers specific for the transgene in the polymerase chain reaction (PCR).

2. Materials

2.1. Isolation of DNA from Mouse Tails

1. Proteinase K buffer: 50 m*M* Tris-HCl (pH 8.0), 100 m*M* EDTA, 100 m*M* NaCl, 1% sodium dodecyl sulfate (SDS). Store at room temperature.
2. Proteinase K: Dissolve Proteinase K powder (available commercially) in ddH_2O for a final concentration of 20 mg/mL. Store at –20°C in 500-µL aliquots.
3. DNase-free RNase: Dissolve pancreatic RNase (available commercially as RNase A) in 10 m*M* Tris-HCl (pH 8.0) and 15 m*M* NaCl for a final concentration of 10 mg/mL. Heat to 100°C for 15 min and allow to cool slowly to room temperature. Store at –20°C in 1-mL aliquots.
4. Phenol:chloroform:isoamyl solution (P:C:I): Available commercially. P:C:I is light sensitive and should be stored in a dark container or wrapped with foil. Phenol is highly corrosive and can cause severe burns. Safety glasses and gloves should be worn. P:C:I should be used in a fumehood.

5. Tris-EDTA (TE): 10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA (pH 8.0). Store at room temperature.
6. Glass rods for spooling DNA: Hematological microcapillary rods (available commercially) heat sealed by rotating tips over a bunsen burner. Store in a covered box, away from dust. 5*M* NaCl: 292 g NaCl dissolved in 1 L ddH_2O. Store at room temperature.

2.2. Southern Blot Analysis

In Southern blot analysis, DNA fragments are transferred from an electrophoresis gel to a membrane support. This membrane is then treated so that the DNA is immobilized, thus reproducing the banding pattern of the gel. The DNA can then be hybridized with a specific probe that will identify specific bands (i.e., the transgene and endogenous gene). The basic protocol for Southern blotting involves upward capillary transfer of DNA from an agarose gel onto a nylon or nitrocellulose membrane. A high-salt transfer buffer promotes binding of DNA to the membrane. DNA is immobilized via ultraviolet (UV) irradiation or baking of the membrane.

1. Ethidium bromide (EtBr) solution: Available commercially.
2. 6X loading dye buffer: Store at room temperature. 0.25% Bromophenol blue, 0.25% xylene cyanol, and 15% Ficoll type 400.
3. TBE (Tris/borate/EDTA) electrophoresis buffer. Store at room temperature. For 1 L of 10X stock: 108 g Tris (890 m*M*), 55 g boric acid (890 m*M*), 40 mL of 0.5*M* EDTA (pH 8.0).
4. Denaturation solution: 1.5 *M* NaCl, 0.5 *M* NaOH. Make solution fresh each time. For 1 L, 87.66 g NaCl, 20 g NaOH; dissolve in 1 L ddH_2O.
5. Neutralization solution: 1.5 *M* NaCl, 0.5 *M* Tris-Cl (pH 7.4). Make solution fresh each time. For 1 L, 87.66 g NaCl, 121 g Tris-Cl; dissolve in 900 mL ddH_2O, add approximately 55 mL HCl to pH 7.4, and bring up to 1 L with ddH_2O.
6. 20X SSC (sodium chloride/sodium citrate). Store at room temperature. For 1 L, 175.3 g NaCl, 88.2 g Na citrate; dissolve in 800 mL ddH_2O, adjust pH to 7.0 with a few drops of 10*N* NaOH, and bring up to 1 L with ddH_2O.
7. Whatman filter paper: Available commercially.
8. Stack of paper towels.
9. 20X PIPES: Store at room temperature: For 500 mL, 87.66 *g* NaCl (3 *M*), 17.12 g PIPES (0.1 *M*), 14.62 g EDTA (0.1 *M*) free acid; add 360 mL ddH_2O, adjust pH to 6.8 with 10*N* NaOH, and bring volume up to 500 mL with ddH_2O.
10. 50X Denhardt solution: Filter sterilize and store at –20°C in 25-mL aliquots. 5 g Ficoll 400, 5 g polyvinyl pyrrolidone, 5 g bovine serum albumin (BSA) (Pentax Fraction V); Add ddH_2O to 500 mL.
11. Denatured salmon sperm DNA: Dissolve 10 mg salmon sperm DNA in 1 mL ddH_2O. Stir for 2–4 h at room temperature to help the DNA dissolve. Pass vigorously through an 18-gage needle several times to shear the DNA. Place in boiling water for 10 min, then cool. Store in 1-mL aliquots at –20°C.

12. Herring sperm DNA: Dissolve 10 mg herring sperm DNA in 1 mL ddH_2O. Extract the DNA five times with phenol : chloroform : isoamyl (pH 8.0). Aliquot the final aqueous layer (i.e., no precipitation). Store in 1-mL aliquots at –20°C.
13. Formamide: Available commercially.
14. Sodium dodecyl sulfate (SDS) 20%: Dissolve 20 g SDS in 100 mL ddH_2O. Stir to help dissolve.
15. Prehybrization solution: 10 mL 20X PIPES, 8 mL formamide, 4 mL 50X Denhardt's solution, 16.4 mL ddH_2O, 0.4 mL of 20% SDS, 400 µL denatured herring sperm, and 400 µL denatured salmon sperm.

2.3. Preparation of a Radiolabeled Riboprobe

Hybridization analysis of immobilized DNA on a nitrocellulose membrane allows single-stranded DNA or RNA molecules of a defined sequence to be base-paired to a DNA molecule that contains the complementary sequence. Hybridization analysis is sensitive and permits the detection of specific genes in genomic DNA. This analysis can be performed with (1) labeled oligonucleotide probes, (2) labeled cDNA probes, or (3) labeled RNA riboprobes. The latter, for which we have provided the protocol, is the most sensitive.

1. Phenol : chloroform : isoamyl solution (P : C : I): Available commercially. P : C : I is light sensitive and should be stored in a dark container or wrapped with foil. Phenol is highly corrosive and can cause severe burns. Safety glasses and gloves should be worn. P : C : I should be used in a fume hood.
2. TE: 10 m*M* Tris-Cl (pH 8.0), 1 m*M* EDTA (pH 8.0). Store at room temperature.
3. Components for transcription reaction—available commercially (e,g., Promega):
   5X transcription buffer
   Nucleotide mix, 2.5 m*M* each UTP, GTP, and ATP
   100 m*M* dithiothreitol (DTT)
   RNasin (ribonuclease inhibitor)
   Appropriate RNA polymerase (e.g., T7, SP6)
   α-^{32}P-CTP (50 mCi/mL; 800 Ci/mmol)
4. Nick column for cleaning radiolabeled probe; available commercially (e.g., Qiagen).

2.4. Isolation of RNA from Small Amounts of Tissue

To measure expression of the transgene, RNA is isolated from the targeted tissue and is subsequently analyzed using Northern blot analysis. A specific probe is used to determine the abundance of RNA of interest and whether it is expressed or overexpressed, presumably from the transgene, in the tissue.

1. Phosphate-buffered saline (PBS): 10 m*M* Na_2HPO_4, 150 m*M* NaCl. Store at 4°C. For 1 L, 2.04 g $Na_2HPO_4 \cdot 7H_2O$, 0.28 g NaH_2PO_4, 8.77 g NaCl; dissolve in 900 mL ddH_2O; pH to 7.4. Bring volume up to 1 L with ddH_2O.

2. Lysis buffer: Store at 4°C. For 500 mL, 10 mL of 1 *M* Tris-Cl (pH 8.8), 20 mL of 5 *M* NaCl, 10 mL of 1 *M* MgC_{l2}. Bring volume up to 500 mL with ddH_2O.
3. Second buffer: Store at room temperature. For 500 mL, 50 mL of 20% SDS, 40 mL of 0.5*M* EDTA (pH 8.0), and 10 mL of 5*M* NaCl. Bring volume up to 500 mL with ddH_2O.
4. TE: 10 m*M* Tris-Cl (pH 8.0), 1 m*M* EDTA (pH 8.0). Store at room temperature.
5. TE + 0.05% SDS: Store at room temperature. 100 mL TE and 0.25 mL of 20% SDS.
6. Phenol : chloroform : isoamyl solution (P : C : I): Available commercially. P : C : I is light sensitive and should be stored in a dark container or wrapped with foil. Phenol is highly corrosive and can cause severe burns. Safety glasses and gloves should be worn. P : C : I should be used in a fume hood.
7. 3M sodium acetate (NaAc): Dissolve 408.10 g NaCl in 900 mL of ddH_2O. Adjust pH to 5.2, then bring volume to 1 L with H_2O. Store at room temperature.
8. 95% Ethanol: Available commercially.

2.5. Immunocytochemical Analysis of Fixed, Frozen Tissue

1. 0.2 *M* phosphate buffer (PB), pH 7.4 stock: 0.2 *M* NaH_2PO_4, 0.2 *M* Na_2HPO_4. Store at room temperature.
2. 2–4% Paraformaldehyde (PF) in PB: For 500 mL: Using a heating stir plate in a fume hood, heat 200 mL ddH_2O to 70°C. Add the quantity of PF that will make a 2–4% PF solution (e.g., 10 g PF for 2% or 20 g for 4%), and add to heated ddH_2O stirring. Cover top. Add 10 drops of 1*N* NaOH so that the PF solution becomes fairly clear. Once the solution has become very clear (i.e., very few fine particles), remove from heat and add 250 mL with 0.2*M* PB. Adjust pH to 7.4 with HCl. Adjust final volume to 500 mL with ddH_2O. Cool solution to room temperature or 4°C and filter. Store at 4°C, wrapped in foil.
3. Sucrose gradients in PB: Weigh sucrose quantity to make 10–30% sucrose solution. Dissolve in 0.1 *M* PB and store at 4°C.
4. Mounting media: Available commercially.
5. PBS, pH 7.4: 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* $Na_2HPO_4{\cdot}7H_2O$, and 1.4 m*M* KH_2PO. Store at room temperature.

2.6. Western Blot Protocol for Brain Tissue

1. Lysis buffer containing protease inhibitors: 137 m*M* NaCl, 20 m*M* Tris (pH 8.0), 1% (v/v) NP-40, 10% glycerol + protease inhibitors (1 m*M* phenylmethyl sulfonyl fluoride [PMSF], 10 µg/mL Aprotinin, 0.2 µg/mL leupeptin, and 1.5 m*M* sodium vanadate). Store 1-L volume lysis buffer at 4°C; add protease inhibitors fresh for each experiment.
2. Protease inhibitor stock solutions: All available commercially.
   PMSF: Make up in ethanol as a 200 m*M* stock and store at –20°C in 1-mL aliquots. Add PMSF to lysis buffer immediately prior to lysing because of its instability in aqueous solution.

Aprotinin: Store at 4°C (2 mg/mL).
Leupeptin: Make up in ddH_2O (1 mg/mL) and store at –20°C in 50-mL aliquots.
Sodium vanadate: For 100 m*M*, dissolve in ddH_2O and adjust pH to 9–10 with HCl. Boil until the solution changes yellow to clear. Cool and adjust pH to 9–10 again and boil. If the pH drifts below 9, start over. Store at 4°C, wrapped in foil.

3. BCA protein assay kit: Commercially available from Pierce.
4. Sample buffer: 2% SDS, 100 m*M* 2-β-mercaptoethanol, 0.05% bromphenol blue, 60 m*M* Tris-Cl (pH 6.8), and 1% glycerol. Store at –20°C in 2X stock solutions.
5. Separating gel: For 1X 15% mini-gel, 5 mL of 30% acrylamide, 2.5 mL of 1.5 *M* Tris (pH 8.8), 100 µL of 10% SDS, 655 µL of 1.5% ammonium persulfate, and 5 µL TEMED. Add ddH_2O up to 10 mL. Make gel fresh for each experiment.
6. Stacking gel: For 1 gel, 0.89 mL of 30% acrylamide, 3.46 mL ddH_2O, 2.5 mL of 1.0*M* Tris (pH 6.0), 100 µL of 10% SDS, 660 µL of 1.5% ammonium persulfate, and 8 µL TEMED. Add ddH_2O up to 5 mL. Make gel fresh for each experiment.
7. Broad-range protein molecular-weight markers (200–7 kDa): Available commercially, store at –20°C.
8. 5X running buffer: For 1 L, 15 g Tris-hydroxymethylamine, 72 g glycine, and 5 g SDS. Dissolve in ddH_2O.
9. Nitrocellulose membrane: 0.2-µm pore size (available commercially). Store in a sealed container and handle with gloves and flat-tipped forceps.
10. Transfer buffer: 0.25 m*M* Tris, 192 m*M* glycine, and 20% (v/v) methanol. Store at 4°C.
11. Transfer unit apparatus: Available commercially (Bio-Rad).
12. Ponceau S: 0.2% Ponceau S and 13% trichloroacetic acid. Store at room temperature.
13. Tris-buffered saline (TBS): 137 m*M* NaCl and 20 m*M* Tris-Cl (pH 8.0). Store at room temperature.
14. Tris-buffered saline (TBS) + Tween (TBST): 0.05% Tween-20 in TBS. Store at room temperature.
15. Blotto: 5% Skim milk powder in TBST. Store at 4°C for no longer than 2 d.
16. Western blotting analysis system (e.g., ECL, available commercially). Store at 4°C, away from light.

3. Methods

3.1. Isolation of DNA from Mouse Tails

1. A 1-cm piece of tail is placed in a 1.5-mL Eppendorf tube (*see* **Note 1**). Tails can either be processed immediately or stored at –20°C.
2. Add 500–700 µL Proteinase K buffer to each tube. Mince tail using small sharp scissors that fit into the bottom of the tube (*see* **Note 2**). Add an additional 35 µL Proteinase K solution.
3. Incubate tubes at 65°C overnight in a water bath or heating block. The next day, cool tubes to 37°C.

4. Add 20 µL of RNase A (*see* **Note 3**). Incubate tubes at 37°C for 1–2 h, then add 200 µL of 5 *M* NaCl to each tube.
5. Fill tubes to the top with P:C:I solution (approximately 700 µL). Cap tubes and shake vigorously for 5–10 s.
6. Spin tubes in centrifuge at 4°C for 10–15 min (*see* **Note 4**). Carefully remove tubes. There should be two phases: The bottom phase contains proteins and the top contains DNA and RNA. Tail debris will form a plug at the interface of these two phases (*see* **Note 5**). Remove the aqueous (upper) phase and transfer to the appropriately labeled Eppendorf tube (set 1; *see* **Notes 6** and **7**).
7. Fill each tube containing the aqueous phase with 95% ethanol. Shake tubes for 10 s to allow DNA to precipitate (*see* **Note 8**). The DNA will form as white, thread-like material. Remove DNA by spooling onto the end of a heat-sealed glass micropipet.
8. Wash the DNA sample by dipping the glass micropipet in 70% ethanol. Repeat this step twice. Let the DNA air-dry by standing the micropipet tips, spooled tip up, in separate labeled tubes.
9. Break off tips and drop into the appropriately labeled Eppendorf tube (second set) containing 150 µL TE. Place tubes on a horizontal rotator to allow DNA to dissolve for at least 1 h. Remove glass tips.
10. Add 1 µL of DNA sample to 999 µL of H_2O (1:1000 dilution). Determine DNA concentration by measuring the absorbance at 260 nm and 280 nm on a spectrometer. The ratio between A_{260} and A_{280} should be 1.8 for pure DNA (*see* **Note 10**).
11. Store the DNA at –20°C until needed.

3.1.1. Restriction Enzyme Digestion of Transgenic Genomic DNA

The restriction enzyme (RE) used to digest isolated genomic DNA depends on the specific RE sites located within a transgene. The usual method involves using an RE that cuts within the transgene construct once. As transgenes tend to concatermerize and incorporate as multiple copies in the genome, restriction digestion with an RE that cuts once in the transgene will bisect the multiple copies, resulting in a repetitive band of a known size. The repetitive band is the intervening transgene sequence between the two RE sites in concatamerized copies of the transgene. The RE will also digest sites within the genomic DNA that flank the transgene. Upon hybridization with a probe specific for the transgene (e.g., coding sequence for BDNF or P75 ICD), those samples that show a band the size of the specific repetitive band along with flanking genomic bands are positive for the transgene, whereas nontransgenic samples should only display the endogenous genomic bands.

To determine the transgene copy number, a comparison of the autoradiographic intensity between the repetitive band and the flanking genomic band will give an estimate of the copy number. Because the flanking genomic band is assumed to have a copy number of 1, the intensity of the repetitive band

relative to the intensity of the flanking band will yield an approximate copy number.

To determine whether an animal is homozygous, heterozygous, or null for the transgene, a quantitative Southern blot must be performed. Equal amounts of DNA must be loaded. If the transgene has been incorporated into the germline of a founder animal, then breeding with a wild-type animal should result in an offspring ratio of 1/2 heterozygote to 1/2 wildtype. Only those animals with the repetitive band and flanking endogenous band will be positive and heterozygote for the transgene. Upon breeding the heterozygote animals with each other, the resulting offspring should be 1/4 homozygote, 1/2 heterozygote, and 1/4 wildtype. A comparison of the autoradiographic transgene band intensities between DNA samples should result in a doubling of intensity in "presumptive" homozygote animals. This type of quantitation assumes that equal amounts of DNA have been used. The real test for homozygosity are the results of subsequent breeding of the presumed homozygotes with wildtype animals that must yield progeny that are 100% positive for the transgene.

3.2. Southern Blot Analysis

3.2.1. Gel Preparation

1. Dissolve agarose in 1X TBE buffer in a glass beaker.
2. Heat the agarose solution in a microwave until the agarose is fully dissolved. Remove and allow the solution to cool until warm to the touch.
3. Add ethidium bromide (1 µL/50 mL agarose solution; *see* **Note 11**). Mix well, pour into gel apparatus, and carefully place in comb (*see* **Note 12**). Let gel harden at either 4°C or room temperature. Once hardened, remove comb and place gel in electrophoresis unit containing 1X TBE. Do not allow the gel to "dry out."
4. Spin the DNA samples in a centrifuge. Add 1 µL of 10X loading dye to every 10 µL of digested DNA sample, mix, and load the samples into their respective well (*see* **Note 13**).
5. Run DNA samples between 70 and 90 V for 2–3 h.
6. Photograph gel with a ruler laid along side the gel so that band positions can later be identified on the membrane. Cut a corner of the gel (e.g., the bottom, right-hand side) for easy orientation of DNA samples.
7. Rinse the gel in distilled water and place in a glass Pyrex dish. *Optional*: Add 0.25 *M* HCL and shake slowly on a platform shaker for 30 min at room temperature (*see* **Note 14**).
8. Rinse gel in distilled water. Pour off water and add denaturation solution. Shake for 20 min, then replace with fresh denaturation solution and shake again for 20 min. Pour off denaturation solution and rinse gel with distilled water (*see* **Note 15**).
9. Add neutralization solution, shake for 1 h, replace solution, and shake for an additional 30 min (*see* **Note 16**).

3.2.2. Transfer of DNA to Membrane Support

1. While gel is neutralizing, start preparing the transfer apparatus.
2. Place a support such as a glass plate that is larger than the gel across an oblong Pyrex glass dish. Fill the bottom of the dish with 10X SSC.
3. Cut two long pieces of Whatmann paper strips. These pieces should be wider than the length of the gel. Wet thoroughly with 20X SSC and place across a glass plate, ensuring the ends are sitting in the 10X SSC solution in the dish.
4. Place two filter papers cut slightly bigger than gel, wet with 20X SSC, and place on top of the first two filter strips.
5. Carefully flip the gel over and place on top of the filter papers (*see* **Note 17**). Roll a glass pipet over the gel to remove any bubbles that are present between the gel and filter paper.
6. Pour some 20X SSC over the gel. Never allow the gel, filter papers, or membrane to dry out.
7. Place nitrocellulose or nylon membrane over the gel (*see* **Note 18**). The membrane should not be larger than the gel and should not have any edges hanging over and touching the filter paper underneath the gel. This will short circuit the transfer. If using nitrocellulose, first soak the membrane in distilled water for 5 min, then soak in 20X SSC for an additional 5 min.
8. Pour some 20X SSC over the membrane; roll carefully with a glass pipet to remove all air bubbles.
9. Place three membane-sized filter papers soaked with 20X SSC on top, then five dry membrane-sized filter papers.
10. Place a stack of paper towels cut to membrane size and place on top of dry filter papers. The stack should be at least 5 cm high.
11. Lay a glass plate on top of apparatus and place a weight on top to hold in place (*see* **Notes 19** and **20**).
12. Leave overnight to transfer.
13. The next day, remove weight, glass plate, paper towels, and filter paper. Most of the paper towels should be wet due to the upward capillary transfer.
14. Take gel with membrane still attached; flip over onto Saran wrap. Cut corner (e.g., bottom right-hand corner) of the gel/membrane unit for easy orientation. Also, mark the wells with pencil for sample position.
15. Take apart the gel and membrane and check under ultraviolet (UV) light to determine if all the DNA was transferred from the gel to the membrane.
16. Rinse the membrane in 6X SSC in a glass Pyrex dish for 15 min. Blot the membrane between dry filter papers.

3.2.3. DNA Immobilization

1. Place the membrane between two Whatmann filter papers, and then between two glass plates.
2. Bake in a vacuum oven at a temperature between 70 and 80°C for 2 h.
3. Remove the membrane and either store between filter papers and covered in Saran wrap at 4°C or proceed to prehybridization step in Southern blot analysis.

3.2.4. Prehybridization/Hybridization of Membrane

1. Carefully place membrane in a Seal-a-Meal bag and heat seal all edges with a heat sealer.
2. Cut a corner of the bag, place a P1000 pipet tip in the opening, and pipet through the prehybrization mixture with a 10-mL plastic pipet (*see* **Notes 21–23**).
3. Carefully remove any air bubbles in the bag and heat seal the cut corner.
4. Incubate at 65°C in a shaking incubator or water bath for a minimum of 2 h to overnight.
5. Cut bag in corner and pour off the prehyb solution. Replace prehyb solution with fresh prehyb containing a radiolabeled DNA/RNA probe (*see* **Subheading 3.3.**). Carefully remove air bubbles. Ensure that the procedure is performed on a padded bench coat, which will be immediately discarded in a radioactive waste container. Reseal corner with heat sealer. Put the bag in water in a sealed plastic container.
6. Incubate the plastic container that contains the plastic bag in a 65°C shaking incubator or water bath overnight.

3.2.5. Washing the Membrane

1. Pour out the hybridization solution, using the appropriate disposal method for radioactive waste.
2. Remove the membrane and place in a plastic box (e.g., Tupperware container) containing 2X SSC + 0.2% SDS. Rinse.
3. Wash the blot in the following order (*see* **Note 24**):
   200 mL of 2X SSC + 0.2% SDS for 15 min at room temperature
   200 mL of 0.5X SSC + 0.2% SDS for 30 min at 55°C
   200 mL of 0.05X SSC + 0.2% SDS for 30–45 min at 65°C
4. Blot the membrane between filter paper without drying completely.
5. Wrap membrane in Saran wrap. Ensure that there are no wrinkles in the wrap.
6. Autoradiograph with Kodak XAR film at –70°C overnight.

3.3. Preparation of a Radiolabeled Riboprobe

1. Linearize the RNA transcription vector containing the sequence of interest with a restriction enzyme that cuts downstream of the cloned fragment (*see* **Note 25**).
2. Clean the linearized DNA by P:I:A extraction and ethanol precipitation.
3. Resuspend in TE buffer (pH 8.0) at a concentration of 1 mg/mL.
4. Transcription reaction: For a 20 µL reaction, 4.0 µL transcription buffer, 4.0 µL nucleotide mix without CTP, 0.7 µL of 100 m*M* DTT, 1.2 µL (20 U) RNasin (ribonuclease inhibitor), 2.0 µL (2 µg) purified linearized DNA, 100–200 µCi [α-^{32}P]CTP (cytosine triphosphate), and ddH_2O to a final volume of 20 µL.
5. Add RNA polymerase (5 units) and incubate for 1 h at 40°C for SP6 or at 37°C for T7 (*see* **Note 26**).
6. Clean the radiolabelled RNA using a nick column (*see* **Note 27**): Add 80 µL ddH_2O to 20 µL labeling reaction; add the total 100 µL to the column and allow to gravity filtrate; add 400 µL H_2O to column to wash out unincorporated

nucleotides; add another 400 µL H_2O to column and collect filtrate. This will contain the radiolabeled DNA. Using a geiger counter, measure the radioactivity of 1 µL from the sample (*see* **Notes 28** and **29**). The final specific activity should be at least 1×10^6 cpm/µL.

