Production of compartmented cultures of rat sympathetic neurons

Robert B Campenot, Karen Lund & Sue-Ann Mok

Department of Cell Biology, University of Alberta, Edmonton, Alberta, Canada. Correspondence should be addressed to R.B.C. (bob.campenot@ualberta.ca).

Published online 3 December 2009; doi:10.1038/nprot.2009.210

The compartmented culture, in which primary neurons plated in a proximal compartment send their axons under silicone grease barriers and into left and right distal compartments, has enhanced the experimental capabilities of neuronal cultures. Treatments can be applied separately to cell bodies/proximal axons or distal axons, and cell bodies/proximal axons and distal axons can be separately harvested and analyzed. Distal axons can be axotomized, and the neurons can be studied while their axons regenerate. Construction of the culture dishes requires 3 h for 48 cultures, and preparing the neurons also requires 3 h. Compartmented cultures provide enough cellular material for biochemical analyses such as immunoblotting. The uses of compartmented cultures have included studies of neurotrophic factor retrograde signaling, axonal transport, and axonal protein and lipid biosynthesis. Here we focus on sympathetic neurons cultured from neonatal rats and provide protocols for the production and some of the uses of compartmented cultures.

INTRODUCTION

In compartmented cultures (Fig. 1), the neuronal cell bodies, dendrites (which we include as part of the cell body) and proximal axon segments of cultured neurons reside in the fluid environment of a proximal compartment. The cell bodies and proximal axon segments are separated from the fluid environment of the distal axon segments contained in the distal compartments. In this system, distal axons can be supplied with culture medium of different composition than the medium bathing the cell bodies and proximal axons, and the distal axons can be harvested and analyzed separately from the cell bodies and proximal axons. As neurons in vivo extend their axons into environments that are separate from the environment of the cell bodies, neurons in compartmented cultures can be studied under circumstances that more directly model the in vivo situation than is possible using conventional mass cultures. For example, neurons in vivo are exposed to neurotrophins such as nerve growth factor (NGF) primarily at their axon terminals, which can be more accurately modeled by applying NGF selectively to distal axons than by applying NGF over the entire surface of the neuron, as is the case in conventional cultures. Drugs that affect signaling mechanisms and other processes can be applied selectively to cell bodies/proximal axons or distal axons to help determine where cellular functions are localized. In addition, the location of the production of proteins and lipids and the transport of molecules along the axons in the direction away from the cell bodies (anterograde axonal transport) or toward the cell bodies (retrograde transport) can be more readily examined in compartmented cultures than in conventional cultures. As the barriers are established first, and the axons cross the barriers by growing under them, it is possible to experimentally investigate what constituents are necessary in the medium supplied to distal compartments for axons to enter and grow within them. Studies using compartmented cultures have shown the requirement for the presence of NGF in distal compartments for the growth of axons of sympathetic neurons. In addition, as there is a restricted extracellular space surrounding the axons under the silicone grease barrier, electric current passed across the barrier is channeled partly through the axons. Therefore, the passage of current between electrodes placed into the medium in the proximal and distal compartments can be used to generate action potentials in axons that cross the barrier.

In compartmented cultures, axons growing from neurons plated in the proximal compartment are guided to the left and right between a series of parallel scratches made in the collagen substrate (Fig. 1). Axons grow poorly on the bare plastic exposed by scratching the collagen, and axonal growth is largely confined to the collagen-coated surfaces between the scratches. The collagen substrate in the scratched region is wet with culture medium before seating a Teflon divider coated with silicone grease onto the substrate (Fig. 2). The silicone grease does not adhere to the collagen, which allows the axons to grow beneath the silicone grease barrier. Although axons can grow under the silicone grease between compartments, medium components do not significantly leak or diffuse across the barrier.

Cultured neurons must produce axons several millimeters long to be used in compartmented cultures. Rodent sympathetic neurons, dorsal root ganglion neurons and retinal ganglion cells have been used. Since the discovery of the Trk family of neurotrophin receptors, the compartmented culture method has been used in studies of the mechanisms by which NGF and other neurotrophins applied to axon terminals produce retrograde signals to the cell bodies. Sympathetic neurons and dorsal root ganglion neurons have been the principal neuron types used in the study of neurotrophins. Sympathetic neurons from neonatal rats are the best performers in compartmented cultures, and they have been the main subject of our research. Using sympathetic neurons, we obtain sufficient cellular materials from three to five cultures per treatment group to carry out biochemical analyses, such as immunoblotting, comparing extracts from cell bodies/proximal axons to extracts from distal axons. Compartmented cultures of sympathetic neurons made with extra large compartments have been used to provide additional material for biochemical analyses, but in our experience, this is unnecessary.