3.4. Isolation of RNA from Small Amounts of Tissue

1. Transfer the tissue (about 5 mg) into a weigh boat kept in ice containing approximately 300 µL cold PBS (*see* **Note 30**).
2. Wash the tissue three times with cold PBS (*see* **Note 31**).
3. Transfer the tissue into an Eppendorf tube (1.5 mL).
4. Rinse the tissue with cold lysis buffer and homogenize in 300 µL lysis buffer using a Teflon pestle or mechanical rotating pestle.
5. Centrifuge for 10 min at 4°C.
6. Transfer supernatant to an Eppendorf.
7. Add an equal volume of the second buffer.
8. Add Proteinase K to a final concentration of 200 µg/mL and incubate at room temperature for 20 min.
9. Following incubation, add 500 µL of P:C:I and shake to mix well.
10. Centrifuge for 5 min.
11. Transfer the aqueous phase (top phase) to a new Eppendorf tube and repeat the P:C:I extraction twice.
12. To precipitate the RNA, add to the final aqueous layer transferred to a new Eppendorf tube, 1/10 volume of 3*M* sodium acetate (pH 5.2) and 3 volumes of 95% ethanol. Let precipitate overnight at –20°C.
13. Centrifuge the tube for 15 min.
14. Remove ethanol/sodium acetate solution and resuspend the pellet in 50 µL TE + 0.2% SDS.
15. Reprecipitate with sodium acetate and ethanol as in **step 12** and leave tube at –20°C for at least 2 h.
16. Centrifuge the tube for 15 min, remove ethanol/sodium acetate, and resuspend pellet in 50 µL TE.
17. Repeat **step 15**.
18. Centrifuge the tube for 15 min, remove ethanol/sodium acetate, and allow the pellet to dry.
19. Resuspend the RNA in 10–15 µL TE. Store at –70°C (*see* **Note 32**). Let thaw on ice before using.

3.4.1. Northern Blot Analysis

Specific RNA sequences within a given tissue sample can be analyzed using Northern blot analysis. The basic protocol for Northern blotting involves upward capillary transfer of denatured RNA from a formaldehyde denaturing agarose gel onto a nitrocellulose membrane. A specific DNA or RNA probe is subsequently hybridized to the membrane to identify specific RNA bands

present in the sample (i.e., products of the transgene and endogenous gene). We will not review the various protocols for Northern blot analysis, as they have been thoroughly described elsewhere ***(36)***.

3.4.2. Analysis of Proteins in Tissue

There are two procedures commonly used to determine whether the transgene has been translated into protein: Western blot analysis and immunocytochemical analysis. Western blot analysis allows the quantitation of proteins isolated from tissue; immuncytochemistry determines where the protein is localized in the tissue.

3.5. Immunocytochemical Analysis of Fixed, Frozen Tissue

1. Fix the tissue by either first perfusing the animal, then dissecting out the tissue or dissecting out the tissue, then drop fixing into the fixation solution (*see* **Note 33**).
2. Postfix the tissue in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 1 h to overnight (*see* **Note 34**).
3. Cryopreserve the tissue with increasing sucrose gradients in phosphate buffer (10%, 12%, 18%, 30%) at 4°C. Transfer the tissue to the next sucrose gradient only when the tissue has sunk. Leave the last sucrose gradient, 30%, overnight at 4°C.
4. Mount the tissue in mounting media and cut on a cryostat. Depending on the tissue, 10–30-µm-thick sections are sufficient. Mount the sections on glass slides that have been precoated with gelatin or equivalent (*see* **Note 35**).
5. Once the sections are done, store in a slide box that is kept at 70°C or proceed to the immunocytochemistry protocol (*see* **Note 36**).
6. Place the slides on a platform in a plastic box lined with wet paper towels. This will provide a humid chamber. Ensure that the slides lay completely flat so that any liquid pipetted onto the slide does not run off. Also, make sure the slides do not touch each other. Alternatively, a pap pen or plastic blocks that are drawn/fitted around the slide can be used. This will prevent the buffer from leaking.
7. Block the tissue with 10% serum in 0.1M phosphate buffer (pH 7.4) for 1 h at room temperature (*see* **Note 37**).
8. Wash three times with PBS for 10 min each wash. Remove liquid by aspiration with a Pasteur pipet connected to a vacuum beaker.
9. Add the primary antibody diluted in 0.1M PB containing 10% serum (*see* **Note 38**).
10. Incubate slides in primary antibody overnight at 4°C (*see* **Note 39**).
11. Wash the slides as described.
12. Add secondary antibody (e.g., a fluorescent secondary antibody such as indocarbocyanine [CY3]) diluted in 0.1M PB containing 10% serum.
13. Incubate slides with secondary antibody in a covered box for 1 h at room temperature (*see* **Note 40**).
14. Wash slides as described in **step 8**.
15. Place approximately 200–300 µL of mounting media in the middle of the slide and gently place a plastic cover slip on top. Avoid bubbles in the mounting media.

16. Aspirate any excess mounting media from the sides of the cover slips.
17. Let the cover slips sit for 1 h, aspirating excess mounting media frequently.
18. To immobilize the cover slip, carefully apply clear nail polish around the edges and let dry.
19. Observe the tissue under a fluorescence microscope with the appropriate filter.
20. Take pictures on color slide film (e.g., Ektachrome ASA 400; *see* **Note 41**).
21. Store slides in a slide box at 4°C.

3.6. Western Blot Protocol for Brain Tissue

3.6.1. Protein Isolation from Tissue

1. Rapidly decapitate animal and quickly remove the brain (*see* **Note 42**).
2. Place brain onto glass plate kept on ice.
3. Dissect brain parts (e.g., hippocampus, cortex, cerebellum) and place in an Eppendorf kept on ice.
4. Either freeze brain tissue on dry ice or immediately add lysis buffer to the tissue (1 mL lysis buffer/10 mg tissue; *see* **Note 43**).
5. Rapidly homogenize tissue with a polytron, 10-s pulse.
6. Incubate the tissue homogenate at 4°C for 20 min on a rocking platform.
7. Microfuge the homogenate at 17,000*g* for 15 min at 4°C (*see* **Note 44**).
8. Remove the supernatant (clear upper phase) only and transfer to another Eppendorf tube.
9. Remove supernatant and transfer to another Eppendorf tube.
10. Take an aliquot (5–10 µL) of the supernatant to measure protein concentration of the sample (*see* **Note 45**). Use the BCA Assay kit (Pierce). Dilute the BSA standard 1:20 and load decreasing concentrations of the 1:20 BSA sample in duplicate.
11. Add 50 µg protein to sample buffer (*see* **Note 46**) for a total volume of 30–40 µL per 10-well comb.
12. Boil the samples for 5 min, then spin for 10 s at 10,000*g* (*see* **Note 47**). Keep samples on ice until the gel is ready to be loaded.

3.6.2. Protein Separation by Gel Electrophoresis

1. Make separating gel: For each gel, add 6.5 mL separating gel solution to a protein mini gel caster with a 10-mL plastic pipet and cover the top of the gel with isobutanol, ddH_2O, or 10% SDS. Let gel harden for at least 40 min. Pour liquid off completely and add the stacking gel (*see* **Note 48**).
2. Add the stacking gel on top of the polymerized separating gel with a 10-mL plastic pipet. Fill to the top of the mini-gel caster, and quickly place in comb. Let gel polymerize for at least 10 min, but no longer than 20 min (*see* **Note 49**).
3. Once the stacking gel has polymerized, remove the gel apparatus and place in an electrophoresis unit. Mark the wells on the glass plate with a felt-tip pen before removing the comb.
4. Fill electrophoresis unit with 1X running buffer.

5. Load the gel with the samples, protein molecular-weight markers, and protein standards (e.g., human recombinant BDNF of known concentrations; *see* **Note 50**).
6. Run samples at 70 V (constant voltage, 15 mA) through the stacking gel. Once the dye front has reached the separating gel, increase the voltage to 100–120 V (20–25 mA).

3.6.3. Protein Transfer

1. Cut one piece of nitrocellulose membrane and two pieces of filter paper to the size of the gel. Soak the membrane in ddH_2O for 10 min, then transfer buffer for another 5 min.
2. Remove the gel apparatus from the electrophoresis unit; carefully remove glass plates.
3. Cut away the stacking gel and place one filter paper soaked in transfer buffer on top of the gel. Lift off the gel and filter paper together.
4. Place the gel, filter paper side down onto transfer unit support pads. Place nitrocellulose membrane on top of the gel, and the final filter paper soaked in transfer buffer. Place the sandwich in a transfer tank kept at 4°C. Ensure that the membrane sits closest to the positive electrode, and the gel closest to the negative electrode.
5. Transfer for 1.5 h at 0.6 A (constant amps).
6. After the transfer, remove the nitrocellulose membrane and rinse in ddH_2O two times for 10 min each. *Optional*: Visualize proteins using Ponceau S (*see* **Note 51**). If using Ponceau S, wash Ponceau S three times for 10 min in ddH_2O.
7. Wash the membrane in TBS (pH 8.0), two times for 10 min each.
8. Block nonspecific binding sites on the membrane with Blotto for 1 h at either room temperature or overnight at 4°C.
9. Add primary antibody to fresh Blotto mixture and incubate for 2 h at room temperature or at 4°C overnight on a rocking platform.
10. Wash the membrane in TBST, four times for 10 min each.
11. Add appropriate secondary antibody (e.g., horseradish peroxidase-conjugated secondary) in fresh Blotto and incubate for 1.5 h at room temperature on a rocking platform.
12. Wash the membrane in TBST, three times for 10 min each.
13. Add ECL and expose over XAR-5 film. The time varies depending on the antibody used (*see* **Note 52**).
14. Develop the film.

4. Notes

4.1. Isolation of DNA from Mouse Tails

1. When cutting the tail, it is important to obtain at least 1 cm. If the tail sample is too small, not enough DNA will be isolated for use in Southern blot analysis.
2. Cutting up the tails will allow easier access of the PK to the tissue resulting in thorough digestion of the sample
3. Stock solution of RNase A is heated to 100°C for 10 min to destroy contaminating DNase.

4. While tubes are spinning in **step 10**, prepare two sets of labeled Eppendorf tubes for each sample. In the second set of tubes, aliquot out 150 µL of TE buffer. The first set of tubes will hold the transferred aqueous phase of the P:C:I extraction and the subsequent ethanol precipitation of the DNA, whereas the TE in the second set of tubes will dissolve the isolated DNA strands.
5. Always withdraw P:C:I from the bottom phase. Do this step under a fume hood, as P:C:I fumes are toxic.
6. The order of labeled Eppendorf tubes containing the tail samples should be noted, as P:C:I can remove felt-tipped labels.
7. Use a P1000 blue tip that has been cut at the end to give a widened end for easier removal of the upper (aqueous) phase of the P:C:I extraction in **step 11**.
8. Samples should be performed in batches (approximately 20 samples) such that all can be processed within 10 min of removal from the centrifuge in **step 11**.
9. Glass micropipets ends are sealed by rotating the tip over a bunsen burner. Always keep the ends clean by standing up on a styrofoam tray.
10. The concentration of DNA can be determined by using the following formula: DNA concentration (µg/mL) = absorbance at 260 nm × Dilution factor (e.g., 1000) × 50 µg/mL (DNA concentration factor).

4.2. Southern Blot Analysis

4.2.1. Gel Preparation

11. Always wear gloves when handling EtBr or any solutions containing EtBr, as it is carcinogenic
12. The gel should not be too thick (less than 7 mm). A thinner gel allows easier transfer of DNA from the gel to the membrane.
13. Load at least 10 µg of DNA. This will ensure that there will be efficient hybridization of the labeled probe to the homologous DNA sequence and maximum discrimination between homologous and heterologous sequences. Equal quantities of DNA should be loaded to compare levels of the transgene between individual animals.
14. In **step 7**, HCl depurinates DNA fragments leading to strand cleavage, reducing the length of the DNA. If efficient transfer of DNA larger than 5 kb is not required, then proceed to denaturation.
15. In **step 8**, denaturing the gel unzips the DNA to give single-stranded molecules with unpaired bases, thus allowing hybridization with a DNA/RNA probe.
16. In **step 9**, neutralizing the gel brings the pH down below 9.0. If the pH is greater than 9.0, transferred DNA will not bind to the nitrocellulose membrane.

4.2.2. Transfer of DNA to Membrane Support

17. The DNA is located closer to the bottom of the gel; therefore, flipping over the gel and placing the membrane on the bottom side requires less gel for the DNA to pass through during the transfer.
18. Never handle the membrane with your hands; use flat-tipped forceps.

19. Once the transfer apparatus has been completed, placing masking tape across the length and width of the Pyrex dish from one edge, over the weight, to the other edge can help to stabilize the structure given that the paper towels are not stacked too high.
20. Ensure the weight on top of the transfer apparatus is not too heavy so that it does not crush the gel and obstruct the transfer.

4.2.4. Prehybridization/Hybridization of Membrane

21. The herring sperm and salmon sperm should be mixed and boiled for 5 min before adding to the prehyb solution. These are used to reduce background hybridization of the probe.
22. Formamide destabilizes nucleic acid duplexes. One percent formamide reduces the T_m by 0.7°C, allowing the incubation to be carried out at a lower temperature than needed with an aqueous solution. However, the rate of the hybridization reaction is significantly slower in the presence of formamide.
23. Denhardt's contains three polymeric compounds that compete with nucleic acids for the membrane binding sites and is, therefore, used to reduce the background hybridization.

4.2.5. Washing the Membrane

24. Membrane washes after probe hybridization are designed to denature those DNA–RNA hybrid bonds that are less stable (i.e., that share a lower degree of homology). Washing is often carried out in sequentially decreasing salt concentrations. The conditions can be manipulated to be more or less stringent. Thus, under very stringent conditions (high temperature, low-salt concentrations), only the most stable DNA–RNA hybrid bonds will remain.

4.3. Preparation of a Radiolabeled Riboprobe

25. The advantages of using an RNA probe instead of a DNA probe are that (1) RNA probes are single stranded and do not need to be denatured before adding to the hybridization solution and (2) the specific activity is generally higher.
26. The DNA fragment which will be transcribed into the labeled RNA probe should be specific for the transgene sequence. For DBH:BDNF mice, the 1.6-kb DBH promoter sequence was used, whereas the 1.1-kb Tα1 promoter sequence was used for the Tα1:P75 ICD mice. These promoters were cloned into vectors with appropriate phage promoters. SP6 RNA polymerase is one commonly used RNA polymerase for preparation of riboprobes.
27. Alternatively, unincorporated nucleotides can be removed by a spin-column procedure provided, for example, by a commercially available kit.
28. When working with radioactive material, always use the appropriate shielding in an area designated for radioactive work. In the case of ^{32}P, use plexiglass shielding.
29. Always wear a lab coat, glasses, and gloves when handling radioactive material. It is good practice to double glove and discard the top layer every time a radioactive container is handled.

4.4. Isolation of RNA from Small Amounts of Tissue

30. One of the major problems associated with isolation and analysis of RNA is the presence of ribonucleases (RNAses). To avoid degradation of RNA samples, it is essential that the following precautions be observed: (1) Gloves are always worn. Skin is one of the primary sources of exogenous RNase. (2) Glassware is baked. Standard protocols call for baking at 400°C for 5–6 h. An optional precaution for avoiding RNase degradation is by treating all solutions and water with 0.1% DEPC, followed by autoclaving.
31. Always keep freshly dissected tissue cold to slow RNases from degrading RNA. If it will be used immediately, place on ice. If to be used later, store at –70°C.
32. On an agarose gel, degraded RNA will appear as a smear, whereas intact RNA will show the 28S (4.7 kb) and the 18S (1.9 kb) ribosomal RNAs.

4.5. Immunocytochemical Analysis of Fixed, Frozen Tissue

33. One of the most important issues for immunocytochemistry on tissue is the type and length of fixation. Some antibodies are very sensitive to fixed tissue. Generally, a 2–4% paraformaldehyde solution in a 0.1*M* phosphate buffer (pH 7.4) is used and the tissue is drop fixed (if the tissue is very small such as an SCG or DRG). Alternatively, the animal is cardiac perfused with the PF solution (if the tissue is large such as the brain), the tissue removed, then postfixed at 4°C for 1 h to overnight.
34. Avoid overfixing the tissue. Overfixation may lead to conformational changes in the target protein so that the primary antibody may not be able to bind to the target epitope.
35. Precoated slides will prevent the tissue from coming off the slide in the subsequent incubations with the antibodies and buffer washes.
36. Never let the tissue thaw to room temperature once it is mounted on the slide. Keep the slides cold (e.g., keep in the cryostat machine) and thaw mount with the heat of your finger on the back of the glass slide.
37. Preincubating the tissue with serum will help to decrease nonspecific binding of the primary or secondary antibody and, therefore, lessen background labeling.
38. When using a new antibody, a serial dilution should be performed in order to determine the optimal dilution. For example, start with dilutions 1 : 100, 1 : 500, and 1 : 1000. There are two types of antibodies: polyclonal and monoclonal. Polyclonal antisera contain antibodies that recognize different epitopes on the antigen. Monoclonal antibodies recognize a single epitope.
39. Because of the problem with background staining and nonspecific labeling, a number of different controls are used: preimmune serum from the animal that produced the antibody, secondary antibody alone, or an antibody that recognizes a protein known to be expressed in the tissue.
40. Cover the slide box with foil to keep out light, as secondary fluorescent antibodies will extinguish when exposed to light. Adding an antifading agent will prolong the life of the antibody fluorescence.

41. When taking pictures of the tissue, it is suggested that different exposure times be used (e.g., 10 s, 15 s, 20 s, etc.), depending on the intensity of staining. This will allow you to determine the optimal exposure to which the staining is observable.

4.6. Western Blot Protocol for Brain Tissue

4.6.1. Protein Isolation from Tissue

42. It is important to work quickly through **steps 1–4** to avoid endogenous protease degradation of proteins.
43. The lysis buffer breaks the cell membranes apart and releases cytosolic proteins. If nuclear proteins are the targets, 0.2–0.5% SDS in the lysis buffer can be used.
44. A pellet that contains cell debris will form at the bottom of the Eppendorf. The supernatant contains protein, whereas DNA and RNA are found at the interface between the cell debris and the supernatant.
45. If comparing different samples, it is important to correct for the total amount of protein in each sample. The BCA assay kit determines protein concentration by comparing sample standard concentrations of BSA to the protein samples. Prepare BSA dilutions in ddH_2O, including a control (0 BSA) using the same volume of lysis buffer in each experimental sample.
46. Ensure that the sample buffer stock solution is completely thawed and free of precipitate before adding to samples. At 4°C, the SDS precipitates and glycerol will not dissolve.
47. Boiling allows some antigens to be exposed under these conditions and negatively charges the proteins in the sample so they will run into the gel.

4.6.2. Protein Separation by Gel Electrophoresis

48. The percentage of gel depends on the size of the protein of interest. For detection of BDNF, a 15% gel is used. For example, proteins of 0–60 kDa can be resolved on a 15% gel, 30–100 kDa on a 10%gel, and above 100 kDa on a 7.5% gel. Gels are made in a mini-gel caster available from Bio-Rad.
49. It is important that all components of separating and stacking gel are mixed thoroughly before and after adding TEMED, or the gel will not polymerize properly.
50. Load gel with long, thin tips that will reach to the bottom of each well. When loading samples, keep the tip at the bottom of the well as you are pipetting the sample out, and slowly withdraw tip when the last of the sample is being pipetted out. Be careful not to pipet sample out hard, as it may spill out to the other wells.
51. Ponceau S determines whether the transfer was efficient, gives a profile of the separated proteins, and whether an equal amount of protein was loaded.
52. A Western blotting detection system, such as ECL (Amersham), is a nonradioactive method for detection of an immobilized specific antigen, which is conjugated directly or indirectly with horseradish peroxidase-labeled antibodies.

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14

Tracing with Radiolabeled Neurotrophins

Christopher S. von Bartheld

1. Introduction

The development of a simple and "gentle" technique for the radio-iodination of proteins with lactoperoxidase ***(1)*** and subsequent modifications of this technique ***(2–6)*** have made it possible to introduce labeled neurotrophins as sensitive tracers into living organisms and to determine their fate. Radiolabeled neurotrophins have been used to examine receptor-mediated internalization, transport, and metabolism, both in vivo ***(7–10)*** and in vitro ***(11,12)***. They have been instrumental in the analysis of functional interactions of neurotrophins and their receptors ***(13)*** and the discovery of retrograde axonal transport ***(7,8)***, a cornerstone of the neurotrophic hypothesis ***(14)***, as well as the discovery of anterograde axonal transport of neurotrophins ***(15)***. The neurotrophin dependence of clinically important neuronal populations has been revealed by transport studies with radiolabeled neurotrophins ***(16,17)***.

Advantages of radiolabeled neurotrophins include their sensitivity, the possibility to easily quantify the signal in a gamma counter, the simple iodination procedure, and relatively low cost. The radioactive signal can be visualized by autoradiography, combined with immunocytochemistry, and observed and quantified at the ultrastructural level ***(11,15,18,19)***. Alternative methods for the labeling of neurotrophins have been proposed, first by biotinylation ***(20)*** and, more recently, by conjugation with a fluorescent tag ***(21)***. Biotinylation is more complicated than iodination and has rarely been used with neurotrophins. To date, there are no studies that have compared the sensitivities of these methods, and advantages of the fluorescent tagging of neurotrophins remain to be shown.

The mechanism of iodination with lactoperoxidase (as described in this chapter) is simple: $Na^{125}I$ is oxidized, which covalently modifies the phenolic ring of tyrosine residues, generating mono- and di-iodo derivates. Accordingly,

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lactoperoxidase-catalyzed labeling of proteins with iodine requires tyrosine residues. The success of the iodination further depends on the access of the iodine to these residues in the three-dimensional structure of the protein ***(22,23)***. Such properties vary among different neurotrophins. One molecule (monomer) of murine nerve growth factor (NGF) contains two tyrosine residues, whereas human brain-derived neurotrophin factor (BDNF) and neurotrophin-3 and -4 (NT-3 and NT-4) contain two to five tyrosine residues. This has implications for the iodination, as discussed below.

An important question is whether the iodination changes the receptor-binding properties or the functional activity of the ligands. For most iodinated neurotrophins, there has been no evidence for changes in structure or bioactivity ***(4,10,23)***. As many as 2–6 mol ^{125}I can be incorporated per mole of dimer NGF without loss of biological activity ***(4,5)***, and 1–3 mol ^{125}I per mole dimer NT-3 ***(10,24)***. Higher specific activities of BDNF (more than 2 mol ^{125}I per mole dimer BDNF) appear to reduce the biological activity of this neurotrophin significantly ***(25)***, presumably because the iodination of both tyrosine residues in position 86 of the BDNF dimer alters the epitope such that it interferes with the binding to the trkB receptor (***23,26***; *see* **Note 1**). This effect appears to be neglible in the BDNF dimer when only 1–2 mol ^{125}I are incorporated per mole of dimer BDNF ***(10,27–29)***. It is prudent to test the biological activity or receptor binding at the beginning of an iodination series to know if, and to what extent, the iodinated molecule may be less active than the native neurotrophin.

After the iodination, the free, not-incorporated ^{125}I has to be removed from the preparation. Several techniques are currently being used for the separation of free iodide from labeled neurotrophins, including dialysis ***(3,6,10,30)***, gel filtration ***(27,28,31)***, and membrane filtration ***(32,33)***. For transport studies, the labeled neurotrophins have to be applied to the region of interest at high concentrations. Therefore, membrane filtration is a convenient method because it can concentrate the labeled protein at the same time as it purifies it ***(33)***. The isotope ^{125}I emits primarily low-energy gamma particles and has a half-life of about 60 d. It can be quantified in a gamma counter and visualized on X-ray films as well as autoradiographic emulsions.

This chapter describes in detail (1) the iodination procedure using lactoperoxidase, (2) purification of the iodinated neurotrophin by membrane filtration, (3) analysis of the labeled product, (4) delivery of the iodinated neurotrophin in vivo, and (5) autoradiography of tissue sections at both the light-microscopic and (6) electron-microscopic levels. Alternative ways of obtaining iodinated neurotrophins (NGF) include its purchase from commercially available sources (e.g., Amersham Life Sciences or Dupont NEN Life Sciences) or the use of different iodination procedures (e.g., IODO-GEN, IODO-BEADS, Pierce). For protocols of these iodination procedures, the reader is referred to **ref.** ***31***, ***34***, or ***35***.

2. Materials

2.1. Iodination

The iodination procedure requires an appropriately ventilated fume hood, monitoring of radiation exposure and waste disposal (institutional approval), a dose meter (e.g., Ludlum Measurements, Inc., Model 18 Analyzer with a 44-3 probe), lead shielding, and access to a gamma counter (e.g., Cobra from Packard, Wizard from Wallac; many older Beckman counters are still in operation, although Beckman does not manufacture them any more).

1. 5 µg neurotrophin at a concentration of 1 mg/mL (=1 µg/µL) (0.1 µg/µL will work; aliquots can be stored at –80°C for 3 yr).
2. 0.5 *M* sodium phosphate buffer, pH 7.0.
3. ^{125}I–Na, 100 mCi/mL, in dilute NaOH (e.g., from Dupont NEN or Amersham, prepared fresh every 2 wk by these companies); store at room temperature in a leaded container ("lead pig") no longer than 1–2 wk, half-life = 60 d.
4. 3 mg/mL lactoperoxidase, from bovine milk (e.g., L8257 from Sigma); aliquots of 10 µL in phosphate-buffered saline (PBS); keep at –20°C for 1–2 yr.
5. 30% Hydrogen peroxide (from fresh stock, less than 3 mo after opening at 4°C).
6. 0.4% Glacial acetic acid (keeps for months at 4°C).
7. Bovine serum albumin (BSA) buffer: 10 m*M* sodium acetate (pH 4.0), 100 m*M* NaCl, 1 mg/mL BSA (make fresh, keeps at 4°C for 1 wk).
8. 50% Trichloroacetic acid (TCA) (keeps for months at 4°C).

Also needed: Crushed ice; 1.7 mL centrifuge tubes; sturdy rack; waste container for radioactive pipet tips; 20-µL pipet; 200-µL pipet; microcentrifuge at 4°C.