Applications of compartmental cultures

The principle applications for which we have used compartmented cultures include determining the action of drugs upon mechanisms...
in the cell bodies/proximal axons versus the distal axons; determining
the trafficking, processing and action of neurotrophic factors
and other natural molecules in the cell bodies/proximal axons ver-
sus the distal axons; and labeling of molecules produced by and/or
residing in cell bodies/proximal axons versus the distal axons.

Kinase inhibitors and other agents can be applied independ-
ently to cell bodies/proximal axons or distal axons. The actions of
membrane-permeant agents applied to cell bodies/proximal axons
or distal axons are confined to the region of the neuron to which
the agent is applied. For example, if a membrane-permeant drug
is applied to the distal axons, it will diffuse across the axonal mem-
brane, and the cytoplasm of the distal axons will rapidly equilibrate
to contain the same drug concentration as the bathing medium.

If a drug were to move retrogradely from the distal axons into the
cytoplasm of the proximal axons, it would rapidly diffuse across the
proximal axon membrane, equilibrating with the vastly larger volume
of the medium bathing them, and therefore would not achieve a
biologically effective concentration in the cell bodies and proximal
axons. In contrast, agents that are enzymatically altered after they
cross the membrane into the cytoplasm so that they are retained
within the neuron, such as BAPTA-AM (1,2-bis-(o-aminophenoxy)-
ethane-N,N,N',N''-tetraacetic acid, tetracacetoxymethyl ester), may
travel through the neuron and produce effects in regions where they
are not directly applied. In addition, it is important to realize that cell
bodies are never completely isolated because they are always accom-
panied by proximal axon segments within the proximal compartment.

Therefore, when a drug has an effect when applied in the proximal
compartment, this may suggest that the drug operates on mechanisms
in the cell bodies, but this requires corroboration by other evidence
because the proximal axons are also accessible to the drug.

Compartmented cultures allow quantitative measurements in
studies of retrograde transport. Retrograde transport of [125I]NGF
has been examined for many years using in vivo experiments where
[125I]NGF is injected at the axon terminal field of NGF-responsive
neurons, and after a suitable incubation, the [125I]NGF accumu-
lated in the neuronal cell bodies is measured. Such experiments can
establish that NGF retrograde transport occurs in the neurons of
interest, but the amount of [125I]NGF retrogradely transported
during the incubation period cannot be determined, as the [125I]NGF
that was degraded and released into the circulation after arriving
at the cell bodies is not included in the measurement. In com-
partmented cultures of rat sympathetic neurons, the [125I]NGF liber-
ated by the breakdown of retrogradely transported [125I]NGF in the
cell bodies is released into the medium in the proximal compart-
ment where it can be measured along with the [125I]NGF in the
cell bodies and proximal axons. Therefore, the amount of
[125I]NGF transported is the sum of the [125I]NGF remaining in the
cell bodies/proximal axons plus the amount released into the bathing
medium. Importantly, at incubation times of ≥ 18 h, the amount of
[125I] that has been released into the medium is a reasonable esti-
mate of the total amount transported as it far exceeds the amount
retained in the cell bodies/proximal axons. Thus, transport can be
repeatedly assessed by determining the amount of [125I] released
into the medium without collecting extracts of the neurons. This
allows transport to be assayed in the same population of neurons
sequentially under different experimental conditions.

Metabolic labeling studies using compartmented cultures can
provide information about the site of synthesis of lipids and pro-
teins. By supplying radiolabeled precursors of phospholipids and
cholesterol to the cell bodies and proximal axons versus the distal