2.2. Purification

1. Ultrafree MC filters (UFC3 LGC 00, Millipore) or Microcon-10 microconcentrators (Amicon, Inc.; now distributed by Millipore).
2. 1% Sucrose in 0.5*M* phosphate buffer or BSA (1 mg/mL) buffer, pH 7.4.
3. Buffer without the BSA.
4. 50% TCA.
5. 1.7-mL centrifuge tubes.
6. Adjustable speed microfuge (e.g., Eppendorf 5415C).
7. Gamma counter.

2.3. Analysis

1. 50% TCA.
2. BSA buffer (see **Subheading 2.1., item 7**).
3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (15–20%).

Also needed: Functional assay [e.g., dorsal root ganglion neurite outgrowth ***(36,37)***], dorsal root ganglion cell survival ***(29)***, survival of trk-transfected fibroblasts ***(38)***, or receptor-mediated axonal transport ***(4,10,34)***.

2.4. Delivery

1. Hamilton syringes (e.g., 701 or 702, gas tight, Luer tip, 10- or 25-µL with gage-28, point-style needles).
2. Disposable insulin syringes (e.g., Becton and Dickinson microfine IV needles 28 G1/2).
3. 500- to 1000-fold excess cold neurotrophin (for competition to determine if transport is receptor mediated).
4. Anesthetic.
5. Dose meter.
6. Water-repellent film (e.g., Parafilm).
7. 0- to 2-µL and/or 1- to 20-µL pipet.
8. Gamma counter.

2.5. Autoradiography at the Light-Microscope Level

1. 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4 (fixative, keeps for weeks at 4°C).
2. Emulsion (NTB-2 or NTB-3, Kodak; *see* **Note 2**), wrap container in several layers of aluminum foil and store at 4°C, or aliquot in 20 or 30 ml; keeps in the dark at 4°C for 8–10 mo. Safe light conditions: red safe light only (e.g., Kodak GBX-2 filter).
3. Dipping chamber for glass slides (e.g., dip miser for slide coating, "10 ml capacity" from Electron Microscopy Sciences actually holds about 35 mL).
4. Humid chamber for slowly drying dipped slides prior to storage.
5. Light-tight slide boxes (e.g., for 25 slides), black electrical tape, aluminum foil.
6. Glass or plastic slide rack, clean slide staining dishes.
7. Silica gel (e.g., Drierite).
8. Developer D19 (Kodak), keeps at room temperature for 3–4 mo.
9. "Stop" solution: 1% acetic acid (make fresh).
10. Fixer (Fixer, Kodak), keeps at room temperature for 3–4 mo.
11. DPX cover-slip mounting medium (a mixture of distyrene, a plasticizer, and xylene; BDH Laboratory Supplies, Poole, UK; Electron Microscopy Sciences, Ft. Washington, PA).

Also needed: Anesthetic/perfusion/fixation setup; paraffin embedding and sectioning or cryoembedding and sectioning; dark room (red safe lights only, e.g., GBX-2 filter, Kodak); clean slides (25 × 75 mm, one end frosted for labeling); water bath at 42°C; microscope equipped with dark-field/bright-field optics.

2.6. Autoradiography at the Electron-Microscope Level

1. 1.5% Glutaraldehyde (EM grade, add glutaraldehyde just before use) and 2% paraformaldehyde (stock 4% paraformaldehyde keeps for weeks at 4°C) in 0.1 *M* sodium cacodylate buffer, pH 7.4 (make cacodylate buffer fresh; toxic: wear gloves!)

2. 1% Osmium tetroxide (OsO_4) from a 2% stock solution; stock keeps for years at 4°C in well-sealed, clean glass container, inside a second glass jar (toxic: blackening vapors!); mix stock and 0.2 *M* cacodylate buffer 1 : 1 for final concentration of 1% OsO_4 in 0.1 *M* cacodylate buffer.
3. Ethanol 50%, 70%, 95%, 100%.
4. Propylene oxide.
5. Spurr's resin ***(39)*** (make up fresh): ERL (10 g), DER (3.5 g), NSA (26 g), DMAE (0.4 g).
6. One water bath each at 38–40°C and 32°C (or one waterbath with a temperature range of 32–40°C).
7. Ilford L4 emulsion (keeps for 2 yr at 4°C in the dark).
8. Platinum wire loop (loop dimensions 1 × 3 cm diameter; platinum wire, 0.008 in. wire; e.g., Fisher Scientific, 13-766-10A) (*see* **Fig. 2**; *see* **Note 3**).
9. Developer D19 (Kodak); keeps for months at room temperature; filter before use.
10. Fixer (Rapid Fixer, Kodak); keeps for months at room temperature; filter before use.
11. Lead citrate stain ***(40)***; make fresh (*see* **Note 4**). In 50-mL volumetric flask, add 1.33 g lead nitrate $Pb(NO_3)_2$, 1.76 g sodium citrate $Na_3(C_6H_5O_7)\cdot 2H_2O$, and 30 mL freshly boiled and cooled distilled H_2O. Shake vigorously for 30 min. A white precipitate will form. Add 8 mL of 1 *N* NaOH, dilute to 50 mL with the H_2O, and mix by repeated inversion until precipitate is dissolved. pH should be about 12.

Also needed: Ventilated fume hood for work with osmium; oven at 60°C; Spurr resin mixture and embedding setup; microtome for thin sectioning with diamond knife (e.g., Diatom 2.4 mm); uncoated copper grids (mesh ranges 200 × 200 to 300 × 300 or 300 × 75 give good section support) and grid box; darkroom with safe light: monochromatic yellow overhead (flaps down); filter paper; light-tight slide boxes; transmission electron microscope.

3. Methods

3.1. Iodination

3.1.1. Labeling of Neurotrophin

All radioactive pipet tips are discarded in radioactive waste!

1. Place the following items in a 1.7-ml microcentrifuge tube in a fume hood at room temperature in sequence (*see* **Notes 5-13**): 3 µL sodium phosphate buffer, 5 µL neurotrophin (5 µg), and 4 µL of ^{125}I–Na (= 0.4 mCi of a 100 mCi/mL stock).
2. Add 4 µL fresh 1 : 1 mix of 1 : 100 lactoperoxidase and 1 : 10,000 H_2O_2; mix by pipetting, cap tube, and let sit for 30 min (*see* **Note 7**).
3. Add 4 µL fresh 1 : 1 mix of 1 : 100 lactoperoxidase and 1 : 10,000 H_2O_2; mix by pipetting, cap tube, and let sit for 30 min.
4. Add 20 µL of 0.4% acetic acid, mix, cap, and let sit for 5 min.
5. Add 100 µL BSA buffer, mix, and cap.
6. Store the tube at 4°C in a leaded container ("lead pig").

3.1.2. Initial Analysis for the Calculation of Specific Activities

1. Dilute 2 µL of the iodination reaction in 0.998 mL BSA buffer; mix.
2. Dilute 20 µL of this further in 0.780 mL BSA buffer.
3. Add 200 µL of 50% TCA, mix, and keep on ice for 5 min.
4. Spin for 2 min at > 10,000*g* at 4°C.
5. Pipet off the supernatant; keep in the gamma counter vial.
6. Determine counts of the pellet (P) and supernatant (S) separately in the gamma counter.
7. Calculate the % incorporation (= $Counts_P/[Counts_P + Counts_S]$). If incorporation is >90%, there is no need for purification. If incorporation is <90%, the neurotrophin should be purified (*see* **Subheading 3.2.**)
8. The iodinated neurotrophin can be stored for several weeks (in a lead pig) at 4°C. Because the half-life of ^{125}I is 60 d, it should be used within 2 wk after the iodination. If used for longer periods of time, the incorporation should be checked by TCA precipitation.

3.2. Purification

Membrane filtration (modified from the protocols of **refs. *32*,*33***, and ***41***). If incorporation is >90%, skip this step and go to **Subheading 3.3.**

1. Pipet 400 µL phosphate buffer with 1–10 mg/mL BSA and 1% sucrose into the upper chamber (insert) of the membrane filtration device.
2. Spin for 10–20 min at about 2000*g* (~4700 rpm in a 5415C Eppendorf microfuge).
3. Pipet off and discard the remaining liquid in the upper chamber as well as the filtrate.
4. In a fume hood, add iodinated neurotrophin (up to 400 µL capacity) to the upper chamber.
5. Fill the rest of the upper chamber with buffer (without the BSA). (The sequence of **steps 4** and **5** can be reversed to generate fewer radioactive pipet tips and reduce radiation exposure).
6. Spin for 30 min at about 2000*g* (approx 4700 rpm in a 5415C Eppendorf microfuge).
7. Add buffer without the BSA to the upper chamber (approximately the amount of the filtrate, approx 250 µL).
8. Discard the filtrate in the lower chamber (in liquid radioactive waste).
9. Spin again for 30 min as in **step 6**.
10. Repeat this one or two more times (depending on the initial incorporation).
11. Check incorporation by using a 0.5-µL aliquot, diluted in 0.7995 mL of BSA buffer, add 50% TCA, and calculate the incorporation as in **Subheading 3.1.2., steps 3–7**.
12. If incorporation is now > 90%, stop. If it is below 90%, repeat **steps 7–9** for another one or two rounds (*see* **Note 14**).
13. Filtrate the neurotrophin to a concentration appropriate for your application: usually about $(1–5) \times 10^6$ cpm/µL for transport studies. This can be done by spinning for the appropriate time (20–30 min) without adding buffer to the upper chamber

(*see* **Note 15**). The volume of the neurotrophin solution can be measured by pipetting (*see* **Note 16**).

3.3. Analysis

3.3.1. Calculation of the Specific Activity

The specific activity of the neurotrophin is calculated by multiplying the ratio of the iodide to neurotrophin in the reaction mixture by the fractionation of ^{125}I incorporated into protein or neurotrophin ***(4,6)***. The incorporation is determined by TCA precipitation (*see* **Note 17**).

Calculate the amount of neurotrophin present in the aliquots used for the determination of incorporation. Example: 2 µL (aliquot volume) × 1/50 (dilution factor) /140 µL (total volume) = 0.0002857 × 5 µg (input of neurotrophin) = 0.0014285 µg = 1.4285 ng (neurotrophin in aliquot). If the samples show 200,000 cpm/2 µL × 1/50 (dilution of aliquot) and the incorporation was 80%, the cpm/1.4285 ng neurotrophin = 80% × 200,000 cpm = 160,000 cpm. Thus, the specific activity of the neurotrophin is 160,000 cpm/1.4285 ng = 160 cpm/1.4285 pg = 112 cpm/pg. This is equivalent to 26 (kDa of NGF) × 112 = 2912 cpm/fmol. Because 100–150 cpm/pg is equivalent to approx 1 mol of ^{125}I/dimer neurotrophin, this preparation resulted in the incorporation of about 1 mol/dimer. The specific activity of the neurotrophin when given in mole form should be calculated for the dimer, because virtually all neurotrophin is present in dimeric form (*see* **Note 17**).

3.3.2. Examination of the Iodinated Neurotrophin by Gel Electrophoresis

In the initial phase of an iodination series, the neurotrophin preparation should be examined by SDS-PAGE using standard 15% or 20% SDS gels (*see* **Notes 18** and **19**).

1. Boil sample of about 10,000–20,000 cpm in gel loading buffer with 100 m*M* dithiothreitol (DTT) for 3 min.
2. Spin down and load along with known molecular-weight species (about 10–100 kDa) and run at 100–160 V for 3–4 h.
3. Stain with Coomassie brilliant blue, destain, dry in a vacuum-gel dryer onto filter paper, and expose to X-ray film for 4–12 h.
4. X-ray film can be scanned or analyzed by laser densitometry to determine the purity of the iodinated protein. Normally, >95% of the radioactivity migrates at 13–15 kDa (neurotrophin monomer). Typical examples are shown in **Fig. 1**.

3.3.3. Bioassay of the Iodinated Neurotrophin

There are several assays available to test the bioactivity of the iodinated neurotrophin (*see* **Notes 1** and **20**). Commonly, a dorsal root ganglion (DRG)

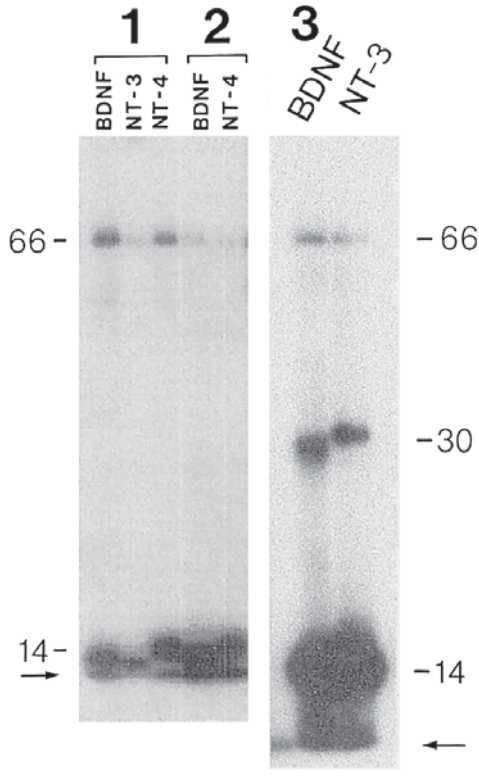

Fig. 1. Migration of iodinated neurotrophins on 15% SDS gels. About 100,000 cpm in 4–8 µL were loaded per lane. The Coomassie-stained gels were blotted to Whatman filter paper, dried, and exposed to X-ray film for 2–3 h. In addition to the major species of monomers (kDa of 14–15), some lanes contain a smaller fraction of labeled dimers (kDa of 30, series #3). Detectable amounts of ^{125}I were incorporated in albumin (kDa of 66), especially in those preparations that had an initial low incorporation into the neurotrophins, because of the use of 50 mCi/mL of ^{125}I (series #1) instead of 100 mCi/mL used in the other series. Initial incorporations in series #1 were 28–40.6% and, after membrane filtration, 73–83%; incorporations were 91.9–93.8% for the neurotrophins in series #2 and 85.9–91.1% in series #3. Molecular weights are shown; the arrows indicate the dye front.

assay is used ***(36,37)***. Alternatively, survival of trk-transfected fibroblasts ***(38)*** or receptor-mediated transport can be examined. Inject iodinated neurotrophin into a target known to contain axon terminals with receptors for the neurotrophin and quantify accumulation in the cell bodies after retrograde transport (e.g., transport from the anterior chamber of the eye to the superior cervical ganglion [NGF; **refs. *7–9*** and ***34***]; from muscle and skin of limbs for

transport to spinal cord and DRG ***(10)***; or from chick embryo eye for retrograde transport to the brain ***(29)***].

3.4. Delivery

Currently, Hamilton syringes (Hamilton, Nevada) are most widely used for the injection of iodinated neurotrophins. We find that for many applications, disposable syringes of the insulin type (e.g., Becton and Dickinson, 1/2 cc) can substitute for more expensive syringes to apply volumes as small as 2–15 µL ***(33)***. To confirm receptor-mediated transport (as opposed to nonspecific uptake and transport), control animals should receive a 500- to 1000-fold excess cold amount of the neurotrophin to compete for the binding to a limited number of receptors (*see* **Notes 21** and **22**). If transport is nonspecific, it will not be competed. Cytochrome-*c* can be iodinated and used as a control. Its transport (if it occurs) should not be competed by excess cold cytochrome-*c*. The injection site should be examined by gamma counting and/or autoradiography at least in some animals to determine if the intended target has been hit and to see if the injected amount remains or diffuses throughout or beyond the site (e.g., stereotactic apparatus may help with unsuccessful injections). For quantitation, it may be relevant to compare the transported amounts to the amounts that were successfully injected ***(15,29)***. To estimate the optimal survival time, measure the length of the pathway of interest. Calculate the time needed for transport: The transport speed is 2–13 mm/h in vivo ***(7–9,42)*** and 10–20 mm/h in vitro ***(12)***. Injected animals will excrete radioactive saliva, urine, sweat, and feces. Keep in an appropriate facility and dispose of excretions in radioactive waste (*see* **Note 23**).

1. Hamilton syringe: # 701 or 702 can be autoclaved (separate needle from syringe); draw from a 1.7-mL tube; inject. Maintain a good supply of extra needles, as clogging will occur.
2. Disposable syringes: Draw volume for injection in a 2- or 20-µL pipet, eject onto parafilm, and draw into disposable syringe; inject.
3. For competition experiments, measure as above; add excess cold; mix; draw in syringe; inject.

3.5. Autoradiography for Light Microscopy

Tissue can be embedded in paraffin for sectioning, frozen for cryosectioning (*see* **Note 24**), or embedded in plastic (*see* **Subheading 3.6.**).

1. Anesthetize the animal.
2. Perfusion-fix with 4% paraformaldehyde in PBS, pH 7.4.
3. Postfix for 2–8 h, dissect tissue of interest, and wash briefly in PBS.

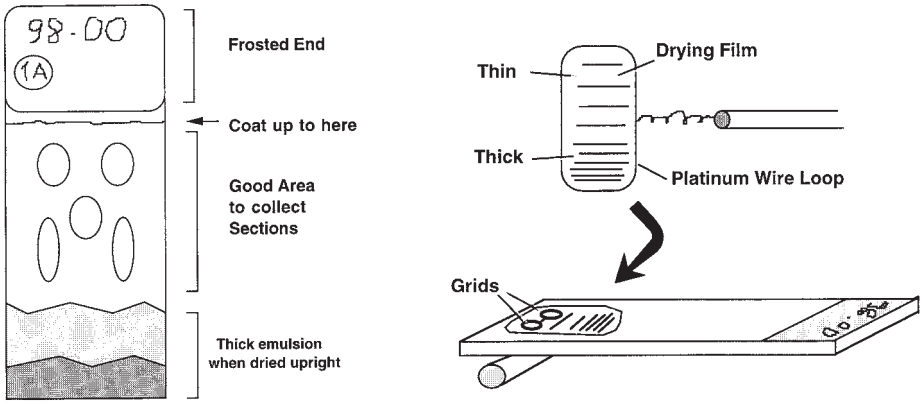

Fig. 2. Illustrations of where to collect tissue sections on slides for light-microscopic emulsion autoradiography (left panel) and where to place grids on slides for coating with emulsion using the platinum wire "loop" method for electron microscopy (right panel).

4. For paraffin embedding, keep tissue overnight in 70% ethanol and dehydrate for 45 min each in 95%, 95%, 100%, 100% ethanol, clearing agent (xylene or methyl salicylate) 100%, 100%, and three changes of paraffin (e.g., Paraplast Plus).

   For cryosectioning, cryoprotect in 20–30% sucrose in PBS overnight; freeze in optimal cutting temperature (OCT)-embedding compound.
5. Section tissue at 10 µm (paraffin), 10–20 µm (cryo), or 1 µm (plastic).
6. Collect sections in the center and upper parts of clean silane- or gelatin-coated slides (*see* **Fig. 2**). Dry sections for 6–12 h at 40–45°C. Collection of several sets of sections is recommended.
7. Deparaffinize the paraffin sections for at least 20 min in two changes of xylene (10 min each), then briefly place in 100% ethanol and air-dry. When dry, wrap the rack in plastic (e.g., Saran wrap) to keep dust-free.
8. One set of sections can be exposed to X-ray film (we routinely expose for 5 d) (*see* **Note 25**).
9. Warm a 1:1 dilution (with H_2O or 0.5*M* ammonium acetate) of NTB-2 or NTB-3 emulsion (*see* **Note 2**) in darkroom (red safe light only!) in a 42°C water bath. This is most easily done by using a 50-mL conical tube, adding 15 mL H_2O (or 0.5*M* ammonium acetate) and marking the 30-mL line with a thick black pen (easier to see in the darkroom). In the darkroom with the red safe light, open the container with the emulsion, scoop out (with a clean spoon) chunks of emulsion that will fit into the tube, and fill up until the level reaches 30 mL. Keep in the waterbath for 20–30 min until dissolved. Do not mix by shaking. Prevent generation of air bubbles. If desired, rock the tube gently to mix.
10. Dip clean test slides by slow emersion into the emulsion twice (about 1 s each dip), drain the bottom briefly (2–4 s) on a paper towel, and check the slides by holding at an angle against the safe light to evaluate the surface. If full of air

bubbles, wait longer (5–20 min). When only a few air bubbles are visible, the emulsion is ready for the dipping of slides with the sections. The quality of the emulsion should be checked occasionally on test slides (*see* **Note 26**).

11. Dip slides with sections in the emulsion by holding the frosted end with your finger tips (*see* **Note 27**). Some investigators prefer to handle slides with bare fingers (which facilitates in identifying the frosted end of the slide); others use gloves when coating slides. Drain the bottom of slides on a stack of paper towels and place the dipped slides in a rack (e.g., empty styrofoam racks for 50-mL tubes); load the racks systematically.
12. When the rack is full, place it in a humid chamber for 30–60 min to prevent drying artifacts ***(43)***.
13. Then, dry slides for another 45–60 min outside the chamber. Mark the darkroom prominently with warning signs if you plan to leave it during this time, to prevent other users from accidentially turning on the lights!
14. Place slides in light-tight slide boxes that have a package of silica gel (Drierite) wrapped in tissue (e.g., KimWipe) on one end, and tape the shut box with black electrical tape. Be careful when dispensing tape from the roll to prevent electrostatic sparks from forming, which could expose the emulsion of slides inside or in the vicinity outside the box. Complete darkness may reveal a burst of sparks not seen when the safe light is in use.
15. Wrap the box in at least one layer of aluminum foil and label it with the protocol number, the current date, and the date you plan to develop the slides in this box.
16. Place boxes at 4°C, away from any radioactive source.
17. Expose for 3–8 wk (different series of slides for, e.g., 4 wk and 6 wk) when examining the transported neurotrophin, and about 5–15 d for the injection site (*see* **Note 28**).
18. Prepare three clearly labeled staining dishes—one with developer (D19, Kodak), one with a "stop" solution (H_2O with 1% glacial acetic acid), and one with fixer (Fixer, Kodak; *see* **Note 29**). Make sure that the fluid level is sufficient to submerge all coated parts of the slides (e.g., 50-mL Coplin jars, 200-mL TISSUE TEK dishes, or 400-mL glass-staining dishes).
19. Place these dishes on crushed ice about 10 min prior to the actual development to reach a temperature of about 14–19°C (*see* **Note 29**).
20. Take slides out of the light-tight boxes in the dark room under a red safe light (remove the tape carefully to prevent sparks), and place them in racks that fit into the dishes.
21. Place the racks for 2 min, 30 s in developer, dunking two to three times to free air bubbles that may have been trapped.
22. Immerse for 30 s in the "stop" bath.
23. Transfer for 5 min to the fixer. Up to three racks with slides can be used without changing solutions. Once in the fixer, the lights can be turned on (make sure that all slides are accounted for and none are left outside).
24. Rinse the rack in H_2O for 10 min, followed by an extensive wash (30 min or more) under running tap water. Do not let slides dry out.

25. Sections now can be counterstained if desired (*see* **Note 30**), followed by dehydration in ethanol (70%, 95%, 95%, 100%, 100%, 3–5 min each), and cleared in xylene 100%, 100% (5 min each) for cover-slipping. We recommend DPX for cover-slipping. Make sure that the sections do not dry out prior to cover-slipping, they should be wet from xylene when the DPX (or other cover-slipping agent) is applied (*see* **Notes 31** and **32**).
26. Scrape off the emulsion on the back side of the slide with a clean razor blade. Removing all the emulsion from the back is advised because pieces of the emulsion will attract dust and dirt and will make it difficult to get clean pictures in the dark field (**Fig. 3a,c**) (*see* **Notes 33** and **34**).

3.6. Autoradiography for Electron Microscopy

1. Perfuse and fix animals with 1.5% glutaraldehyde (EM grade) and 2% paraformaldehyde in 0.1*M* cacodylate buffer, pH 7.4.
2. Trim the tissue of interest to pieces of about $2 \times 2 \times 1$ mm in the fixative. Prepare one nonradioactive control piece of tissue in parallel to determine the level of background.
3. Immersion-fix for 90 min in the same fixative.
4. Count samples in the gamma counter. (They should have levels of radioactivity at least 10–20 times higher than background; *see* **Note 35**).
5. Wash three times for 15 min each in 0.1*M* cacodylate buffer, pH 7.4.
6. Postfix in 1% OsO_4 in 0.1*M* sodium cacodylate buffer for 60 min (use fume hood and gloves; osmium is very toxic).
7. Wash three times for 10 min each in H_2O.
8. Dehydrate 15 min each in 50%, 70%, 95%, 100%, 100% ethanol.
9. Dehydrate two times for 15 min each in propylene oxide (100%).
10. Infiltrate 30 min in 1 part Spurr's and 2 parts propylene oxide (*see* **Note 36**).
11. Infiltrate 30-60 min in 1 part Spurr's and 1 part propylene oxide.
12. Overnight infiltration in 2 parts Spurr's and 1 part propylene oxide.
13. Infiltrate in fresh Spurr's for 8 h.
14. Flat-embed and put in +60°C oven overnight. Trim area from hardened block for analysis.

Fig. 3. *(see facing page)* Neurons containing radiolabeled neurotrophins after retrograde transport shown in the dark field **(a, c)** and the bright field **(b, d, e)**. **(a)** Dark-field view of five heavily labeled oculomotor neurons after injection of ^{125}I BDNF into the medial rectus muscle of a 15-d-old chick embryo. Survival time = 20 h; exposure time = 6 wk. **(b)** Two of the labeled neurons are shown at high magnification in the bright field. **(c)** Dark-field view of ectopic (boxed, bottom) and orthotopic isthmo-optic neurons (upper right, ION) after injection of ^{125}I NT-3 into the eye of 15-d-old chick embryos. Survival time = 20 h; exposure time = 6 wk. Note that several ectopic isthmo-optic neurons (arrows) are clearly visible in the dark-field image. **(d)** Bright-field view of the four neurons indicated with arrows. Note that they are not as clearly distinguished

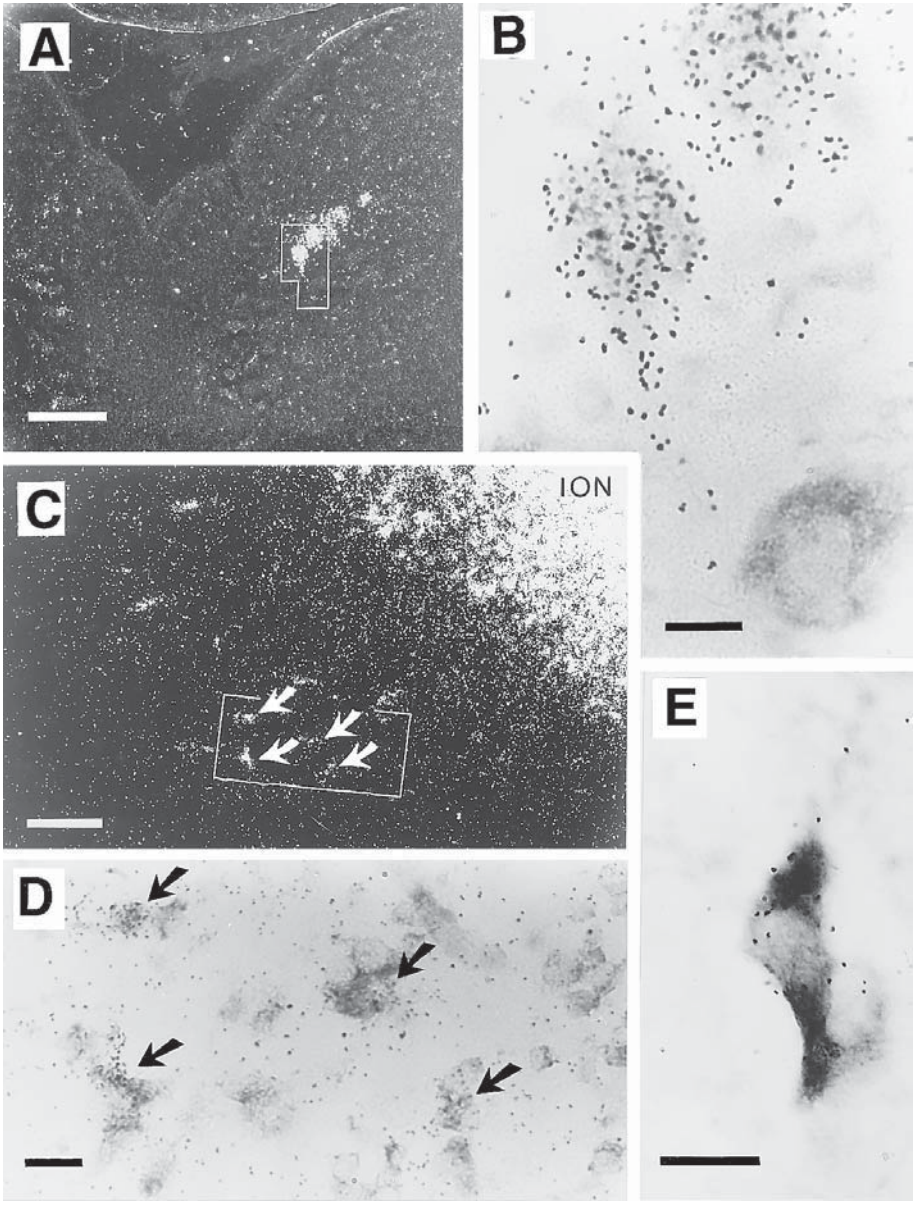

Fig. 3. *(continued)* from background grains in the bright-field view. **(e)** Bright-field view of a noradrenergic locus coeruleus neuron double-labeled with ^{125}I NGF after retrograde transport from an injection site in the telencephalon of a 17-d-old chick embryo, and immunolabeled with an antibody to dopamine-β-hydroxylase. Survival time = 20 h; exposure time = 6 wk. Scale bars = 100 μm (a, c), 10 μm (b, e), and 20 μm (d).

15. Cut semithin sections (1 µm) to evaluate tissue quality. Collect some 1-µm sections onto clean glass slides and process for autoradiography in parallel with thin sections. This also helps for the orientation within the thin sections (*see* **Note 37**).
16. Cut thin sections (95 nm) and collect on uncoated mesh grids (*see* **Note 38**).
17. In a darkroom with a low-intensity, monochromatic yellow safe light or red safe light, dilute the emulsion (Ilford L4):H_2O at a ratio of 2:3 in a 50-mL tube in a water bath at 38–40°C. Allow several hours for thorough melting of emulsion. Seal the tube containing the emulsion with parafilm to prevent drying out and mix gently (*see* **Note 34**).
18. Place grids on slides (two to three grids per slide; section side up, near one end of slide; **Fig. 2**) and label the other, frosted end of slide with pencil (this is best done outside the darkroom).
19. Elevate grid end of slide using a glass rod (**Fig. 2**).
20. Reduce the temperature of the water bath to 32°C and dip a platinum wire loop (1 × 3-cm loop size) in emulsion ***(44)***, carefully lift out keeping film oriented vertically, drain excess emulsion on wet filter paper, and let the upper (thin) end begin to harden (becomes shiny). This takes 20–40 s. Apply film to grids on a slide by tipping it carefully until it is parallel to the slide and close to the grids. Aim to put an area of a thinner film over the grids (**Fig. 2**). Film must be partly hardened to be uniform over the grid, but not completely dry so that it will "jump" over to grids (*see* **Note 3**).
21. Clean the wire loop in water and reflatten between slides (using two clean slides).
22. Let films dry for 10–15 min. Dried emulsion should hold the grids to the slide for **steps 23–27**.
23. Place slides in light-tight slide box with silica gel (Drierite) package. Tape shut, wrap in aluminum foil, and label the box; place in a refrigerator at 4°C.
24. Expose for 6–12 wk.
25. To develop, carefully immerse the slide with grids in the following solutions: for 75–90 s in D19 developer (Kodak, stock strength, filtered) in a Coplin jar at room temperature. Rinse in double-distilled H_2O.
26. Fix for 3 min in Rapid Fixer (Kodak) (filtered).
27. Wash for 5 min gently in running H_2O.
28. Pick up each grid with forceps and immediately immerse in a drop of fresh lead citrate ***(40)*** on parafilm for 5–15 min (*see* **Note 4**). This adds electron density to the section by staining from below through the open mesh grid and helps to "clear" the emulsion so that it is translucent in the electron microscope (*see* **Note 40**).
29. Wash by dipping each grid in three small beakers with double-distilled H_2O for approximately 5 s per rinse.
30. Drain grids on filter paper and dry on lens paper (e.g., Ross).
31. File in a grid box.
32. Examine in a transmission electron microscope. Identify grains by their "wormlike" shape (**Fig. 4**). They can be seen at × 6100 magnification and distinguished from artifacts. Take pictures at × 6100 magnification or higher (*see* **Notes 41** and **42**).

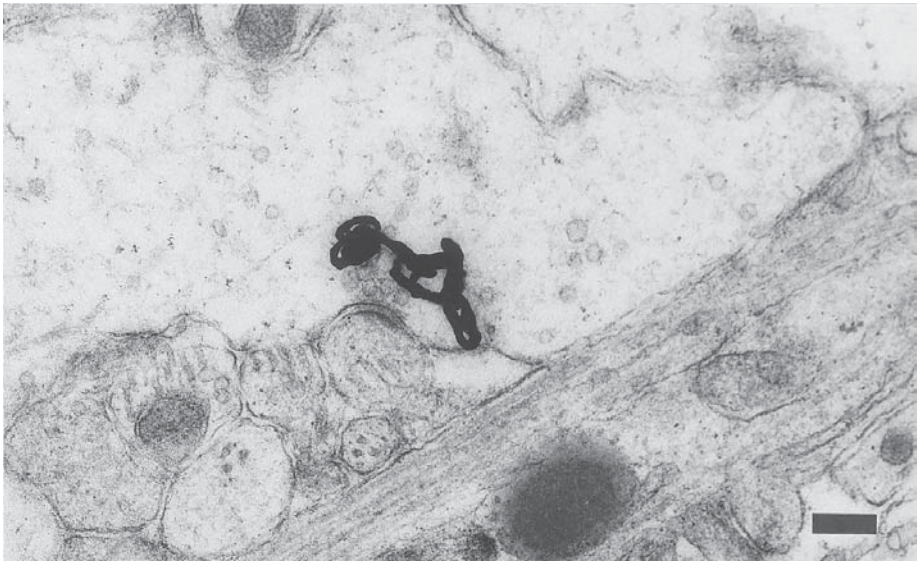

Fig. 4. Silver grains indicative of ^{125}I NT-3 in a retinotectal synapse after anterograde transport from the eye in a 16-d-old chick embryo. Exposure time = 3 mo. Scale bar = 200 nm. (From **ref. *15***, with permission.)

33. For quantitative analysis, use a probability distribution circle. Center a circle with a diameter corresponding to approx 170 nm around each grain and score all organelles that fall within this circle. A 50% circle has a 50% probability of containing the radioactive source responsible for the grain ***(11,15,45)***.

4. Notes

1. Tests for bioactivity have shown that lactoperoxidase-iodinated BDNF can have significantly reduced bioactivity ***(23,25)***. Other investigators, however, reported only a slight loss of bioactivity ***(10,27–29,48)***. The critical factor appears to be the ratio of ^{125}I to neurotrophin and the efficiency of the reaction. When high ratios are used in an efficient reaction, many BDNF dimers will have both tyrosine residues iodinated at position 86 ***(23)***. This renders a loss of bioactivity of one magnitude for the "dead" BDNF. With lower ratios, however, most of the iodinated dimers will not have both monomers iodinated at position 86, and the loss of bioactivity is minor (9–17%). The loss of bioactivity appears to require the iodination of both tyrosine residues at position 86, a residue which may be involved in trk receptor binding ***(23,26)***. Analysis of subsequent reports shows that the loss of bioactivity would be substantial if *one* iodinated tyrosine at position 86 per dimer was sufficient to reduce bioactivity. Sixty-one percent of BDNF dimers have at least one tyrosine residues iodinated at position 86, but only 14%

would have both tyrosine 86 iodinated, assuming independent probabilities of tyrosine iodination and assuming the following values for the four types of monomers: 50% for peaks P1, 12.5% for P2, 25% for P3, and 12.5% for P4 (compare with **Fig. 1** of **ref. *23***). The reported loss of bioactivity ranging from 9–17% ***(10,29)*** is consistent with 14% of the iodinated BDNF being inactive, but not 61%.

2. Kodak recommends NTB-3 emulsion for ^{125}I because it produces larger silver grains; however, most investigators use NTB-2. My comparison of the two emulsion types with adjacent sections coated with NTB-2 and NTB-3 did not reveal visibly increased silver grains with NTB-3.
3. The gage of the platinum wire is critical to making films of the correct thickness: too thin films break; too thick wires do not produce monolayers of emulsion ***(44)***.
4. Freshly prepared Reynolds lead citrate stain is always clean and worth the extra 30 min to assure dirt-free grids.
5. When several proteins are iodinated, it helps to color code the tubes.
6. Once the ^{125}I has been pipetted, the finger tips of gloves easily become contaminated by opening and closing the tubes. Gloves should be monitored after each handling of hot tubes and when contaminated, they should be discarded in the radioactive waste. Changing gloves is much easier when double-gloved.
7. To speed up the addition of the lactoperoxidase/H_2O_2 mix, separate tubes can be prepared with H_2O in which the lactoperoxidase and H_2O_2 are diluted: a tube with 495 µL of H_2O for the 1 : 100 dilution of 5 µL H_2O_2 and a second tube for the further dilution of 5 µL to 1 : 10,000 (495 + 5 µL). The second tube can then be mixed with a third tube containing the 5-µL aliquot of lactoperoxidase diluted in 495 µL water.
8. Some investigators carry out the iodination at 4°C ***(2)*** or 0°C ***(5)***, or for less than 10 min ***(4)***. Some vortex the sample ***(4)***. We find that gentle pipetting (one or two strokes) is sufficient.
9. We use 0.4 mCi ^{125}I for 5 µg neurotrophin (= 0.08 mCi/µg). Some investigators use a higher ratio of ^{125}I to neurotrophin [e.g., 0.85 mCi/µg NGF ***(4)*** or 0.17 mCi/µg NGF ***(5)***]. For reasons detailed in **Note 1**, high ratios are not recommended for the iodination of BDNF. We find that 100 mCi/mL yields more than twice the incorporation (75–95%) compared with the dose of 50 mCi/mL (30–45%, with this protocol).
10. Some investigators use matrix-bound lactoperoxidase. Because the enzyme cannot diffuse freely, this method is thought to be less efficient ***(22)***. Indeed, higher ratios of ^{125}I to neurotrophin [e.g., 0.75 mCi ^{125}I/µg neurotrophin rather than 0.08–0.20 mCi ^{125}I /µg] appear to be necessary to achieve comparable specific activities ***(27)***.
11. None of the reagents used for the iodination should contain sodium azide, as this inhibits the reaction ***(31)***.
12. The PB molarity is not critical; we have successfully iodinated neurotrophins with a 10-fold higher molarity.
13. Low incorporation can be the result of insufficient mixing of the iodination reaction (e.g., by having a droplet with ^{125}I remaining on the tube wall). We usually

get incorporations of 40–70% for NGF and 75–95% for BDNF, NT-3, and NT-4. It can be worth keeping or ordering one "backup" volume of ^{125}I in case one of the reactions yields low incorporation; thus, it can be repeated immediately.

14. We find that when the initial incorporation is less than 20%, it is usually not worth the time trying to purify the iodinated neurotrophin. When analyzed on SDS-PAGE, most of the counts are incorporated into BSA and lactoperoxidase rather than the neurotrophin.
15. When separating the free iodine by membrane filtration or concentrating the neurotrophin, one should avoid running the membrane dry, because this may result in a loss of neurotrophin by sticking to the membrane when reconstituted.
16. The microfuge will become lightly contaminated during purification; the fuge can be simply kept in the hot fume hood until the radioactivity is decayed or it can be cleaned up after use.
17. The calculation of the specific activity by TCA precipitation is not totally exact, but an approximation. Inaccuracies arise because of the estimation of input neurotrophin (pipetting error, loss by sticking to pipet tips and tubes). Inaccuracies also arise because of the gamma counter efficiencies and variabilities in the TCA precipitation and incorporation into lactoperoxidase and BSA.
18. The iodination protocol will also result in some iodination of the BSA (kDa = 66) and lactoperoxidase (kDa = 77) ***(46)***. BSA may be visible on the SDS gel, especially when the incorporation into the neurotrophin is low (**Fig. 1**).
19. Not all of the neurotrophin dimers may be separated into monomers by boiling in the presence of dithiothreitol (DTT); some dimers of the iodinated neurotrophin may persist or reaggregate (**Fig. 1**).
20. Because neurotrophins bind with different epitopes to p75 or trk receptors ***(47)***, binding to one or the other receptor type can be differentially impaired. In vitro assays may not detect functionally important deficits in axonal transport ***(13)***, whereas transport studies with neurotrophins that bind primarily to p75 neurotrophin receptors during axonal transport [e.g., NT-4 ***(49)***] may not detect deficits in the activation of trk receptors. Furthermore, mutant neurotrophins can retain high levels of receptor binding, but show low biological activity ***(49)***.
21. When calculating the dose of neurotrophin delivered, it has to be considered that neurotrophins are very sticky molecules ***(50)***. A significant fraction of the protein may be stuck to pipet tips, needles, and "dead space" in syringes. The effective doses of the hot neurotrophin (and the excess cold) thus may be lower.
22. Doses delivered for axonal transport usually are about $(2–10) \times 10^6$ cpm in volumes of 0.5–10 µL ***(29,48)***.
23. There is no need to keep the neurotrophins sterile for experiments with < 24 h survival. For long-term survival, sterile techniques may be necessary.
24. Paraffin or plastic embedding preserves the morphology better than cryoembedding. Dehydration prior to embedding may also remove free iodine or degradation products. In cryosections, especially when used for double-labeling procedures, the signal tends to be less clean than with paraffin sections. Dehydra-

tion may remove some of the background radioactivity, but leaving the accumulated neurotrophin behind, especially in cellular structures.

25. Sections can be exposed to X-ray film to verify radioactivity in the tissue, but they do not match the sensitivity and resolution achieved with the emulsion autoradiography. Quantification of signals on X-ray film is problematic, unless all samples are exposed to the same film, because the results vary from one exposure to the next much more than with emulsion autoradiography.
26. Emulsion batches should be routinely tested both for sensitivity and for background. This is done by coating two blank slides, letting the emulsion dry, and then light-flashing one slide (by carrying it out of the darkroom), and developing both slides. The light-flashed slide should have a smooth black emulsion, whereas silver grains on the background test slide should be within standard limits ***(43)***.
27. To save emulsion, slides can be dipped back to back. This practice bears the risk of coating the wrong side of the slide.
28. The difference in grain accumulation between 4 wk and 6 wk exposure is visible, but not tremendous. Differences between 6 wk and 8 wk are barely visible. Remember that ^{125}I has a half-life of 60 d.
29. Instead of the Kodak Fixer, some researchers develop the emulsion in Rapid Fixer. Also, some investigators develop, stop, and fix the slides at room temperature.
30. Do not counterstain too heavily if you want to observe the silver grains at dark-field illumination. Although it takes longer for the counterstain to penetrate the emulsion, overstaining can occur.
31. Artifacts can form in the coating/developing/cover-slipping process. Sometimes, they are mysterious, but they may correlate with drying of sections after coating or during cover-slipping. We avoid air-drying of sections during cover-slipping.
32. We find that DPX is a much cleaner mounting medium than Permount.
33. Lightly labeled cells are more readily visible (and documented) in the dark field than in the bright field (**Fig. 3c,d**). One has to use high magnification to make silver grains visible (and impressive) in the bright field, unless a cell is heavily labeled and the background is particularly clean (**Fig. 3a,b**). Oil-immersion objectives with a long focal plane can be used to obtain a sharp resolution of both section and overlying silver grains.
34. Sections can be immunolabeled and then dehydrated and dipped in emulsion as described. Cryosections ***(48)*** as well as paraffin sections ***(51)*** can be double-labeled (**Fig. 3e**).
35. At least a 10-fold higher than background level of radioactivity in the samples is necessary for successful autoradiography at the ultrastructural level.
36. Araldite/Spurr, a different set of resins, has been used with similar success for ^{125}I–neurotrophin autoradiography (M.R. Byers and C.S. von Bartheld, unpublished).
37. Test slides with 1-µm sections can be coated in Ilford L4 emulsion and developed as described above (**Subheading 3.5., steps 18–26**). The 1-µm sections are helpful to locate the radioactivity in the thin sections and can serve as a reference for orientation within the thin sections. They also cover a wider tissue area than can be sampled in the electron microscope.

38. One will always lose some grids during development and, therefore, should have several "extra" slides with grids.
39. The temperature of the emulsion is critical for achieving a monolayer of emulsion for the grids. The film quality can be tested by applying the semidry film to a blank glass slide and then viewing its interference colors outside the darkroom. Usually, one must make many films before the correct broad band of the primary red–green zone is consistently achieved (monolayer zone; for details, *see* **ref. *44***).
40. We do not stain the sections with uranyl acetate, as it is unnecessary and often causes dirt and other artifacts.
41. It can be difficult to identify how many silver grains are contained in a cluster of three or more. Examine several "singles" to get a feel for their size, shape, and length.
42. On the scope, it can be more difficult to distinguish some dirt artifacts from silver grains. It helps to go to a higher magnification. On prints, it is much easier to distinguish details, even at a lower magnification.

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15

Toxin-Induced Death of Neurotrophin-Sensitive Neurons

Ronald G. Wiley

1. Introduction

Selective destruction of neurons based on the use of targeted toxins has proven successful for several types of neurons ***(1)***. This chapter will describe the use of an immunotoxin to selectively destroy rat neurons that express the low-affinity neurotrophin receptor ($p75^{NTR}$) ***(2)***. This immunotoxin consists of a monoclonal antibody disulfide coupled to a ribosome inactivating protein. The most extensively used and studied version uses the antibody 192 IgG originally developed as an antibody to a rat NGF-binding protein ***(3)***. 192 IgG has been extensively used to study rat $p75^{NTR}$. Results of these studies have demonstrated $p75^{NTR}$ expression on a variety of neurotrophin-responsive cells, including sympathetic ganglion neurons, some primary sensory neurons, and cholinergic neurons of the basal forebrain. $p75^{NTR}$ also is expressed on cells not known to be responsive to neurotrophins, such as cerebellar Purkinje neurons and numerous other tissues during development ***(4)***. Thus, producing selective lesions using 192 IgG also requires restricting application of the immunotoxin to the region of the target cells.

The use of targeted toxins to make highly specific neural lesions, nicknamed "molecular neurosurgery" generally relies upon arming selective targeting vectors with a ribosome-inactivating protein (RIP). The targeting moiety is required to mediate binding to the target cell surface and endocytosis of the toxin conjugate. Once inside a cell, the RIP must escape the endosome and gain access to the cytosol, where it catalytically inactivates the large ribosomal subunit ***(5)***. After about 10% of ribosomes are inactivated, protein synthesis fails and the cell goes on to die by apoptosis. It is likely that one molecule of

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the RIP free in the cytoplasm is sufficient to kill a cell ***(6)***. Several RIPs have been used to make targeted toxins ***(7,8)***. Saporin is a RIP purified from the seeds of *Saponaria officinalis* ***(9)***. As mentioned later, saporin is the RIP of choice because of its superior in vivo activity.

Various approaches have been used to achieve selective targeting, including immunotoxins (monoclonal antibodies conjugated to RIP [i.e., 192 IgG–Saporin ***(10)***], peptide–RIP toxin conjugates [substance P–Saporin] ***(11,12)***, or neurotrophin–toxin conjugates or fusion proteins (NGF–Diphtheria toxin ***(13)***). Some of the requirements for such toxins include the following:

1. The targeting vector must retain selective binding to the cell-surface target molecule when conjugated to RIP.
2. The targeting moiety must mediate efficient endocytosis after selectively binding to the surface of the target cell.
3. The targeting vector and RIP must be able to part company inside the cell, which usually means reduction of a disulfide bond.

Initial studies using immunotoxin consisting of 192 IgG coupled to the ricin A chain were disappointing ***(14)***. However, intraventricular injections of 192 IgG into rats clearly showed selective uptake into cholinergic neurons of the basal forebrain ***(15,16)***. Because saporin conjugates to the anti-Thy 1 monoclonal antibody, OX7, were shown more effective in vivo than ricin A conjugates ***(17)***, we made 192 IgG–Saporin to selectively destroy the rat cholinergic basal forebrain. Subsequent experience with 192 IgG–Saporin has repeatedly confirmed the efficiency and selectivity of the immunotoxin in vivo for rat neurons expressing $p75^{NTR}$ ***(9)***.

192 IgG–Saporin can be applied in several ways to make neural lesions, including stereotactic pressure microinjection into brain parenchyma (most selective), intracerebroventricularly, into specific peripheral organs such as salivary gland or intravenously (least selective). This chapter describes use of 192 IgG–Saporin in rat brain (192 IgG is specific for rat $p75^{NTR}$). This chapter describes a simple method for assessing the lesion made with 192 IgG–Saporin in vivo, an essential part of using targeted toxins to make neural lesions. 192 IgG–Saporin and related toxins also are very useful in vitro for eliminating specific cell types from mixed cultures.

2. Materials

1. 192 IgG–Saporin: Commercially available from Advanced Targeting Systems, San Diego, CA in sterile phosphate-buffered saline (PBS). A protocol for making the immunotoxin has been published elsewhere ***(18)***. Keep sterile in aliquots stored frozen until use and keep aliquots on ice after thawing. Minimize repeated freezing and thawing. Add Fast Green dye to a final concentration of 0.1% (w/v) to permit visualization of injections and leaks.