PROTOCOL

Figure 1 The compartmented culture system. (a) Photograph and
(b) schematic diagram of a three-compartment culture dish. A Teflon
divider, seated to the floor of a 35-mm tissue culture dish with silicone
grease, partitions the dish into three media compartments—left and right
distal compartments (III), which are separated by a septum, and a proximal
compartment (I) consisting of a slot in the septum that forms an inlet
from the perimeter of the culture dish. Neurons are plated in the proximal
compartment and extend axons to the left and right on collagen tracks
formed between scratches in the substratum (indicated by horizontal parallel
lines), underneath the Teflon barriers and into the distal compartments. The
locations of the cell bodies and proximal axons (CB/PAx) and distal axons
(DAx) are indicated. (c) Photomicrograph of neurons cultured for 7 d in a
compartmented culture, which were immunostained for tubulin. The upper
panel shows neurons in 4 out of a total of 20 tracks in a compartmented
culture. Dotted lines indicate the borders of the grease barriers separating
the compartments. Axons under the grease barriers are unstained. The lower
panel is a higher magnification of the area boxed in the upper panel. The
arrow indicates a cable of axons and the arrowhead indicates a scratch in the
collagen substratum. (d) Compartmented cultures with intermediate
compartments. The location of cell bodies and proximal axons, intermediate
axons (IAx) and distal axons are indicated. The diagram on the left is a
culture constructed using a three-compartment Teflon divider. A cylinder of
silicone grease is laid down before attaching the divider to create a restricted
area near the Teflon barrier in the proximal compartment (I) in which the
neurons are plated (also see Fig. 7). Axons extend to the right, across one
barrier, through the intermediate compartment (II) and into the distal
compartment (III). The diagram on the right is a culture constructed with a
five-compartment Teflon divider (Camp 13, Tyler Research Corporation). Axons
extend outward from a proximal compartment in the center (I), across the left
and right intermediate compartments (II) and into the distal compartments
(III). Panel c is reproduced with permission from ref. 5. All experiments using
live animals must conform to national and local regulations.
Figure 2 | Assembly of three-compartment cultures. (a) A collagen-coated, 35-mm tissue culture dish is scratched and wet with a droplet of medium. Note the position of the scratches within the culture dish area. The droplet of medium should be spread to cover the scratched region where the barriers will be situated. (b) Image of the position of the Teflon divider in relation to the hemostatic forcepts. Diagrams are the side view (left) and top view (right) of the forcepts and divider when the back of the divider is placed on a work surface. The forcepts should clamp the divider at the solid portion of the septum. (c) Applying silicone grease to the divider. Cylinders of grease are applied to the Teflon divider in the pattern and direction indicated by the blue arrows in the left diagram. The right diagram depicts a divider after the grease is applied. Note that in areas where two cylinders of grease intersect, the cylinders are touching and slightly overlapping. (d) Attaching the divider to the dish. The diagram depicts a top view through the stereo microscope of the prepared tissue culture dish after it is flipped upside-down and seated onto the divider-forcepts assembly. To seal the grease to the dish, a pair of fine-tipped forcepts (or other pointed implement) is used to press down gently on the bottom of the dish along the cylinders of grease, except in the areas where the grease contacts the droplet of medium. (e) Example of proper and improper grease seals. The left diagram depicts a section of the divider showing a cylinder of grease before seating the divider. The right diagrams depict the grease after seating the divider. The clear region running along the center of the grease indicates correct sealing of grease to the culture dish in the diagram marked with a check. The diagram marked with an X depicts a gap in the grease seal, which will lead to medium leakage. (f) The completed compartmented culture. A small mound of grease is applied to the opening of the proximal compartment. Two drops of the medium have been added to each distal compartment (shown in red).

Figure 3 | Use of fluid gradients to study the effects of fluid movement on the growth of axons. A narrow glass partition is used in this approach, making the distance across the barrier between compartments shorter than the 1 mm typically used in conventional compartmented cultures. In this way, short axons originating from cell bodies positioned near the barrier can cross into the distal compartment. This approach is amenable for techniques involving small numbers of neurons such as immunohistochemical analyses, but the amount of cellular material is too small for conventional analyses of proteins and lipids. A second approach involves retinal ganglion cells, which are central nervous system neurons that produce the long axons in the optic nerve, plated in conventional compartmented cultures. Although certainly more challenging than using sympathetic neurons, retinal ganglion cell cultures can provide enough material for biochemical analyses. Also, the protein composition of spinal cord axons has been studied in compartmented cultures, and compartmented cultures have been used in a study of axon guidance in the chick retinotectal system.

Recently, a technique has been employed using a different principle from compartmented cultures to create a barrier. Microfluidic barriers are created, which consist of narrow channels through which axons grow, such that the extracellular space is kept to a minimum. Movement of substances across the barrier is controlled by establishing a flow through the extracellular space in the channels driven by a pressure gradient produced by maintaining different fluid levels in the compartments on each side of the barrier. For example, using this kind of barrier it would be possible to supply distal axons with NGF while maintaining a higher fluid level in the proximal compartment, thereby creating a flow of culture medium not containing NGF into the distal compartment. This flow is, presumably, sufficient to oppose diffusion of NGF into
the distal compartment, but small enough to only slightly dilute the NGF supplied in the distal compartment. We have no experience with this approach, but it would seem to be problematic when both the proximal compartment and distal compartment contain substances that must be confined to the compartment in which they are supplied. Major advantages are that the culture substratum is on glass, which permits the use of inverted confocal microscopy, and shorter axons are compatible with this approach. A disadvantage is that the amount of cellular material is too small for conventional analysis of proteins and lipids.