2. Stereotactic apparatus: Widely used rat version available from David Kopf Instruments, Tujunga, CA. Use electrode carrier to hold injection pipet.
3. Glass micropipets: Initially pulled on a standard micropipet puller such as made by David Kopf using volumetric micropipets (Clay Adams, Accu-Fill 90, #4614). For typical intraparenchymal injection, 5-µL pipets are suitable. Before use, the tip is broken back to 25–50 µm outside diameter using microscissors under a dissecting microscope.
4. Polyethylene tubing: Use two sizes, PE20 in lengths of 12–18 in. and PE160 in 0.5- to 1-in. segments. First, glue the PE160 to the back of a micropipet using fast epoxy cement; then, glue the PE20 to the PE160 in the same fashion. The PE160 is used as a cuff or connector to join the glass micropipet to the PE20.
5. Sterile water for injection. Fill the tubing and pipet with water; then, attach a water-filled 10-µL Hamilton microsyringe. Purge all bubbles and then draw back about 0.5 µL of air into the pipet tip before drawing up toxin. This bubble will serve to separate the toxin solution from the water in the pipet. During injection, movement of the lower meniscus indicates the amount injected. The Accu-Fill pipets are marked in 1-µL increments.
6. Nitrite–heparin–saline: Prefixative wash consisting of 100–200 mL of phosphate-buffered normal saline containing 1 g/L sodium nitrite and 500 units/L heparin sodium.
7. Formaldehyde fixative: Stir 40 g paraformaldehyde into 500 mL deionized water heated to 70°C and add 1*N* sodium hydroxide dropwise until clear, mix with 500 mL of 0.2*M* sodium phosphate buffer (pH 7.6), cool, and vacuum filter before use.
8. Sucrose solution: Dissolve 30 g of sucrose into a mixture of 1 mL of 0.5 *M* sodium phosphate buffer (pH 7.6) and deionized water to make 100 mL.

3. Method

1. Anesthetize the rat by intraperitoneal injection of a mixture of acepromazine (3 mg/kg) and ketamine (150 mg/kg).
2. Position the rat in the stereotactic, shave the head, and drill a burr hole at the appropriate location. Typical coordinates from bregma for the medial septum are AP 0.4 mm, lateral 1.5 mm, vertical –7.6 mm below the dural surface at 12° from vertical and for the nucleus basalis AP –0.3 to –0.6 mm, lateral 2.8 mm, Vertical –7.5 mm below the dural surface.
3. Using an electrode holder, lower the pipet to the correct position and slowly inject 100 nL to 1 µL containing 50–200 ng of 192 IgG–Saporin (*see* **Note 1**). The precise dose depends on the extent of lesion and other factors best determined in the individual laboratory by pilot experiments (*see* **Note 2**).
4. Allow animals to recover for 10–14 d. Binding and uptake of the immunotoxin is rapid (minutes), but neurons continue to function for 1–3 d after toxin injection ***(1)*** (*see* **Note 3**). Resolution of the histological reaction to the lesion takes 10–14 d (*see* **Note 4**).
5. At the conclusion of any behavioral testing, sacrifice the animals using high-dose pentobarbital (100 mg/kg, ip). Before the rat stops breathing,

perfuse transcardially with nitrite–heparin–saline until clear, followed by formaldehyde.
6. Remove the brain from the skull after fixation and place in sucrose overnight at 4°C.
7. Section the brain at 40 μm on a freezing sliding microtome-collecting sections in PBS.
8. Mount sections on gel-coated slides, dry and stain with acidic cresyl violet dye, then dehydrate, clear in xylene, and cover-slip (*see* **Note 5**).
9. A lesion will be evident even within 1–-2 d because Saporin causes prompt and dramatic chromatolysis (dissolution of Nissl substance), which abolishes cresyl violet staining of the neuronal cytoplasm even though the nucleus may remain visible for several more days (*see* **Note 6**).
10. Immunohistochemical stains may be applied to sections prepared as above to assess loss or preservation of markers known present on the target cells (i.e., $p75^{NTR}$) or restricted to nontarget cells, respectively (*see* **Note 7**).

4. Notes

1. The immunotoxin requires care to avoid microbial contamination. It comes sterile and should be initially aliquoted using sterile technique. When aliquots are thawed for use, keep them on ice. The most common reason for failure to obtain a lesion with adequate injection technique is contaminated toxin.
2. Immunotoxin can be applied either directly to cell bodies, dendrites, or axon terminals because $p75^{NTR}$ is on all surfaces of the neuron. If applied into a terminal field such as neocortex, a lag of several hours will occur before toxin arrives in the cell body. Transport of immunotoxin to the cell body involves fast axonal transport and can be blocked by microtubule toxins such as colchicine or vincristine ***(19,20)***.
3. Optimum results with intraparenchymal injections require attention to immunotoxin concentration. Injections of 100–200 ng in 100 nL to 1 μL have worked consistently in several laboratories. High concentrations may result in nonspecific, bulk, fluid-phase endocytosis at the injection site that is independent of specific receptor binding. If this happens, a necrotic lesion forms at the injection site, affecting many cell types including glia.
4. Physiologic experiments often require measurements at various times after toxin injection to separate the direct effects of failure of the targeted neurons from the secondary effects, such as plastic remodeling of surviving neural systems. Injection of 192IgG–Saporin into the medial septum caused progressive decrease in the hippocampal theta amplitude beginning on the third day after injection reaching a maximum effect by the seventh day ***(21)***. Secondary effects seem to show even longer interval to onset ***(22)***.
5. Fast Green dye in the toxin solution is very helpful in revealing the spread of toxin at the injection site. This is useful when injecting superficial sites such as neocortex or peripheral targets such as nerves, ganglia, or parenchymal organs.
6. Appropriate control experiments include injecting a mixture of unconjugated antibody and Saporin which can be achieved by incubating 192 IgG–Saporin in

20 m*M* dithiothreitol for 1 h at room temperature prior to injection. Other controls may include injection of only antibody, only Saporin, or an irrelevant immunotoxin.

7. Assessment of the lesion by immunohistochemistry is best done 10–14 d after toxin injection to allow clearing of cellular debris that may stain for the marker of interest. In the cholinergic basal forebrain of rats, staining for $p75^{NTR}$ and choline acetyltransferase begin to change after about 72 h and gradually disappear over the next week. A mononuclear infiltrate is present over the same period (3–10 d after injection). By 14 d, little visible change is evident in the target except loss of the target neurons. The mononuclear infiltrate is gone and astroglia do not change significantly ***(23)***.

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Oligonucleotides as Inhibitors of Protein Synthesis

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1. Introduction

Duplex formation between an oligonucleotide and a strand of mRNA can effectively inhibit the expression of a specific gene via interfering with the cellular protein synthesis process. The antisense technology ***(1)*** is regarded as a powerful tool in molecular biology. Advances in the field have also led to the development of many pharmaceutical/therapeutic applications, with a number of oligonucleotides undergoing human clinical trials at present ***(2)***. Making the best use of this approach requires a good understanding of the subject, careful planning, a knowledge of limitations and pitfalls, and a capacity to go beyond the basic antisense techniques.

Antisense technology is now widely used as a research tool in the neurosciences. Among the recent examples of neurotrophins and receptors on which antisense oligonucleotides have been used include nerve growth factor (NGF) ***(3,4)***, brain-derived neurotrophin factor (BDNF) ***(4)***, NT-3 ***(4)***, TrkA ***(5)***, TrkB ***(6)***, and $p75^{NGFR}$ ***(7)***. In this chapter, we will describe our efforts at using the antisense technology in investigating neurotrophins and their receptors. In our investigations, we have found the need to use modified oligonucleotides with more favorable properties than customary unmodified phosphorothioate oligonucleotides. Techniques described are not specific for neurotrophins only; they are broadly applicable to antisense investigations of neuronal cells, and we have concentrated on describing the information that is essential to carry out antisense studies irrespective of the precise gene that is being targeted. The methodology section is divided into three parts. The first section (**Subheading 3.1.**) discusses the factors to be considered in planning antisense experiments. The second part (**Subheading 3.2.**) is on the synthesis of the oligonucleotides and strategies to improve the efficacy of antisense action. This describes the

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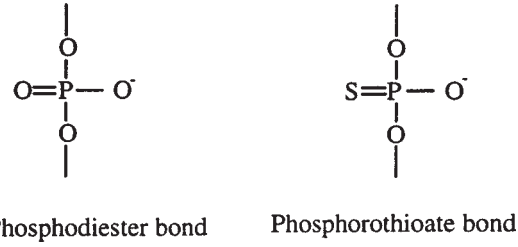

Fig. 1. Phosphorothioates.

synthesis of phosphorothioates, mixed-backbone second-generation oligonucleotides, and improving cellular uptake by conjugating to poly-L-lysines, cholesterol groups, and complexing with cyclodextrins. The third section (**Subheading 3.3.**) deals with the measurement of antisense efficacy.

1.1. Determinants of Antisense Action

Antisense action depends on a number of factors. Among them are the nuclease stability of the oligonucleotide, cellular uptake, affinity and specificity toward the target sequence, and the ability to inhibit the expression of the gene. Phosphodiester oligonucleotides are rapidly degraded within the cell, mainly because of 3′ exonuclease activity. In phosphorothioates (**Fig. 1**), one of the oxygen atoms of the phosphodiester bond is replaced with a sulfur atom, which increases its stability toward nucleases. However, the presence of sulfur also increases non-sequence-specific effects of the oligonucleotide *(8)*. Reaching the target sequence is a major problem in antisense usage. Although both phosphodiester and phosphorothioate oligonucleotides are internalized to an extent through endocytotic mechanisms, most of the oligonucleotides end up trapped in endosomes or lysosomes *(9)*. Thus, the amount of oligonucleotides available to the target may be merely the fraction that escapes or leaks from the endosomes. The affinity of the oligonucleotide toward the target mRNA is also important in increasing antisense effects: As the affinity increases, duplexes become more stable and there is a greater chance for mechanisms that inhibit gene expression to be operative. The inhibition of gene expression basically occurs through two mechanisms: the RNase H cleavage mechanism and translational arrest through steric blocking. The RNase H degrades the RNA part of the RNA–DNA duplex and is considered to be the major antisense mechanism. Physical blocking of the translation is not as effective as the duplex formation is reversible and thus it does not permanently remove the mRNA from the system. The phosphorothioate backbone is the only known synthetic backbone that does not block RNase H activity.

2. Materials

2.1. DNA and Organic Synthesis Reagents

1. All the DNA synthesis reagents are available from Perkin-Elmer (Australia) or Glen Research (Sterling, Virginia, USA). Long-chain alkyl amine controlled-pore-glass (CPG) solid support (pore size 500 Å) was obtained from Sigma (Castle Hill, Australia).
2. Oligonucleotide purification cartridges used for purification and desalting were obtained from Perkin-Elmer (Australia). NAP columns used for desalting were obtained from Pharmacia Biotech (Australia).
3. DNA syntheses were carried out on an Applied Biosystems model 392 synthesizer.
4. All the organic synthesis reagents were obtained from either Sigma or Aldrich (Castle Hill, Australia). Flash chromatography ***(10)*** for the purification of organic compounds was done using flash-chromatography-grade silica gel (particle size 40–63 μm) obtained from BDH Lab Supplies (Poole, UK).
5. Proton nuclear magnetic resonance (NMR) spectra were obtained on a Varian 400-MHz NMR spectrometer.

2.2. HPLC Conditions and Buffers

A Waters 510 high-performance liquid chromatography (HPLC) system equipped with a Waters 486 detector and a U6K injector, and controlled by a Beckmann System Gold software package was used for separations. Analyses were done on a Phenomenex Jupiter C-18 (250 × 4.6 mm) reverse-phase column; purifications were done on a Phenomenex Jupiter C-18 (250 × 10 mm) reverse-phase column. Solvent systems used were acetonitrile and 0.1*M* triethylammonium acetate.

3. Methods

3.1. Conducting Antisense Experiments

Antisense experiments are well suited to in vitro experiments on cell lines and primary cell cultures. We have found them to be much less effective in experiments using thick brain-slice explants, probably because of poor permeation of tissue barriers. Even in the much more favorable setting of monolayer cell cultures, great care is needed to ensure that the oligonucleotides work as planned. Successful use of antisense oligonucleotides in one cell type does not accurately predict success in a different cell type. In particular, for any new combination of oligonucleotide chemistry and cell type, cellular uptake must be verified. Phosphorothioate (PS) oligonucleotides have been found to be efficiently taken up by a number of cell lines, including HeLa, V79 cells, H9 leukemic cells ***(11)*** and fibroblast-derived cell lines ***(12)***. They have also been shown to be taken up by peripheral blood monocytes ***(11)***. Although uptake may be impressive in numerical terms, efficacy may be poor because the oligo

is principally sequestered in endosomes. Many workers have successfully used lipofection to increase the effective uptake in such cases. However, lipofection agents are notoriously fickle for cell types and culture conditions (such as the presence of serum). We have found that phosphorothioate (PS) oligonucleotides are readily taken up by freshly dissected and disaggregated dorsal root sensory neurons *(7)*. This is probably partly the result of the reversibly damaged state of these neurons, which have undergone axotomy as part of their dissection. In cultured PC12 cells, which have many similarities to peripheral neurons, the same PS oligonucleotides were much less effective, but cholesterol-conjugated PS and cholesterol-conjugated mixed-backbone oligonucleotides gave much better results.

3.1.1. Choice of the Target Sequence

Because of the presence of secondary structures, many potential annealling sites on an mRNA are not accessible for hybridization with the oligonucleotide. The translation initiation region is often targeted (and the termination region less frequently), but this does not necessarily guarantee success.

Generally, a sequence 15–20 bases long is chosen because it is considered that a sequence of that length is statistically unique *(13)* for the gene. In addition, the ability to elicit a response also depends on the affinity of the oligonucleotide to the target sequence, which increases with the length of the oligonucleotide. However, as the length of the sequence increases, nonsequence-specific effects may also increase. A similar increase in nonspecific effects could also be seen if the G and C content of the sequence is too high. On the other hand, it may be possible to elicit a response from less accessible sites by choosing an antisense modification that increases the effective concentration of the oligonucleotide within the cell or a modification that increases affinity toward the target sequence. In cases where a PS oligonucleotide is only marginally effective, a derivatized oligonucleotide of the same sequence may induce a significant degree of downregulation, primarily because of higher intracellular levels or more stable duplex formation.

3.1.2. Choice of Controls

In any study purporting to draw conclusions based on the use of antisense oligonucleotides to cause specific gene downregulation, it is imperative that downregulation is actually demonstrated and quantified. Unfortunately, this has not always been adhered to and the reputation of antisense work has suffered as a result. The biological effects of antisense oligonucleotides do not usually reflect purely antisense effects. Many oligonucleotides (specially phosphorothioates) can interact in a nonpredictable way with proteins and other cellular structures, leading to so-called nonspecific effects. Biological effects

could also be produced from bases of the degraded oligonucleotide (degradation products of the antisense and control oligonucleotide would be similar) and this often is a reason for observing biological effects from control oligonucleotides.

Thus, the use of appropriate controls is crucial in antisense experiments. The control sequences should contain the same base composition as the antisense sequence. Two types of such controls are commonly used. "Nonsense" oligonucleotides (with scrambled base sequences) and "mismatches" (with sequences similar to the antisense, but generally with two to four "switched" bases) are the two types. "Sense" oligonucleotides with sequences complementary to the antisense have also been used. As there is a remote possibilty that the "sense" sequence could get involved in triplex formation (and as the base composition is different from the antisense), "nonsense" and "mismatch" controls are more appropriate. Ideally, all control oligonucleotides should be compared to the EMBL or Genebank sequence databases to ensure against inadvertent targetting of other genes.

3.2. Synthesis of Antisense Oligonucleotides

*3.2.1. Synthesis of Phosphorothioate Oligonucleotides (***Fig. 2***)*

As the solid-phase synthesis of oligonucleotides has been covered well in a previous volume of the series ***(14)***, it will be described only briefly here. Solid-phase synthesis of oligonucleotides is an efficient process and can be performed on an automated DNA synthesizer. The basic steps are illustrated in **Fig. 2**. The 3′-nucleoside [**1**] is attached via a linker to the solid support. The 5′-hydroxyl protecting dimethoxytrityl group (DMT) is cleaved first with acid to give [**2**]. The next base (in the form of a phophoramidite [**3**]) is coupled to this. The resulting phosphite [**4**] is oxidized to the phosphodiester (with iodine) or sulfurized to the phosphorothioate [**5**] [with tetraethylthiuram disulfide (TETD)]. Any remaining unreacted 5′-hydroxyl groups of the first nucleoside are capped at this stage. The new DMT group is cleaved again with acid and the next base is added as the synthesis cycle continues. At the end of the synthesis, the final DMT group is cleaved and the oligonucleotide [**6**] is cleaved from the solid support with concentrated (30%) ammonia. Cleaving from the solid support is also performed on the automated synthesizer.

3.2.1.1. Deprotection

The exocyclic amino groups of adenine, cytosine, and guanine are protected (with benzoyl for A and C, and isobutyryl for G) to prevent side reactions during the DNA synthesis (*see* **Note 1**). Removal of the these groups is done by heating at 55°C in concentrated (30%) ammonia for 8 h. After cooling to room

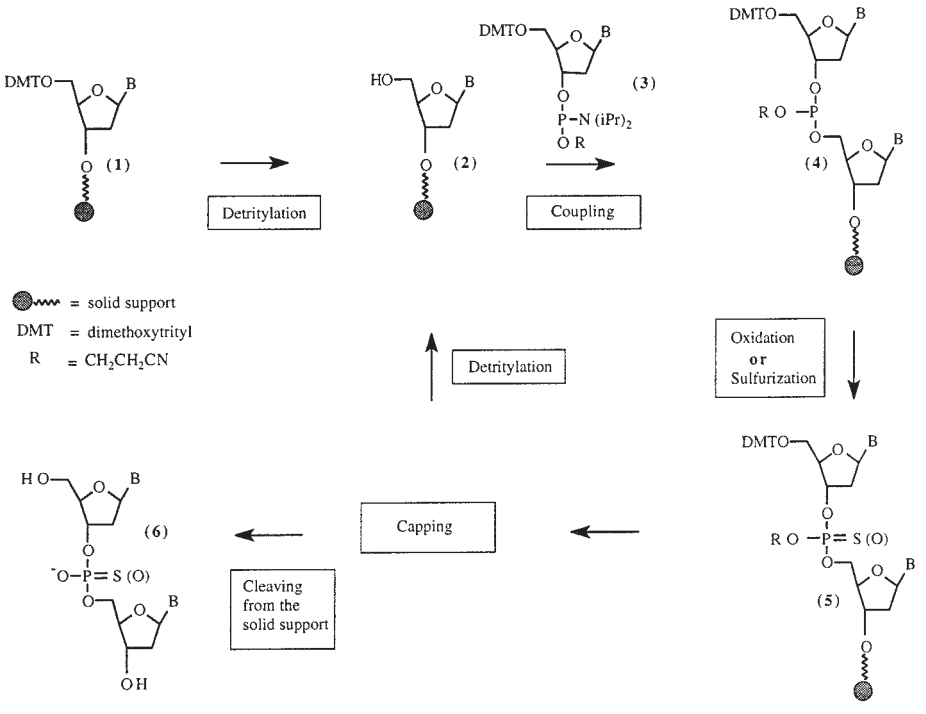

Fig. 2. Steps in solid-phase DNA synthesis.

temperature, the deprotected oligonucleotide solution is transfered to a 1.5-mL Eppendorf tube and evaporated to dryness using a vaccum evaporator.

3.2.1.2. Purification and Analysis

Purification of the oligonucleotide can be carried out by HPLC or polyacrylamide gel electrophoresis (PAGE) (*see* **Note 2**). Larger quantities are more coveniently purified using reverse-phase HPLC. Having the final trityl group on helps the HPLC purification by making the oligonucleotide more hydrophobic than the failure sequences. In such cases, the final trityl group of the purified oligonucleotide is cleaved manually by dissolving in 200–500 µL of 80% acetic acid for 20 min. An equal volume of 95% ethanol is added to the detritylated oligonucleotides and lyophilized. The sample is desalted (NAP columns) to remove the cleaved DMT group and any other remaining salts. Alternatively, the oligonucleotides can be readily purified or desalted using oligonucleotide purification cartridges according to manufacturer protocols.

The HPLC-purified oligonucleotides can be readily characterized with electrospray mass spectrometry (ESMS) ***(15)***. Characterization and structure verification with ESMS is more important for modified oligonucleotides.

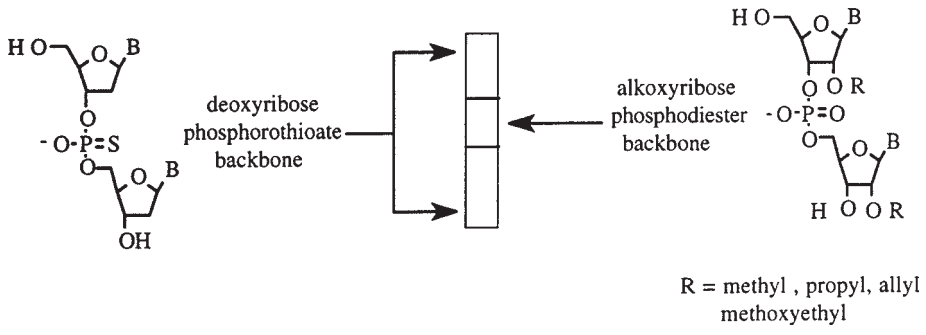

Fig. 3. Second-generation gapmer oligonucleotides.

3.2.1.3. Quantification

The purified oligonucleotides are usually quantified by the measurement of ultraviolet (UV) absorbance at 260 nm. Absorbance of a 1-mL solution (water), at 260 nm, in a 1-cm path length cuvet is measured with optical density units (ODU). Approximately 33 µg of single-stranded DNA would show an absorbance of 1 ODU. The sample can be quantified by considering that 1 µmol of oligonucleotide will absorb 10 ODU per base.

*3.2.2. Synthesis of Second-Generation "Gapmer" Oligonucleotides (***Fig. 3***)*

Replacing the middle part of the PS backbone with a non-PS backbone (that shows higher affinity toward RNA) should reduce undesirable sulfur-dependent non-sequence-specific effects while increasing the antisense effects. RNase H activity should be maintained as a phosphorothioate sequence as short as five to six bonds is sufficient to activate RNase H ***(16)***. 2′-Alkoxyribose phosphodiester backbone has been used successfully as the non-PS section ***(17)***. This backbone shows a higher affinity toward RNA as compared with a PS backbone. Alkoxy groups can also increase the resistance of the phosphodiester bond toward endonucleases, whereas 5′- and 3′-end phosphorothioates give the oligonucleotide stablity against the exonuclease promoted degradation. Methoxy, allyloxy, propyloxy, and methoxyethoxy groups are among the alkoxy modifications that have been used ***(18)***. Although the methoxyethoxy group appears to be the best modification, only methoxyribonucleosides are commercially available at present.

3.2.2.1. Synthesis of Deoxyribose Phosphorothioate/Methoxyribose Phosphodiester Gapmers

Mixed-backbone "gapmer" oligonucleotides are synthesized in such a way that the first and last six bases contain phosphorothioate bonds with normal

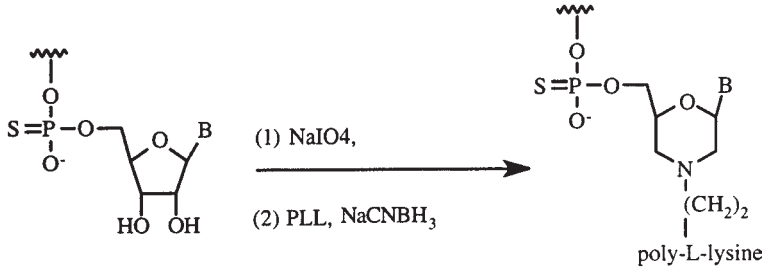

Fig. 4. Conjugating poly-L-lysines.

deoxyribose nucleosides and the middle section contain phosphodiester bonds with methoxyribose nucleosides. For example, with a 19-mer, the first six bases are synthesized using standard phosphorothioate chemistry, the next seven bases are synthesized with methoxyribose phosphoramidites with iodine as the oxidizing agent, and the final six bases are synthesized using phosphorothioate chemistry. Deprotection, analysis, and purification are as described previously.

3.2.3. Increasing the Cellular Uptake by Masking the Negative Charge

As the cell surfaces are relatively negatively charged, masking the charge of the oligonucleotides should decrease the repulsion between oligonucleotides and cell membrane, resulting in increased internalization.

3.2.3.1. Conjugating Poly-L-lysines

Conjugating cationic poly-L-lysines (PLL) has been shown to increase cellular uptake ***(19)*** of oligonucleotides (*see* **Note 3**). The oligonucleotide is synthesized on a ribonucleoside solid support, and the 3′-ribose diol of the oligonucleotide is oxidatively cleaved with sodium periodate. The resulting dialdehyde is immediately reductively aminated by PLL amine groups in the presence of sodium cyanoborohydride. This yields an oligonucleotide conjugated to PLL via a morpholino ring (**Fig. 4**).

1. Synthesize a phosphorothioate oligonucleotide on a ribonucleoside-derivatized solid support.
2. Dissolve 100 nmol of the dried oligo in sodoium periodate (1 µmol in 0.1*M* sodium acetate buffer, pH 4.75). Incubate the reaction mixture in the dark at 0–4°C for 2 h.
3. Bring the solution back to room temperature and add the PLL solution (100 nmol in 100 µL of 0.2*M* phosphate buffer). Incubate the reaction mixture at room temperature, overnight.
4. Purify the oligonucleotide–PLL conjugate using a Sephadex G-50 column equillibrated with 0.1*M* ammonium acetate buffer. Pool the fractions that contain the oligonucleotides by monitoring the absorbance at 260 nm (*see* **Note 4**).

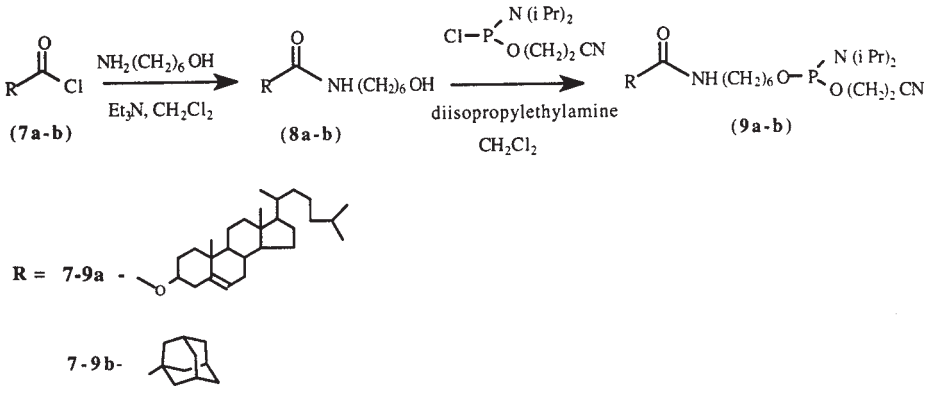

Fig. 5. Synthesis of cholesteryl and adamantyl phosphoramidites.

3.2.3.2. Lipofection

Cationic lipids can associate with negatively charged oligonucleotides, yielding net positively charged complexes. These complexes can interact and fuse with the cellular membrane to increase the cellular uptake. Lipofection ***(20)*** is widely used in the delivery of genes and has been used with oligonucleotides as well. Among the drawbacks are cellular toxicity, serum instability, and the variation of efficiency between different cell types. Improved cationic lipids have been reported ***(21)***. A number of transfection agents are commercially available with detailed protocols and therefore will not be described in here.

3.2.4. Increasing Uptake by Increasing Lipophilicity: Cholesterol-Conjugated Oligonucleotides

Among the lipophilic groups that have been conjugated to oligonucleotides to increase uptake, most success have been achieved with cholesterol groups ***(22)***. The increased uptake compared to other lipophilic groups has been attributed to the possiblity of low-density lipoprotein (LDL) receptor-mediated endocytosis following association with LDL. Conjugation of the groups to either ends should also increase the stability of the oligonucleotides toward exonucleases.

3.2.4.1. Conjugation of Cholesterol Groups to the 5′ End of the Oligonucleotide (**Fig. 5**)

To prevent steric hindrance for the duplex formation, the cholesterol group is coupled to the olgonucleotide via a six-carbon spacer. This requires the coupling of cholesterol to the spacer [**8a**] first and the addition of **8a** to the

oligonucleotide in the form of a phosphoramidite [**9a**] during solid-phase DNA synthesis. All modern DNA synthesizers will have positions in the instruments for the attachment of additional phosphoramidite reagent bottles.

1. Synthesis of cholesterol-3-carboxyaminohexan-6-ol [**8a**]. To a cooled (0°C) mixture of 6-aminohexanol (0.177 g, 0.0015 mol) and triethylamine (0.24 mL, 0.0017 mol) in anhydrous dichloromethane (5 mL), add cholesteryl chloroformate [**7a**] (0.615 g, 0.00137 mol) slowly (*see* **Note 5**). After stirring overnight, add dichloromethane (25 mL) and wash with aqueous sodium bicarbonate (2 × 25 mL) using a separatory funnel. Concentration of the organic layer *in vacuo* after drying with anhydrous sodium sulfate and further purification with flash chromatography (elute with 10–50% ethyl acetate in hexanes) yields the product [**8a**] (0.648 g) as a white foam upon drying. ^{1}H-NMR (nuclear magnetic resonance) ($CDCl_3$): δ 5.4 (crude d, 1H), 4.6–4.4 (m, 2H), 3.6 (dd, 2H), 3.15 (m, 2H), 2.05–1.75 (m, 4H), 1.6–0.6 (m, 45H).
2. Synthesis of cholesteryl-3-carboxyaminohexyl-2-cyanoethyl *N*,*N*-Diisopropylphosphoramidite [**9a**]. To [**8a**] (0.35 g, 0.00066 mol), in anhydrous dichloromethane (25 mL), add *N*,*N*-diisopropylethylamine (0.345 mL, 0.00198 mol) and 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (0.192 mL, 0.00086 mol) under an inert atmosphere with stirring (*see* **Note 6**). After 4 h, add dichloromethane (25 mL) and wash with aqueous sodium bicarbonate using a separatory funnel. Dry the organic layer with anhydrous sodium sulfate and concentrate *in vacuo* (the workup of the reaction mixture should be done as fast as possible to minimize the exposure to water). The product can purified by flash chromatography (column equillibrated with 10% ethyl acetate in hexanes/1% triethylamine, elute with 10–25% ethyl acetate in hexanes/1% triethylamine). Evaporation of the solvent and drying should yield [**9a**] as a thick oil (0.4055 g) (*see* **Note 7**). ^{1}H-NMR ($CDCl_3$): δ 5.4 (crude d, 1H), 4.6–4.4 (m, 2H), 3.95–3.5 (m, 6H), 3.2 (m, 2H), 2.4–2.2 (m, 2H), 2.05–1.75 (m, 4H), 1.2–1.5 (2d, 12H), 1.6–0.6 (m, 45H)
3. Synthesis of the 5′-cholesteryl oligonucleotide. Dissolve the phosphoramidite [**9a**] in anhydrous dichloromethane at a concentration of 0.1*M*. Attach the phosphoramidite at the designated position on the synthesizer. Synthesize the oligonucleotide as in **Subheading 3.2.1.** The cholesterol group is added last. For the step for the addition of the phosphoramidite [**9a**], increase the coupling time to 10 min. The column should be washed with additional dichloromethane before and after the coupling step to prevent the precipitation of cholesterol residues in the reagent delivery lines. Sulfurization time also could be increased to 20 min to ensure complete coupling. After synthesis and cleaving from the line, deprotect and purify as described previously. HPLC purification can be done readily because of the hydrophobicity of the 5′-cholesterol group.

3.2.4.2. Synthesis of 3′-Cholesteryl Oligonucleotides (Fig. 6)

Modifications of the 3′ end of the oligonucleotides require the derivatization of the solid support. This requires a "three-pronged molecule" such as 3-amino-

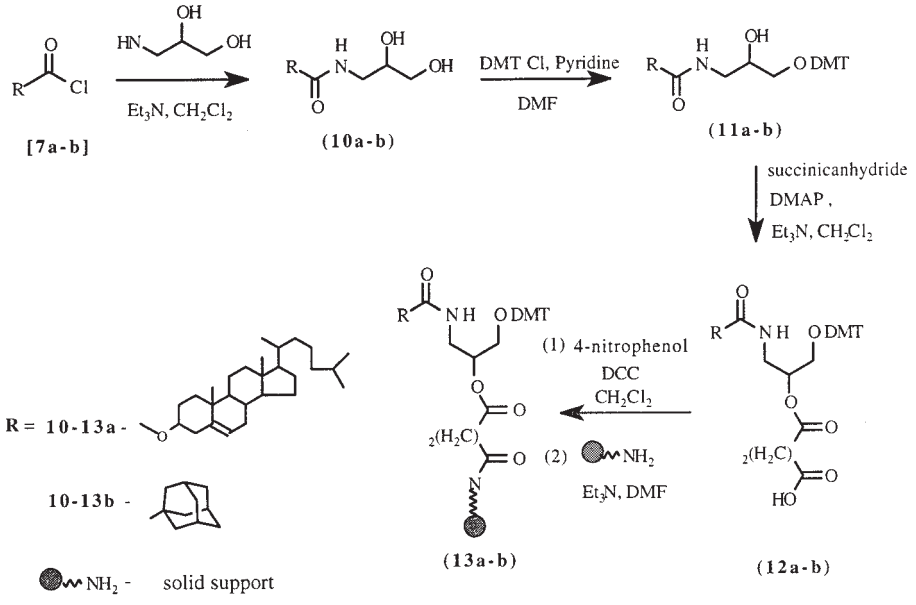

Fig. 6. Derivatizing the solid support for 3′ functionalization.

1,2-propanediol where the three ends are attached to the cholesterol group, to the solid support, and to a DMT group at the position where chain extension is to occur ***(23)***. Synthesis of the oligonucleotide is carried out on this modified solid support.

1. Cool a mixture of 3-amino-1,2-propanediol (0.253 g, 0.0028 mol) and triethylamine (0.465 mL, 0.0033 mol) in dichloromethane (10 mL) and add cholesteryl chloroformate (1.0 g, 0.0023 mol). Stir the reaction mixture overnight at room temperature. Work up the reaction mixture by adding ethyl acetate (25 mL), washing with water (two times in 15 mL). Dry the organic layer with anhydrous sodium sulfate. Evaporation of the organic layer should give a foam [**10a**] (1.15 g). This can be directly used in the next reaction.
2. Dissolve the crude product [**10a**] (1.16 g, 0.0023 mol) in dry dimethylformamide (DMF) (10 mL), add 4-dimethylaminopyridine (DMAP) (0.028 g, 0.00023 mol, 10 mol%), triethylamine (0.959 mL, 0.0069 mol), followed by dimethoxytrityl chloride (DMTCl) (0.973 g, 0.0029 mol) (*see* **Note 8**). After overnight stirring, cool the reaction mixture and quench with aqueous sodium bicarbonate. Wash the reaction mixture in a separatory funnel with aqueous sodium bicarbonate and aqueous saturated ammonium chloride. Dry the organic layer with anhydrous sodium sulfate and concentrate *in vacuo*. Flash chromatography (10–30% ethyl acetate in hexanes) should yield 1.1 g of [**11a**], as a slightly colored crystalline solid (*see* **Note 9**). ^{1}H-NMR ($CDCl_3$): δ 7.4–6.8 (m, 13 H), 5.35 (crude d, 1H),

4.9 (crude t, 1H), 4.4 (m, 1H), 3.8 (m, 1H), 3.75 (s, 6H), 3.4 (m, 1H), 3.2 (m, 3H), 2.8 (crude t, 1H), 2.2 (m, 2H), 2.0–0.6 (m, 41 H).

3. The following two steps can be carried out without chromatographic purifications.
   a. To [**11a**] (1.3 g , 0.0016 mol) under an argon atmosphere, add DMAP (0.02 g, 0.00016 mol, 10 mol%), triethylamine (0.328 mL, 0.0024 mol), succinic anhydride (0.235 g, 0.00235 mol), and stir overnight. Monitor the reaction with thin-layer chromatography (TLC) (*see* **Note 10**). Work up the reaction by adding ethyl acetate (25 mL) and washing with water and aqueous sodium bicarbonate. Drying with anhydrous sodium sulfate and concentrating *in vacuo* should give [**12a**] (1.34 g) as a light yellow foam. ^{1}H-NMR ($CDCl_3$): (all the peaks are broadened) δ 7.4–6.8 (m, 13H), 5.35 (broad m, 1H), 5.05 (broad m, 1H), 4.8 (crude t, 1H), 4.4 (m, 1H), 3.8 (s, 6H), 3.5 (m, 1H), 3.4 (m, 1H), 3.2 (m, 2H), 2.6 (m, 4H), 2.3 (m, 2H), 2.0–0.6 (m, 41H)
   b. Dissolve the crude [**12a**] in dry dichloromethane (10 mL). Add 4-nitrophenol (0.202 g, 0.0016 mol), dicyclohexylcarbodiimide (DCC) (0.6 g, 0.00291 mol), and stir for 7 h. Filter the precipitated dicyclohexylurea (DCU) and evaporate the reaction mixture. If more DCU precipitate appears, filter that as well. Dissolve the crude nitrobenzoate in dry DMF (5 mL), add pyridine (1.0 mL), triethylamine (1.0 mL), and long-chain alkyl amine CPG resin (1.0 g). Shake the reaction mixture overnight. Filter the derivatized CPG using suction and wash with DMF (five times in 10 mL), dichloromethane (five times in 10 mL) and finally with ether (five times 10 mL). First dry under suction and then under vacuum.
4. Capping. The unreacted amino groups must be capped to prevent the formation of (*n*–1) sequences. Add tetrahydrofuran (THF) (2 mL) to the derivatized CPG (1.0 g) from above and add acetic anhydride (0.75 mL, 1.0*M* in THF) and *N*-methyl imidazole (0.4 mL, 2.0*M* in THF). Shake for 3 h. Wash the CPG as earlier with THF and dichloromethane (five times in 10 mL each) and dry under vacuum as earlier (*see* **Note 11**).
5. Quantification. The extent of the derivatization can be determined by a spectrophotometric assay ***(24)***. Prepare a solution of 70% perchloric acid (25.7 mL) and methanol (23 mL). Carefully weigh the derivatized CPG (4 mg) to a small beaker and add 8 mL of the perchloric acid/methanol solution. Measure the absorbance of the released DMT cation at 498 nm. The loading (μmol/g) can be calculated by using the following formula (generally the loading should be between 25 and 30 μmol/g):

$$\frac{\text{Absorbance at 498 nm} \times \text{volume of perchloric/methanol solution (mL)} \times 14.3}{\text{Weight of the CPG (mg)}}.$$

6. Synthesis of the 3′-cholesteryl oligonucleotide. The derivatized solid support is loaded into suitable reactor vessels available commercially (Perkin-Elmer or Glen Research). Synthesis, deprotection, and purification is carried out as described previously.

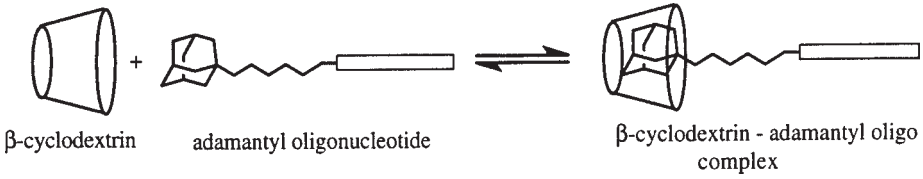

Fig. 7. Adamantane–oligonucleotide–cyclodextrin complexes.

3.2.5. Cyclodextrin-Mediated Uptake of Oligonucleotides

Cyclodextrins are cone-shaped cyclic oligomers composed of six to eight glucose units. The interior of the cyclodextrin molecule is relatively hydrophobic and the exterior is hydrophilic. Because of this, cyclodextrins could both interact with polar molecules as well as form inclusion complexes with suitably sized hydrophobic molecules.

3.2.5.1. Oligonucleotide–Cyclodextrin Complexes

More soluble derivatives of β-cyclodextrin, hydroxyethyl β-cyclodextrin (HECD), and hydroxypropyl β-cyclodextrin (HPCD) have been used to increase the uptake of oligonucleotides ***(25)***. Generally, an oligonucleotide solution with the desired concentration is first prepared and HECD or HPCD is added to make the sample 1–10% (w/v) with respect to the cyclodextrin.

3.2.5.2. Adamantane–Oligonucleotide–Cyclodextrin Complexes (**Fig. 7**)

Adamantane is a hydrophobic cage molecule that can fit inside the cavity of β-cyclodextrins. By conjugating the adamantane molecule to either 3′ or 5′ ends of the oligonucleotide, the oligonucleotide could complex strongly with the cyclodextrin via the adamantane molecule. This results in an increase of the cellular uptake ***(26)***. Adamantane can be conjugated to the oligonucleotide by following the same procedures used to conjugate cholesterol to the oligonucleotide (**Figs. 5** and **6**).

3.2.5.3. Conjugation of Adamantane to the 5′ End of the Oliginucleotide

Again, the adamantane group is coupled to the olgonucleotide via a a six-carbon spacer. The adamantane molecule is coupled to the spacer first [**8b**] and then converted to the phosphoramidite form [**9b**]. This phosphoramidite is coupled to the oligonucleotide after adding the final (5′) base (**Fig. 5**) during solid-phase DNA synthesis.

1. Synthesis of adamantyl-carboxyaminohexan-6-ol [**8b**]. To a cooled (0°C) mixture of 6-aminohexanol (0.352 g, 0.003 mol) and triethylamine (0.452 mL,

0.00325 mol) in dry dichloromethane (2 mL), add adamantane carbonylchloride [**7b**] (0.5 g, 0.0025 mol) slowly. After stirring overnight, add dichloromethane (25 mL), and wash with dilute HCl (25 mL), followed by aqueous sodium bicarbonate (three times in 25 mL) using a separatory funnel. Drying the organic layer with anhydrous sodium sulfate and concentration *in vacuo* yields [**8b**] (0.553g) as a white crystalline material. Chromatographic purification is not necessary and can be directly used for the next reaction. ^{1}H-NMR ($CDCl_3$): δ 5.5 (broad s, 1H), 3.6 (dd, 2H), 3.2 (q, 2H), 2.0 (broad s, 3H), 1.8 (s, 6H), 1.7 (m, 6H), 1.6–1.2 (m, 8H).

2. Synthesis of adamantanecarboxy-6-aminohexyl-2-cyanoethyl *N*,*N*-disopropylphosphoramidite [**9b**]. Adamantyl-carboxyaminohexan-6-ol [**8b**] (0.2 g, 0.00072 mol), 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (0.21 mL, 0.000932 mol) and diisopropylethylamine (0.38 mL, 0.00216 mol) are used for the reaction following the same procedure described in **Subheading 3.2.4.1., step 2**. The product is purified by flash chromatography (column equillibrated with 25% ethyl acetate in hexanes/1% triethylamine, eluted with 25–50% ethyl acetate in hexanes/1% triethylamine). Evaporation and drying *in vacuo* yields [**9b**] as a thick oil (0.208 g). ^{1}H-NMR ($CDCl_3$): δ 5.5 (broad s, 1H), 3.9 (m, 2H), 3.2 (q, 2H), 2.6 (t, 2H), 2.0 (broad s, 3H), 1.8 (s, 6H), 1.7 (m, 6H), 1.6–1.2 (m, 8H), 1.15 (dd, 12H).
3. Synthesis of the 5′-adamantane derivatized oligonucleotide. The exact procedure and synthesis cycles used for 5′-cholesteryl oligoucleotides can be followed (**Subheading 3.2.4.2., step 3**). HPLC purification is again simplified because of the presence of the hydrophobic adamantane group.

3.2.5.4. Synthesis of 3′-Adamantyl Oligonucleotides (Fig. 6)

Again, exactly the same procedure that was used for synthesizing 3′-cholesteryl oligonucleotides could be followed.

1. Add adamantane carbonylchloride (0.994 g, 0.005 mol) to a cooled mixture of 3-amino-1,2-propanediol (0.456 g, 0.005 mol), triethylamine (0.00625 mol, 0.87 mL) in dichloromethane (3 mL) and stir under an argon atmosphere overnight. Work up the reaction mixture by adding dichloromethane (25 mL) and washing with water (two times in 15 mL). Drying the organic layer with sodium sulfate and evaporation should give [**10b**] (0.826 g) as a foam. This is directly used for the next step.
2. Dissolve the crude product [**10b**] from above (0.826 g, 0.0033 mol) in dry DMF (5 mL), add DMAP (0.04 g, 0.00033 mol, 10 mol%) and triethylamine (1.36 mL, 0.0098 mol), followed by DMTCl (1.38 g, 0.004 mol). Stir the reaction mixture overnight and work it up following the procedure for [**11a**]. Flash chromatography (20–50% ethyl acetate in hexanes) yields [**11b**] (1.195 g) as a slightly colored crystalline solid. ^{1}H-NMR ($CDCl_3$): δ 7.6-6.8 (13H, m), 5.9 (crude t, 1H), 3.9 (m, 1H), 3.8 (s, 6H), 3.5 (m, 1H), 3.4 (m, 1H), 3.3 (m, 1H), 3.2–3.0 (m, 2H), 2.0 (broad s, 3H), 1.7 (s, 6H), 1.9–1.6 (m, 6H).

3. The following two steps can be carried out without chromatographic purifications:
   a. To [**11b**] (1.18 g, 0.00213 mol) under an argon atmosphere, add DMAP (0.026 g, 0.00021 mol, 25 mol%), triethylamine (0.371 mL, 0.00027 mol), succinic anhydride (0.266 g, 0.0027 mol) and stir overnight. Work up and purify the reaction mixture as in **Subheading 3.2.4.2.** ^{1}H-NMR ($CDCl_3$): δ 7.6–6.8 (13H, m), 5.9 (crude t, 1H), 5.1 (crude t, 1H), 3.8 (s, 6H), 3.6 (m, 1H), 3.4 (m, 1H), 3.2(m, 1H), 2.6 (m, 4H), 2.0 (broad s, 3H), 1.7 (s, 6H), 1.9–1.6 (m, 6H).
   b. Dissolve the crude product [**12b**] in dry dichloromethane (10 mL) and add 4-nitrophenol (0.325 g, 0.0023 mol), dicyclohexylcarbodiimide (0.877 g, 0.0043 mol), and stir overnight. Filter the dicyclohexylurea precipitate as described in **Subheading 3.2.4.2.** Dissolve the crude nitrobenzoate in dry DMF (8 mL), add pyridine (0.8 mL), triethylamine (0.8 mL), and long-chain alkyl amine CPG resin (1.5 g). Shake the reaction mixture overnight. Filter the derivatized CPG using suction and wash with DMF (five times in 10 mL), dichloromethane (five times in 10 mL), and finally with ether (five times in 10 mL). First dry using suction and then under vacuum.
4. Capping and quantitation can carried out as described in **Subheading 3.2.4.2.**, **step 4**.
5. Synthesis of the 3′-adamantyloligonucleotides. The derivatized solid support is first loaded into suitable reactor vessels, and synthesis, deprotection, and purification are carried out as described previously.

3.2.5.5. Formation of Adamantane–Oligonucleotide–Cyclodextrin Complexes

An adamantane–oligonucleotide solution with the desired concentration is prepared as in **Subheading 3.2.5.1.** and HECD or HPCD is added to make the sample 1–10% (w/v) with respect to the cyclodextrin.

3.3. Measurement of Efficacy

Biological effects alone are not a good measure of antisense efficacy. Antisense efficacy is best quantified by the measurement of amount of target mRNA (Northern blotting) and of target protein (Western blotting), and by the measurement of new protein synthesis by pulse labeling. Downregulation should be expressed by comparing the level of mRNA or protein obtained with antisense treatment to levels obtained by treating with the control oligonucleotides (nonsense and/or mismatch oligonucleotides). This minimizes the contribution of effects which arise from nonantisense mechanisms. A control in which cells are not treated with any oligonucleotide should also be performed, and it will often be found that the level of target mRNA or protein is quite different in this control compared to the nonsense oligonucleotide treated controls.

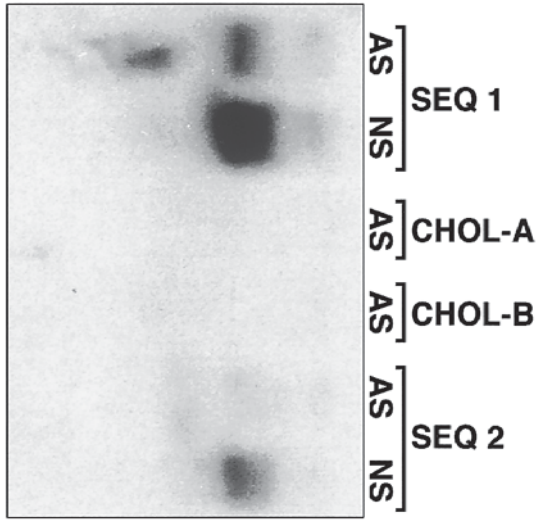

Fig. 8. Downregulation of $p75^{NGFR}$ mRNA levels in neuronally differentiated PC12 cells measured by northern hybridization after treating with phosphorothioate oligonucleotides (at 5μM for 48 h). AS: antisense; NS: nonsense; SEQ 1: 18-mer sequence directed toward the transltion termination region; SEQ 2: 19-mer sequence directed toward the translation initiation region; CHOL-A, B: AS SEQ 2 oligonucleotides modified at the 5′ end with cholesterol groups with variations in the linker. Equal amounts of RNA were loaded in each lane. The figure shows the increased downregulation of the 5′-cholesteryl oligonucleotides as compared to both unmodified antisense and nonsense sequences. An example of the potential for nonspecific effects of phosphorothioates is shown by different levels obtained with the two different nonsense oligonucleotides.

*3.3.1. Assessing Downregulation by mRNA (***Fig. 8***)*

Downregulation of mRNA requires that the RNase H mechanism is operative; this will occur for PS oligonucleotides and for oligonucleotides based on the PS backbone. We have found that PS-based oligonucleotides downregulate mRNA in neuronally differentiated PC12 cells, indicating that RNase H is present. This is true for unmodified PS oligonucleotides, cholesterol-conjugated PS oligonucleotides, and adamantane-conjugated PS oligonucleotides complexed with cyclodextrins. In measuring the mRNA levels, it is important to know the time-point at which the downreulation is most pronounced. This requires a time-course experiment, although we have rarely seen downregulation within 12 h of applying the oligonucleotides. Briefly, the cells are incubated with the antisense and nonsense oligonucleotides for 12–48 h, after which time, the medium is removed, the cells are washed, and the RNA is

extracted. The desired mRNA is measured by Northern hybridization with a ^{32}P labeled cDNA (to the desired mRNA) probe, as described in **ref. *27***. After exposing the Northern blot to a X-ray film, quantification can be performed by densitometry or scanning methods.

*3.3.2. Assessing Downregulation by Protein Levels **(28)***

It is particularly important to select the optimal time-point at which the level of target protein is to be measured. Depending on the half-life of the target protein, it may take longer for protein downregulation to become apparent. Briefly, the cells are incubated with the oligonucleotides and, at the appropriate time-point, the cells are washed and lysed. Proteins are then extracted and separated with SDS-PAGE. The proteins are then transferred electrophoretically and incubated with an antibody raised against the desired protein. Amplification, detection, and quantification are done according to the established procedures.

Because cellular protein stores may make it difficult to detect protein downregulation, pulse labeling may be required to demonstrate that synthesis of the target protein is indeed inhibited. Cells are treated in culture with ^{35}S-labeled methionine or cysteine either concomitantly with or following application of the oligonucleotides. After a period of time (which depends on the level of expression and needs to be determined empirically for each protein), the cells are harvested and immunoprecipiation is performed, using an antibody against the target protein. The immunoprecipitated proteins are analyzed by PAGE, using molecular-weight markers to determine size. Autoradiography or phosphorimaging then allows visualization of the proteins and assessment of the degree of downregulation of the rate of new protein synthesis.

Antisense oligonucleotides are now widely used as agents for sequence-specific inhibition of gene expression. Although considerable advances in the field have been made since the idea was first proposed two decades ago ***(29)***, it is not yet at a stage that it can be routinely undertaken following a simple protocol. Rather, a systematic investigation with proper controls needs to be conducted. Advances in the field in the form of chemical modifications have yielded newer generations of oligonucleotides with increased potency and reduced toxicity. These modifications can now be applied even by the nonspecialist researcher. Such advances are making the antisense technology a more reliable and effective molecular biology tool and should also lead to the eventual development of potent therapeutic agents.

4. Notes

1. Replacing the isobutyryl (iBu) protecting group of guanosine with dimethylformamidine (DMF) allows the deprotection to be complete in 2 h of

heating at 55°C (the benzoyl protectin groups of A and C will be completely cleaved during this time period as well).
2. As HPLC purifications and desaltings are done in 0.1*M* triethylammonium acetate, the dried oligonucleotide could contain triethyammonium residues, which are toxic to cells (and interfere with the antisense results). These residues can be removed by repeated (three to four times) coevaporation with water.
3. Medium-molecular-weight PLL (9000-14000) is the best for cellular uptake purposes. Low-molecular-weight PLL is less effective and high-molecular-weight PLL could be cytotoxic ***(30)***.
4. Complete seperation of free PLL from the conjugate may be difficult to achieve using a Sephadex column.
5. Dichloromethane, triethylamine, pyridine, and *N*,*N*,diisopropylethylamine are dried by distilling over calcium hydride.
6. All the reactions are best done under an argon atmosphere. Certain reactions (such as synthesizing the phosphoramidites) are extremely moisture sensitive.
7. The phosphoramidite should be stored at 0–4°C under an argon atmosphere.
8. DMAP is generally used in catalytic (1–10%) amounts.
9. During the flash chromatography, the product elution is monitored with thin-layer chromatography with phosphomolybdic acid (20 wt% in ethanol) as the visualization agent.
10. The adamantyl phosphoramidite [**9b**] (also the precursor [**8b**]) visualization on TLC may be difficult. Therefore, pooling of the product containing fractions after flash chromatography needs to be done carefully.
11. During derivatization of the CPG resin with cholesterol or adamantane reagents (**Subheadings 3.2.4.2.** and **3.2.5.2., steps 3** and **4**), the CPG resin is shaken with the reagents, as stirring could damage the resin.

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17

The IODO-GEN Method for Labeling and the Use of Retrograde Axonal Transport to Assay Neurotrophins

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1. Introduction

In order to look at the retrograde transport of neurotrophic factors from the target tissue to the innervating cell body, the neurotrophin needs to be labeled to a high specific activity. This chapter outlines methods used to bioassay the labeled neurotrophins and the advantages and disadvantages of the various methods. Conventional methods to test whether the labeled protein retains its biological activity consist of bioassays using the material to keep explanted ganglia ***(1,2)*** or dissociated neuron cultures ***(3,4)*** alive. These have two main drawbacks. First, there is a requirement to use large amounts of radioactive material in the cultures creating a safety hazard. Second, it cannot be determined what proportion of the neurotrophin molecules are, in fact, labeled. If the product is a mixture of labeled inactive and unlabeled active protein, the bioassay will give the erroneous result that the labeled material is active or has a reduced activity (also see Note 1 of Chapter 14). More satisfactory methods consist of receptor-binding assays ***(5)*** or the retrograde axonal transport of the ^{125}I-labeled neurotrophin ***(6,7)*** as a quality control assay and a semiquantitative bioassay.

We have used a retrograde transport assay to determine the extent of radiolabeling of nerve growth factor (NGF), NT-3, and NT-4/5 with ^{125}I. This chapter will outline our labeling procedures and go through the steps required to determine the extent of the labeling of the material.

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2. Materials

1. βNGF was purified from male mouse salivary glands in our laboratory by the method of Mobley et al. ***(8)***; NT-3 was a kind gift from Amgen and Regeneron Pharmaceuticals. Use 10 µL at a concentration of 1 mg/mL.
2. ^{125}I–Na, 100 mCi/mL, in dilute NaOH.
3. 0.05*M* sodium phosphate, 150 m*M* sodium chloride (pH 7.4), phosphate-buffered saline (PBS).
4. PBS with 2% bovine serum albumin (PBS/BSA).
5. Biogel P2 preswollen in PBS/BSA for 1 h before use.
6. IODO-GEN: 10 m*M* of 4.3 mg/mL in chloroform. Store in a dark bottle wrapped with foil at 4°C.
7. Protein-free glass tube.
8. Label 12 plastic tubes to collect the eluate from the column.
9. Geiger counter suitable for measuring ^{125}I.
10. Glass wool.
11. Bags for radioactive waste.
12. Dry nitrogen cylinder.
13. Ice bucket and ice.
14. Pasteur pipets.
15. Hamilton syringes, 10 or 25 µL with fixed needles.
16. Anesthetic for mice: Use 4 µL/g of a mixture of 580 µL saline, 220 µL of 100 mg/mL ketamine, and 200 µL rompun (20 mg/mL xylazine).
17. Gamma counter.
18. BME Eagle's basal medium sterile.
19. 10X BME, a 10-fold concentration of BME.
20. Collagen solution (*see* **Note 1**).

3. Methods

The NGF and NT-3 can be labeled with ^{125}I using a number of different methods, including chloramine T, Bolton and Hunter, and lactoperoxidase ***(9)***, (*see* von Bartheld, Chapter 14), but the one we find simplest and best is labeling using the IODO-GEN method.

3.1. ^{125}I–NGF Labeling Using the IODO-GEN Method

1. Cut the end off a Pasteur pipet and pack with a small pledget of glass wool. Fill with PBS/BSA and Biogel and allow it to pack to create a column 65 mm long. Wash with 2 mL PBS/BSA.
2. Prepare IODO-GEN-coated tubes by placing 40 µL IODO-GEN solution in an acid-washed glass test tube and dry down by putting tube under a stream of dry nitrogen for about 5 min (*see* **Note 2**).
3. Set up an ice bucket behind an adequate radiation shield in a good fume cupboard. Place 10 µL of PBS, 10 µL containing 1 mCi ^{125}I–Na, and 10 µL PBS

containing 10 µg neurotrophin into the IODO-GEN-coated tube on ice. Leave for 10 min with occasional gentle agitation.
4. Remove contents of the tube carefully to avoid contamination and place on top of Biogel column and allow to run dry and then wash it with 100 µL PBS followed by 1 mL PBS/BSA. The peak of radiolabeled protein will come after the first 10–20 drops. Collect in plastic tubes at 3 drops per tube and monitor radioactivity of each tube to determine when first peak comes off. Labeled neurotrophin should all be present in one or two tubes. Counts should start to fall when column runs dry, leaving unreacted iodine still in the column, which can be disposed of in an appropriate decay storage area before final disposal.
5. Count 1 µL from each tube in a gamma counter and pool tubes with highest counts and count 1 µL from pool. Calculate the concentration of the pool and dilute to 1µCi/µL.
6. The specific activity of the neurotrophin is calculated by multiplying the ratio of the iodide to neurotrophin in the reaction mixture by the fractionation of ^{125}I incorporated into protein or neurotrophin ***(10,11)*** determined by trichloroacetic acid (TCA) precipitation and is described in Chapter 4.
7. We usually obtain between 100 and 600 µCi of product with a specific activity for ^{125}I–NGF of between 2 and 10 Ci/*M*.

3.2. Bioassays

3.2.1. Dissociated Cell Cultures

1. Dissect out appropriate ganglia for assay of neurotrophic factor and dissociate as described in Chapter 5 of **ref. *12***.
2. Prepare polyornithine/laminin-coated dishes ***(13)***.
3. Make up serial dilutions of unlabeled and labeled neurotrophin in the wells and dispose of all radioactive waste carefully.
4. Add cells to wells and leave overnight at 37°C in a 5% CO_2 incubator.
5. Count phase bright cells at X200 using an inverted phase microscope.
6. Calculate dilution with 50% of maximal number of surviving neurons.
7. Compare concentrations of unlabeled and labeled neurotrophin and determine the concentration of the labeled compound.

3.2.2. Chick Dorsal Root Ganglion Explant Cultures

1. Dissect dorsal root ganglia from 8-d-old chick embryos.
2. Place ganglion one per well in 96-well plates.
3. Prepare a collagen solution using 0.8 mL rat tail collagen (*see* **Note 1**) and 210 µL of a mixture of 455 µL of 10X BME, 112 µL of 7.5% $NaHCO_3$, 50 µL of 200 m*M* L-glutamine, 55 µL fetal calf serum (FCS), and 383 µL of 0.15*M* NaOH.
4. Place a drop of the collagen mix in each well.
5. Center the ganglion with a watchmaker's forceps.

6. Make up serial dilutions of neurotrophin in a separate plate containing 100 µL BME in each well by taking 11 µL of neurotrophin into the first well and diluting 11 µL serially into each subsequent well.
7. Transfer diluted neurotrophin to wells containing ganglia in collagen.
8. Incubate in standard cell culture incubator at 37°C for 16–24 h.
9. Examine fiber outgrowth under dark-field or phase contrast microscopy.
10. Score fiber outgrowth on a semiquantitative scale from 0 to 5+ ***(1)***.

3.3. Retrograde Transport

1. In mice, the best anesthetic we have found for this procedure is 88 µg/g ketamine and 16 µg/g rompun (i.p.), use 4 µl/g of the mixture.
2. After 10–20 min, the animals are completely immobile and could be injected in the anterior eye chamber. We use a 10-µL Hamilton syringe whose tip has been sharpened on a fine-needle stone.
3. 0.1–1 µL is drawn up into the syringe, and under a dissecting microscope, the needle tip pushed forward through the sclera until it can be seen through the pupil in the right anterior eye chamber and the iodinated material injected (*see* **Note 3**).
4. At a standard time between 16 and 20 h, the animals are killed and both the superior cervical ganglion and trigeminal ganglion are dissected out (*see* **Note 4**).
5. The ganglia are placed in tubes suitable for gamma counting and accumulated radioactivity determined using a gamma counter.
6. The amount of ^{125}I–βNGF transported is calculated by subtracting the counts accumulating on the noninjected side, which represents systemic spread, from those on the injected side, which represents both systemic and transported material.
7. The retrograde axonal transport of ^{125}I–neurotrophins in sympathetic neurons to the superior cervical ganglia (SCG) and sensory neurons to the trigeminal ganglia (TGG) can be expressed as a percentage of the injected number of counts.

3.4. Use of Retrograde Transport to Assess the Labeled Neurotrophin

The amount of active labeled material can be roughly assessed by noting the amount of material injected and the amount transported, as shown in **Fig. 1**. The percentage of the material transported at a specific time (we use 16 h) should be constant. Higher values represent good labeling and lower values represent poor labeling; this can be used as a quality control for each iodination. For example, in a series of our iodinations, the range of ratios of injected to transported neurotrophin to the SCG at 16 h was as follows:

NGF	*NT-3*
0.086 good	0.0104 good
0.056	0.0031
0.025 bad	0.0013 bad

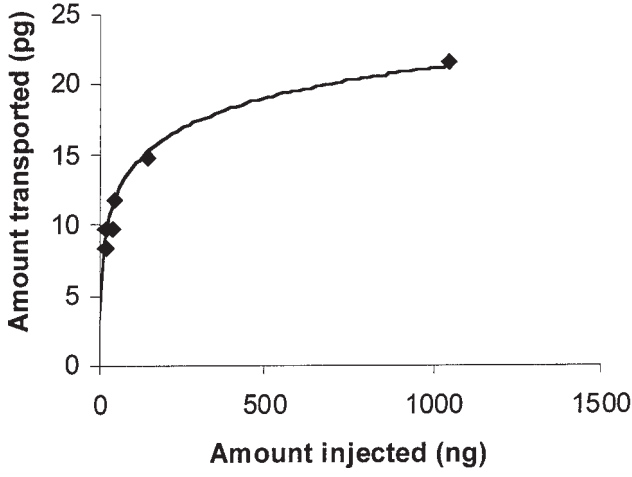

Fig. 1. Dose relationship between amount of NGF injected and amount transported to the SCG. Graph of amount of injected material verses amount of neurotrophin transported to superior cervical ganglion.

However, a more accurate measurement of the specific activity of the active labeled neurotrophin can be obtained using the method in **Subheading 3.5.**

3.5. Estimate of Specific Activity of Injected Material

1. Make up two dilutions of the radiolabeled neurotrophin, one by diluting fivefold in PBS (**Table 1**, Low) and the other by diluting in cold neurotrophin to give a final concentration of 20 µg/µL (**Table 1**, Low + 20 µg).
2. Inject three groups of four to six animals in the anterior eye chamber with 1 µL of the radiolabeled neurotrophin (**Table 1**, High), the 1 in 5 diluted neurotrophin (**Table 1**, Low) and the cold diluted neurotrophin (**Table 1**, Low + 20 µg).
3. After 16 h, the animals are killed and both the superior cervical ganglia and the trigeminal ganglia are dissected out, and the radioactivity in both left and right ganglia counted.
4. The amount of radioactivity transported is obtained by the average of the difference between the left and right sides.
5. An estimate of the specific activity can be obtained if the difference in concentrations between the Low +20 and High is relatively small. In this case, the assumption is that the increase in the amount of material transported is linear and a calculation can be made as to the slopes between the low and the two higher values.
6. Set up an "Excel" spreadsheet as shown in **Table 2**.
7. Enter the data on an Excel spreadsheet as shown in **Table 2**.

Table 1
Sample Data and Calculation for Determination of Specific Activity of NGF Following the Method as Described in Subheading 3.5. Output of the "Excel" Spreadsheet Using the Format and Formulas as Shown in Table 2

	Initial data from gamma counter			
	Injected		Transported	
Unknown NGF injected	CPM/μL	ng/μL	*fg*	CPM
Low	1,881,300	12.4	6.5	987
High	5,031,100	33.3	8.5	1,288
Low+20μg	1,881,300	32.4	8.4	489
ng/μL injected		33.3		
Volume from column		156		
Recovery (μg)		5.2		
Calculated specific activity	(mCi/μg)	137.2		
	(mCi/mol)	5.28		
Counter efficiency (%)		50		
	(μCi/μL)	4.57		
MW of neurotrophin		26,000	for NGF	
Slope				
High–Low	0.0954			
Low20–Low	0.0954			
	0.0000	Set to 0 using "goal seek" in tools 3.5.8		

8. Use "goal seek" on the tools menu to set cell C23 to 0 by changing cell D11.
9. This sets the slopes of the two lines, Low–Low+20 and Low–High to be the same.
10. The specific activity is the iterative value required establishing this condition.

4. Notes

1. Rat tail collagen can be prepared by acetic acid digestion of sinues from adult rat tails described in Chapter 4 of **ref. *12***.
2. IODO-GEN solution is usually quite stable in the refrigerator, but we have experienced that on some occasions, it loses its activity. It is therefore best to make this up fresh.
3. The site of injection into the anterior eye chamber is critical, because if this is not carefully monitored, it is possible to inject into the lens or into the vitreous humor. In both sites, the injection remains sequestered and only a small percentage is available for transport.
4. The time for maximal transport levels to be reached in mice is between 8 and 16 h.

Table 2
Set up "Excel" Spreadsheet Using the Template and Formula Shown Below; These Are Required for the Calculation of the Specific Activity of the Neurotrophin in the Example Shown in Table 1

A	B	C	D	E	F
3		Injected			Transported
4		CPM/µl	ng/µl	fg	CPM
5	Low	1,881,300	=+D11*C5/C6	=+F5/C5*D5*1000	987
6	High	5,031,100	=+D11	=+F6/C6*D6*1000	1288
7	Low+20 µg	1,881,300	=+D5+20	=+F7/C7*D7*1000	489
10					
11	ng/µL injected		33.3		
12	Volume from column		156		
13	Recovery (µg)		=+D11*D12/1000		
14	Specific activity (mCi/µg)		=+D17/D11*1000		
15		(mCi/mol)	=+D14/D18*1000		
16	Counter efficiency (%)		33		
17	µCi/µL		=+C6*100/D16/2,200,000		
18	MW of neurotrophin		26,000		
19					
20	Slope				
21	High–low	=+(E6–E5)/(D6–D5)			
22	Low20–low	=+(E7–E5)/(D7–D5)			
23		=+C21-C22			

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18

Stereological Estimation of Neuronal Number

The Optical Disector/Cavalieri Combination

Victor Nurcombe, Nigel G. Wreford, and John F. Bertram

1. Introduction

In recent years, we have witnessed an explosion in the number of techniques available for selectively perturbing the genes and molecules that regulate tissue growth and maintenance. From drugs and neutralizing antibodies, we have progressed to gene deletion, "dominant negative," antisense, and ribosymal strategies. As advances are made in chromosomal mapping and analysis, we are progressively more capable of creating accurate animal models of disease by means such as the site-directed mutagenesis of DNA and the creation of transgenic animals. Allied to this have been rapid developments in the means by which additional genes can be engineered into cells in tissue culture prior to their transplantation or integration into living animals. This latter technique often includes an inducible means of identifying the foreign cells in tissues by such methods as staining for β-galactosidase. In these studies, a commonly asked question is, "What has happened to the foreign cells?" Have they increased or decreased in number? Have they induced changes in the numbers of cells surrounding, or been influenced by them?

The enormous power now afforded by genetic knockouts by homologous recombination is already being superseded by more refined techniques that allow for tissue-specific and developmental-stage-specific gene deletion. Advances made possible by the development of methodologies such as the Cre/loxP system should allow for more targeted mutations and, thus, easier analysis. However, for many molecules, particularly growth factors, it can remain almost impossible to predict how a deletion will affect the often multiple-organ systems in which they are expressed. Both means of gene deletion

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have presented and will thus continue to present interesting problems for tissue analysis. Other means of knocking out genes at the level of translation have been developed, such as antisense oligonucleotides. Many of the earliest gene knockouts have included such powerful tissue regulators as growth and trophic factors and the transcription factors affected by such growth factors. The field has further developed as larger and larger genes have been cloned and sequenced and their 5′ regulatory regions characterized. As the sophistication of the methods used for knocking out genes in defined times and places increases, it can be anticipated that increasingly sophisticated means of analyzing the effects on cell numbers will be even more necessary.

The emergence of new molecular and genetic strategies for studying roles of specific molecules in vivo should not hide the fact that there still remains an enormous amount to be learned about the development and maintenance of normal tissues. Knowledge of the numbers of cells that appear, differentiate, and disappear during the development of different tissues will improve our knowledge of both tissue construction in embryogenesis and tissue breakdown in disease states.

The foregoing indicates that for the full potential of the new molecular and genetic strategies to be realized, accurate (without bias), precise (with low variance), and efficient (in terms of time and money) methods for counting defined cell populations in tissues and organs are required. Stereology, which can be defined as "the discipline concerned with the quantitative analysis of 3-dimensional structures" ***(1)***, has grappled with the issue of counting cells in tissues for the better part of a century. In that time, numerous stereological cell-counting methods have been developed, but as described in brief below, these methods were extremely limited. Fortunately, however, the emergence of the new molecular and genetic tools has coincided with the development of powerful new stereological methods for counting cells in tissues. These methods, when used correctly, provide accurate and precise estimates of total cell number and are proving to be surprisingly efficient. For example, using the optical disector/Cavalieri combination (the current method of choice for counting cells, which is described in full in this chapter), we estimated the total number of motor neurons in the lateral motor column of the chick spinal cord. Estimates with a coefficient of error of 10% or less were obtained in just 30 min, following sectioning and staining ***(2)***.

This chapter continues with some necessary definitions and then considers the reasons for quantifying cell number. Next, the limitations of the traditional stereological counting methods are described and the emergence of the new stereological counting methods is discussed. Then, the important issue of numerical density versus total number estimates discussed, the Cavalieri method for estimating volume is presented, and the optical disector method for

counting cells is described. Following the discussion of the optical disector/Cavalieri combination, equipment requirements, and sampling issues, a step-by-step description of how to estimate the total number of motor neurons in the lateral motor column using an optical disector/Cavalieri combination is presented. Finally, some helpful technical notes are provided.

1.1. Definitions

Cavalieri Principle: A method for obtaining an unbiased estimate of the volume of any structure. Briefly, the volume of any three-dimensional (3-D) object can be estimated by exhaustively sectioning or slicing the object, and multiplying the sum of the section/slice areas by mean section/slice thickness. The method is unbiased if the position of the first section/slice is chosen at random within the first sampling interval.

Disector: A 3-D sampling probe. The disector is the only sampling probe that samples particles uniformly (regardless of their size, shape, or orientation) in 3-D space. Once particles have been sampled with the disector, they can be counted.

Estimate: Stereological methods provide estimates of true values because data are normally derived from a sample of the tissue. The accuracy of the estimate is therefore critically dependent on the sampling regime.

Numerical density: The number of the feature of interest (e.g., cells, nuclei) per unit volume of the reference compartment.

Optical disector: An unbiased 3-D sampling probe that utilizes optical sectioning to sample particles. Once particles are sampled, they can be counted.

Particle: A 3-D object, such as a cell or nucleus.

Profile: The 2-D representation of a particle once it has been sectioned. Thus, nuclear profiles are normally seen in histological sections and electron micrographs, not nuclei.

Reference compartment: The containing space or tissue compartment (e.g., hippocampus, cerebellum) that contains the feature of interest (e.g., neurons, a subpopulation of neurons). The reference compartment is defined by the investigator.

1.2. Why Quantify?

What are the advantages of a quantitative estimation of cell number over a subjective description? One answer might be, to paraphrase Bertram ***(1)***, that "the era of qualitative morphology is over and if you want your grant application to be successful you had better make some measurements!" However, perhaps the most significant advantage concerns the enhanced ability to identify or detect structural differences between specimens. Although stereology is not

needed to identify unusual or pathological features in cells and tissues, when properly applied it gives us the ability to detect small changes in the quantity (e.g., number, volume) of structural features normally present in tissues. Stereological analysis allows a reproducible determination of quantitative data describing tissue structure. Here, we refer to the reproducibility of data collected by an individual on different occasions as well as the reproducibility of data collected by different individuals. Put simply, through the use of objective quantitative techniques, it should be possible to increase the reproducibility of data, eliminating interobserver and intraobserver variation. Thus, the subjectivity that can bedevil qualitative histology and electron microscopy can be largely overcome. Another advantage of a stereological analysis over a qualitative analysis of cell populations is the potential to integrate quantitative morphological data with quantitative physiological, biochemical, and molecular data, and the fact that the full power of statistics can be used to analyze data.

1.3. The Emergence of Stereology

As alluded to earlier, one of the major unsolved problems in biological stereology until recently was the unbiased determination of cell number (the cell number is generally equated to nuclear number assuming one nucleus per cell). The stereological methods available (*see* **ref.** ***3*** for complete descriptions) required information about cell/nuclear size distribution and/or shape. Accordingly, the methods were only well suited to counting those nuclei with simple geometric shapes (e.g., spheres). For nuclei with complex shapes or varying size distributions, complex assumptions with respect to shape and nuclear size distribution were required. To the extent that these assumptions deviated from the true value, the number estimates were biased.

The traditional stereological cell counting methods were also extremely vulnerable to technical artifacts, including shrinkage and swelling changes that occur in tissues during processing for microscopy and section compression. Finite section thickness also influenced estimates obtained with many methods. It is not much of an exaggeration to state that with traditional stereological cell-counting methods, often as much time was spent measuring, correcting, and coping with technical artifacts (estimating tissue shrinkage, measuring section thickness, section compression, and so on) as in actually making the measurements on the biological feature of interest!

The absence of a robust cell-counting method thus had profound implications for the emergence of stereology as a mainstream technique in biology. However, publication in 1984 of the *disector* method ***(4)*** revolutionized biological stereology and led to the development of a new generation of stereological methods, including a new generation of stereological counting methods.

The disector is the only known unbiased 3-D probe that samples particles uniformly with respect to their number. In other words, all particles regardless of their size, shape, orientation, or position have the same chance of being sampled. As stated in **ref. *1***, "with the disector, small particles have the same chance of being sampled as large particles; round particles have the same chance as long particles." Once particles are sampled uniformly with the disector, they can be counted, or other measurements made. The so-called new unbiased stereological counting methods include the *optical disector **(5)***. With the disector, no knowledge or assumptions of particle size, size distribution, shape, or orientation are required in order to sample and subsequently count particles. The only absolute requirements are that the particles of interest can be unambiguously identified and that the boundaries of the reference compartment can be identified in three dimensions. Other problems associated with traditional counting techniques are also overcome—depending on experimental design, it is possible to avoid measuring section thickness or calculating correction factors for shrinkage/swelling artefacts.

However, not all biologists have adopted the new stereological counting methods. This is despite the fact that no lesser authorities than Luis Cruz-Orive and Ewald Weibel advised in 1990 that the "traditional stereological counting methods should no longer be used" ***(6)***. Unfortunately therefore, the literature in a number of areas, particularly in the neurosciences, is still dominated by cell counts obtained using traditional, biased methods. Worse, many biologists are still counting profiles. The persistence of these older methods led *The Journal of Comparative Neurology* in 1996 to establish an editorial policy stating, "stereologically unbiased estimates are always preferable for establishing absolute counts or densities of structures in tissue sections. We expect that any papers that use simple profile counts, or assumption-based correction factors will provide adequate justification for these methods, which will stand up to critical review. Referees are urged to consider carefully this justification, and to insist on unbiased counts when it is not adequate" ***(7)***. Coggeshall and Lekan ***(8)***, writing in the same issue, reviewed articles published in major neuroscience journals in 1994. They reported that 18% of all articles reported the use of histological sections and 30% of these articles reported data for cell or synapse number. Studies reporting numerical data used profile counts (76%), serial reconstruction (11%), assumption-based methods such as the Abercrombie ***(9)*** method (7%), and unbiased stereological methods (5%). Coggeshall and Lekan ***(8)*** recommended that in the long term "assumption-based methods not be accepted unless the counts are calibrated." They also emphasized the importance of uniformly sampling the anatomical region under study (*see* **Subheading 3.1.**).

1.4. Numerical Density Versus Total Number

A consideration of stereological cell-counting methods would be deficient if the issue of numerical density estimation was not included. Numerical density was defined in **Subheading 1.1.** Numerical densities relate the number of, for example, neurons to a unit volume of the reference compartment (e.g., the number of neurons per unit volume of cerebral cortex, or the number of Purkinje cells per unit volume of cerebellum). Typical units are number/mm^3 or number/cm^3. Perhaps more than any other parameter, the stereological literature is littered with reports of cell numerical density that have been misinterpreted by authors and readers as changes in absolute cell number. In other words, in numerous published studies, it has been changes in the *denominator* of the numerical density estimate (i.e., the volume of the reference compartment) that have actually occurred, rather than changes in the *numerator* (the number of cells) that actually occurred as authors have reported. It is unfortunate that the majority of these misinterpreted data have occurred in neuroscience, where studies on cell loss are so important for obtaining, for example, a full understanding of brain changes in aging, as well as the pathogenesis of such conditions as Alzheimer's disease, Parkinson's disease, and alcoholism ***(10–16)***.

Two strategies are available for obtaining estimates of total cell number, rather than numerical density. The first involves multiplication of cell numerical density (estimated, for example, using optical disectors) by the volume of the reference compartment. The volume of the reference compartment can be estimated using fluid displacement techniques or weighing and density strategies, or alternatively using the Cavalieri Principle (*see* **Subheading 1.5.**). Multiplication of numerical density by the volume of the reference compartment provides an unbiased estimate of absolute cell number. Ideally, the volume of the reference compartment will be determined from the same sections as those used to determine numerical density. The advantage of doing this is that dimensional changes in cells and tissues that occur during processing for microscopy, such as shrinkage and swelling, become irrelevant when estimating total number, because changes in the dimensions of the reference space cancel.

The second strategy involves estimating the number of cells of interest in a known fraction of the reference compartment. Once the number of cells in a *known* fraction is determined, the total number can be determined by multiplying by the inverse of the sampling fraction. This is called a *fractionator* experimental design ***(5)***. When estimating *total* cell number (e.g., the total number of neurons in the hippocampus) with either of these approaches, the effects of shrinkage/swelling artifacts can be completely eliminated. Provided the cells

of interest can be unambiguously identified and that the boundaries of the reference compartment can be identified in three dimensions, they can be counted. It does not matter whether the reference space has swollen or shrunk—the experimental design is such that the estimates of number are not influenced.

1.5. The Cavalieri Method for Estimating Volume

The principle of Cavalieri is ideally suited to estimating the total volume of objects. With this principle, unbiased estimates of the total volume of any object can be obtained ***(17,18)***. The object of interest can even be totally embedded inside another object (e.g., layer CA1 in the hippocampus, or the lateral motor column in the spinal cord). To estimate the volume of an object with the Cavalieri principle, the object must be exhaustively sliced or sectioned into slices/sections of approximately equal thickness. The sum of the areas of the slices/sections multiplied by the interval between sections provides an unbiased estimate of tissue volume. The only requirement is that the position of the first slice/section must be selected at random in the interval between the point at which sectioning commences and the thickness of the sampling interval. Typically, no more than 10–15 slices/sections, uniformly spaced through the object, are required to obtain a volume estimate with an acceptable precision (coefficient of error between 5% and 10%). Methods for estimating slice/section area and thickness are presented next.

1.6. The Optical Disector

The most important innovation in the disector family of counting methods is the optical disector ***(2,19–27)***. The technique involves cutting thick (at least 20 μm) sections, typically plastic or frozen sections, and then viewing sampled fields within sections using an oil-immersion objective lens with a high numerical aperture (the microscope must also be fitted with a high-numerical-aperture condenser lens). The high-numerical-aperture objective lens has a very short depth of focus, allowing one to focus progressively through the section and, thus, potentially through many nuclei. The tissue is thus "optically sectioned." Nuclei sampled by an unbiased counting frame ***(28)***, are counted when a *unique event* occurs, such as when they first appear, disappear, or come into sharp focus. (Alternatively, if the nucleus of interest contains a single nucleolus, then nucleoli can be counted when they come into focus.) In practical terms, it is usually easiest to count the nuclei when they first *come into focus*, which generally corresponds to the equatorial region of the nucleus. This has been the *unique event* counted in most publications to date.

The important point here is that the nuclei are counted in 3-D space. The counting frame can either be located in an eyepiece graticule or overlaid and

displayed on a computer monitor. Multiplication of the area of the counting frame by the height of the disector (depth of section analyzed; given by a microcator fitted to the microscope) gives the *volume* of the tissue in the disector; hence, the number of nuclei that come into focus in the volume (numerical density) can readily be determined. For example, if the disector has a height of 10 μm (from the microcator) and an area of 1000 μm^2 (calibrated from a grid overlaid on the section), and 6 nuclei come into focus, then the numerical density for this disector sample is 6 nuclei/10,000 μm^3. A series of unbiased samples gained using the sampling strategies described in **Subheading 3.1.** will give an overall tissue estimate of average numerical density. This, multiplied by the volume of the reference compartment (determined using the Cavalieri principle) gives an unbiased estimate of the total number of the cells of interest. This experimental design constitutes the so-called optical disector/Cavalieri combination.

Generalizing the above, the numerical density of particles is estimated using

$$N_V = \frac{Q^-}{\text{Frame area} \times h}$$

where Q^- is the number of nuclei actually counted in a disector, frame area is the area of the unbiased 2-D counting frame, and h is the height of the disector. Because any estimate of the number of cells in a tissue involves counting multiple disectors, it is normal to associate each unbiased counting frame with one or more points. If the associated point hits the reference space, the volume of that disector is included in the total volume counted. Thus, the sum of points hitting the reference space (P) multiplied by the area of the counting frame associated with each point [a(p)] multiplied by the height of the disector is the total volume counted in a tissue and

$$N_V = \frac{Q^-}{\Sigma P \cdot a\,(p) \cdot h}$$

In addition to its theoretical advantages over the model-based, potentially biased, traditional stereological counting methods, the optical disector enjoys significant practical advantages over the physical disector and serial section reconstruction. The major advantage is that the optical disector does not require alignment of sections. The optical disector is a single section (at a time) technique. Physical alignment of sections, as required by the physical disector and serial section reconstruction, is a very time-consuming process, even at the light-microscopic level, and is often further complicated by small local distortions of tissue architecture caused by shrinkage/swelling and section compression artifacts.

1.7. The Optical Disector/Cavalieri Combination

To obtain an unbiased estimate of the number of a specified cell type in a defined reference compartment, the estimate of cell numerical density obtained with the optical disector is multiplied by the volume of the reference compartment, obtained with the Cavalieri Principle. The optical disector/Cavalieri combination has been described in a number of review articles ***(1,6,29–33)*** and has been used to estimate the following:

- The total number of neurons in rat brain cortex ***(34)***
- The total number of neurons in the human hippocampus ***(20)***
- The total number of neurons in the neocortex of alcoholics ***(27)***
- The total number of pigmented and nonpigmented neurons in the substantia nigra of patients with Parkinson's disease ***(35)***
- The total number of neurons in the neocortex of patients with senile dementia of the Alzheimer's type ***(15)***
- The total number of dying, surviving, and proliferating neurons in the rat dorsal root ganglion ***(36)***
- The total number of various cell types in rat and human cerebellar cortex ***(26,37)***.

2. Materials

2.1. Optical Disector

Establishing an optical disector facility requires histological hardware as well as the optical disector itself. The histology laboratory requires the following:

1. The facilities for processing and embedding tissue in glycolmethacrylate, or alternatively, the facilities for producing frozen blocks of tissue.
2. A microtome for cutting thick (20 μm at least) glycolmethacrylate sections, or a cryostat.
3. A glass knife maker.
4. The optical disector requires the following
   a. A bright-field microscope.
   b. A high-numerical-aperture (preferably 1.4) oil-immersion objective lens.
   c. A high-numerical-aperture condenser lens. Although optical sectioning has its greatest depth resolution when an oil-immersion condenser is used, in practice a dry condenser (numerical aperture approximately 0.9) is normally used for convenience.
   d. A microcator for measuring the height of the disector. Most groups use an electronic microcator fitted to the microscope and connected to a display unit.
   e. Nuclei can be counted directly at the microscope using the eyepiece lenses; alternatively, the microscope can be fitted with a video camera and the image viewed on a monitor. In this latter situation, a graticule containing an unbi-

ased 2-D counting frame can be placed in the projection lens and it will be seen on the monitor screen; alternatively, a stereological software program can be used to overlay a counting frame on the computer monitor. Ideally, the microcator reading can also be displayed on the monitor.

f. A motorized microscopic stage is ideal for obtaining a systematic uniform random sample of fields on sections. In the most advanced configurations, computer software drives the motorized stage in a sampling pattern predetermined by the user.
g. Several companies manufacture and sell apparatus for counting cells with optical disectors. The authors are familiar with, and recommend the following: The Olympus Denmark C.A.S.T. - Grid System and Stereo Investigator™ from MicroBrightField Inc. (USA).

3. Methods

3.1. Sampling

The successful implementation of these new stereological methods depends on appropriate sampling of the tissue of interest. Sampling is the most critical step of any stereological study, because unless the sample is representative of the tissue, there is little to be gained from a quantitative analysis. Indeed, because most scientists associate more importance to quantitative data than to qualitative data, quantitative data from a nonrepresentative sample can be particularly misleading.

Three sampling regimes have been used in stereological studies. Many early studies used fixed-point sampling where a section at a defined part of the tissue was assessed as typical. This type of sampling does not allow the determination of parameters describing the tissue as a whole. Data determined with fixed-point sampling only describe what is happening in the area sampled. Random sampling has been used in a number of studies, and so long as it is properly applied, it will always give an unbiased estimate. The major limitation of random sampling is that it can be inefficient, requiring sampling of a large number of fields to derive a stable estimate. The preferred method of sampling used in contemporary studies is the systematic uniform random (SUR) approach. This sampling approach is both unbiased and efficient. With this technique, which can be applied at all sampling levels (blocks, sections, fields), a sampling interval is first chosen (e.g., every 10th section). The first section is chosen at random from sections 1–10 (e.g., the seventh section). Subsequent sections are then sampled by adding 10 to the first section sampled (e.g., 7, 17, 27, 37, etc.) until the tissue has been completely sampled. A similar regime can be applied to sampling fields in sections.

3.2. How to Estimate the Total Number of Motor Neurons in the Lateral Motor Column Using an Optical Disector/Cavalieri Combination

We used an optical disector/Cavalieri combination to estimate the total number of motor neurons in the lateral motor column of the chick spinal cord at embryonic days 6, 8, 10, and 12 ***(2)***. The method is detailed as follows:

1. Fix the embryonic spinal cord. We immersion-fixed the spinal cords in Carnoy's fixative (*see* **Note 1**).
2. Process the spinal cords for embedding in glycolmethacrylate. Our laboratories currently use Technovit Embedding Resin from Kulzer Inc. (Germany) (*see* **Note 2**).
3. Exhaustively section the blocks at a nominal thickness of 20 µm, keeping count of the number of sections cut. Pick up and mount every 30th section with the first section chosen at random (in the interval 1–30) using a random number table (*see* **Note 3**).
4. Estimate mean section thickness (*see* **Note 4**).
5. Stain the sections, bearing in mind that it is essential to stain nuclei throughout the full thickness of the section. We used hematoxylin (Mayer's, 2 h) and eosin (0.1%, 1 h) (*see* **Note 5**).
6. Estimate the area of lateral motor column in the sampled sections. Point counting can be used, either through placing a grid in a microscope eyepiece, projecting sections onto a grid on a table in a semidarkened room, or overlay a grid on the image on a computer screen (*see* **Note 6**).
7. Estimate the volume of the lateral motor column (V_{lmc}) using the Cavalieri Principle:

$$V_{lmc} = 30 \cdot ta \cdot (p) \cdot \Sigma P$$

   where 30 is the inverse of the section sampling fraction, t is the mean section thickness, $a(p)$ is the area associated with each point in the stereological grid, and P is the total number of points counted on the lateral motor column (*see* **Note 7**).
8. Count neuronal nuclei with optical disectors. We used a ×63 oil immersion lens (numerical aperture 1.4) and disectors with a height of 10 µm (*see* **Fig. 1**). Fields were viewed via a color video camera and the image was displayed on a color monitor. A motorized stage was used to obtain a random systematic uniform sample of fields on the columns (*see* **Note 8**).
9. Estimate the numerical density of motor neurons in the lateral motor column ($N_{V_{neu,lmc}}$) using:

$$N_{V_{neu,lmc}} = \frac{Q^-}{10 \cdot \Sigma P \cdot a(p)}$$

   where Q^- is the actual number of neurons counted, 10 µm is the height of the disector, P is the sum of grid points lying on the sampled fields, and $a(p)$ is the area associated with each grid point (*see* **Note 9**).

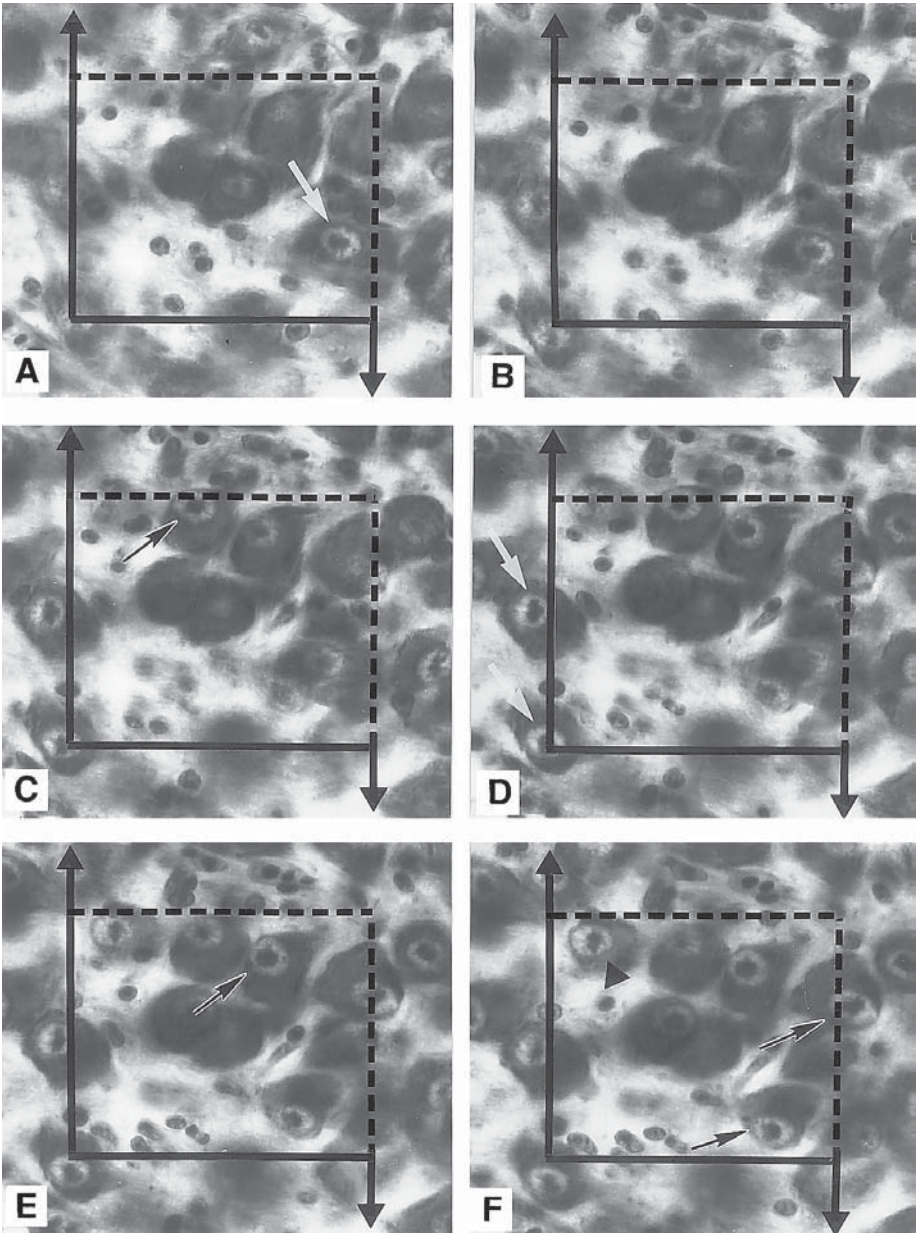

Fig. 1.

10. Estimate the total number of neurons in the lateral motor column ($N_{neu,lmc}$) using (*see* **Note 10**).

$$N_{neu,lmc} = N_{V_{neu,lmc}} \times V_{lmc}$$

3.3. Summary

The optical disector/Cavalieri combination has emerged as the method of choice for counting cell nuclei, and thereby cells in tissues. It is indeed fortunate that this unbiased cell-counting method was developed at around the same time as the new molecular and genetic methods for studying cell proliferation, cell fate, and cell population dynamics. Nevertheless, significant advances in stereological counting methods can be expected in the future. Perhaps the most immediate aim is to develop and optimize methods for counting subpopulations of defined neurons, identified on the basis of specific immunostaining. Here, issues of antibody penetration and staining optimization are critical.

At the same time, it is important that the new unbiased methods quickly replace many of the traditional methods used to quantify neuronal populations.

Fig. 1. *(see opposite page)* Photomicrographs of a series of optical sections (separated by 2 µm) through a 20-µm glycolmethacrylate section of a chick lateral motor column. The optical sections were obtained using a high-numerical-aperture oil-immersion lens and a matching condenser lens. An unbiased counting frame ***(28)*** is superimposed on each optical section, with the solid line representing the forbidden line. Starting at the first section, **A**, neuronal nuclei are counted when they *come into focus*. Those nuclei that touch the solid line when they come into focus are not counted. In A, one nucleus is in focus (white arrow), but is not counted because it did not come into focus in the disector—it was already in focus. In **B**, *no* nuclei come into focus. In **C**, one nucleus comes into focus (black arrow). It is sampled by the sampling frame and is therefore counted. In **D**, two nuclei come into focus (white arrows). Both touch the forbidden line and are therefore not sampled by the frame and not counted. One nucleus comes into focus in **(E)** and two nuclei come into focus in **F** (black arrows). All three nuclei are sampled by the frame and are therefore counted. Between sections A and F, a distance of 10 µm has been analyzed—this is the height of the disector. Four nuclei (black arrows) have been counted. The numerical density of motor neurons in this sample is equal to four nuclei in the disector volume (the product of the disector height [10 µm] and the area of the sampling frame). It is important to note that the division of the total disector height into these discrete steps of 2 µm is only for the purpose of illustration. In practice, one focuses down smoothly through the section and counts nuclei when they *come into focus*. Note also that one nucleus is close to focus in F (arrowhead). The guard region below the disector volume can be used to check whether this nucleus is in focus in F or comes into focus below F. If this nucleus is in focus in F, then the total number of nuclei counted in this disector would be five.

In particular, the use of profile counts and numerical densities has, unfortunately, led to the publication of enormous amounts of misleading and inaccurate data. The international Neuroscience community is now well aware of this, and presumably the continuation of these past practices will cease in the very near future.

4. Notes

1. Carnoy's fixative is a particularly fast-penetrating fixative for neural tissue. Tissue shrinkage is substantial with Carnoy's but has no effect on number estimation with this experimental design. It is best to avoid glutaraldehyde as a fixative, unless tissue is also to be taken for electron microscopy. In our experience, glutaraldehyde fixation frequently causes problems when counting nuclei in thick sections.
2. Janson and Moller ***(21)*** performed their immunohistochemical procedures on free-floating frozen sections. However, in our experience, glycolmethacrylate sections are superior with respect to morphology and staining of nuclei.
3. This can be varied with experience, as long as a minimal number of sections is sampled.
4. This is easily done using a hand-held micrometer. Knowledge of the number of sections cut from a block measured for thickness, both before and after the sectioning process, allows the calculation of the average thickness per section, a measurement more accurate than the nominal thickness indicated by the microtome. Janson and Moller ***(21)*** used sections that were cut at a mean thickness of 30 µm. However, following staining, dehydration, embedding, and cover-slipping, mean section thickness was just 16.7 µm. This change in section thickness was accounted for by the experimental design, but failure to measure the thickness of the sections actually used for the optical disector counts would have led to a significant overestimation of cell number. We have used a micrometer to check mean section thickness of stacks of 20 and 25 µm sections cut using a Reichert–Jung Supercut microtome and found consistent agreement between the nominal and the actual thickness. Section thickness measured from the top to the bottom of individual sections, with a high-numerical-aperture oil-immersion lens on a microscope equipped with a microcator was also in good agreement.
5. Nuclear staining is critically important. It is important to ensure that nuclei throughout the section thickness are stained and therefore visible and countable, and that nuclei are not understained or overstained. In pilot studies, it is advisable to stain trial sections for varying periods of time and to check stain penetration. Typically, much longer staining times are required than when staining paraffin sections of standard thickness. Optimum staining requires not only satisfactory stain penetration but also staining of nuclear material such that it is crisp and sharp, as the optical disector counting most often depends on identifying when the nucleus comes into sharp focus. Understained or overstained nuclei are difficult to count. A variation of this technique has used immunohistochemical staining in combination with cresyl violet staining. Janson and Moller ***(21)*** performed their

immunohistochemical procedures on free-floating frozen sections. Sections were then mounted and carefully counterstained with cresyl violet to obtain a violet staining still allowing for visualization of the immunohistochemical reaction product.

6. As described in **Subheading 3.2.**, 10–15 sections evenly spaced through the lateral motor column with a random start should be sufficient to obtain an estimate of volume with an acceptable level of precision. If the section area is to be estimated using point counting, which, in the interest of speed and accuracy, we recommend, no more than a total of 100–200 points need be counted on the entire lateral motor column to estimate volume with the Cavalieri method.
7. Section thickness means actual (not nominal) section thickness, as measured from the micrometer readings. For example, instead of the nominal thickness of 20 µm, as read off the microtome, our sections tended to be of the order of 19.5 µm.
8. How many cells do you need to count? 120–150. There are a number of important procedural matters to consider when counting nuclei with optical disectors:
   - It is important to define a guard region of say at least 5 µm at the top and bottom of the section. The disector should not extend into these guard regions. The guard regions are used because (1) the surface of the section may be irregular and (2) nuclei may be pulled out of sections during sectioning, and would therefore not be counted.
   - When counting nuclei when they come into focus, those nuclei in focus in the top plane of the disector volume are not counted because they did not come into focus in the disector—they were already in focus. In contrast, those nuclei in focus in the bottom plane of the disector are counted because they did come into focus.
   - A nucleus is only counted if it is sampled by the unbiased counting frame at the moment the unique event occurs. Again, if this unique event is defined as when the nucleus comes into focus, then it does not matter whether the nucleus is not sampled by the counting frame before or after it comes into focus, what matters is if it is sampled at the precise moment that it comes into focus.
   - It is generally more efficient to count one or two nuclei per frame over a larger number of frames than the converse. This is especially important when nuclei are unevenly distributed in the reference space.
9. Of course the height of the disector can vary with section thickness in different studies, provided the guard region is observed.
10. In our study on the lateral motor column ***(2)***, approximately 10 min was required to estimate V_{lmc}. Between 16 and 62 optical disectors were counted per lateral motor column. The total number of neurons counted per column ranged from about 105 to 230. Approximately 20 min was required to count this number of neurons in each column. Estimates of neuronal number had coefficients of error of approx 10%.

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