Limitations of the protocol

The presence of the Teflon divider produces menisci in the surface of the culture medium that interfere with the light path in inverted, phase-contrast microscopy. This can reduce the contrast, making visualization of the neurons difficult, especially in the compartment and close to the barriers in the distal compartments. This arises because the menisci distort the ring of light produced by the phase condenser so that it is not circular and therefore cannot be aligned on the target annulus in the phase telescope. We have solved this problem by constructing a rotating shield that blocks ~80% of the ring of light allowing only a segment of the ring of light to pass through. Also, we have modified the mechanical support of the light source/condenser to increase the range of movement for adjustment in the XY plane. These modifications make it possible to rotate the shield and move the light source/condenser such that the segment of the light ring that is not blocked by the shield projects nearly completely onto the target annulus, which results in a reasonable phase-contrast image (Fig. 3). As the distortion arising from the meniscus varies with location in the culture dish, adjustments must be made whenever the field of view is moved. Also, the adjustments produce better phase-contrast images at higher magnification, as the curvature of the segment of the meniscus that must be compensated is smaller, and therefore more uniform, when the field of view is smaller. The need for this modification varies for different microscopes. This modification for our Nikon Diaphot inverted microscope has been provided by Tyler Research Corporation. A limitation of compartmented cultures is that plating the neurons on the floor of a plastic tissue culture dish makes it difficult to perform inverted confocal microscopy.

Compartmented cultures require about twice the time to produce as conventional cultures, and medium changes are also more time-consuming. The compartmented culture technique is sufficiently complex that it may not be practical for a laboratory to produce these cultures for use in a few studies. However, investigators should not hesitate to embark on a research program involving the extensive use of compartmented cultures, as the capabilities of the system will justify the effort.

Experimental design

Compartmented cultures, in their most commonly used configuration, consist of a 35-mm culture dish in which a Teflon divider is seated with silicone grease on the culture substratum, dividing the culture dish into a center compartment and left and right distal compartments (Fig. 1a, b). The center compartment is a narrow slot occupying half of the septum that forms the partition between the distal compartments. The center compartment is open to the perimeter of the culture dish, which provides an additional volume of medium of up to 2.5 ml and facilitates changing the medium, as the medium in the dish perimeter can be replaced without disturbing the neurons. When it is desirable to have a low volume (≤80 µl) of medium in the center compartment, such as during incubations with isotopes, the center compartment can be supplied with medium and the dish perimeter left empty. Although the center compartment is open to the dish perimeter, a mound of silicone grease placed on the dish floor at the mouth of the center compartment during dish assembly prevents medium in the center compartment from flowing out into the dish perimeter. However, the mound of grease does not interfere with medium in the center compartment connecting with medium supplied in the dish perimeter. The distal compartments can each hold about 0.75 ml of medium, but can be supplied with 200 µl when a low volume is desired.

We have tried many variations in the procedure, and we now present our refined protocols. Although our production techniques have been published in reasonably up-to-date form, there is no single publication where the features of the cultures themselves are described in detail so that investigators know their full potential, their limitations and can assess the performance of the cultures produced in their own laboratories. There are several techniques for experimental manipulation of the cultures described here which have never been previously described in detail. Using our methods, compartmented cultures are easily and reliably constructed. It is routine in our laboratory for an experimenter to construct 48 cultures in 3 h.

Production of compartmented culture dishes

Stereo microscope: The major requirement for general equipment, in addition to the equipment ordinarily available in a cell culture laboratory, is a
Before assembling the cultures, the Fig. 4: In order to cross between compartments, the culture substratum is a film of dried silicone grease barriers, greatly decreasing the amount of axons crossing into distal compartments and increasing the amount of distal axons remaining in the compartment containing the cell bodies. The width of the collagen tracks is critical for axons to cross into the distal compartments. The optimal track width for rat sympathetic neuron cultures is about 200 µm. Extremely narrow tracks are not conducive to axonal growth, and if tracks are too wide the growth cones have enough room to make U-turns away from the barrier. If axons grow across the scratches and elongate perpendicular to them, it defeats the purpose of the tracks, and crossing of distal axons into the distal compartments is much reduced.

**Teflon dividers:** Reusable dividers machined from Teflon (Tyler Research Corporation) form the compartments. The dividers are acid washed and sterilized before their initial use and between uses (see EQUIPMENT SETUP).

**Silicone grease:** In order to cross between compartments, the axons grow on the collagen substratum underneath a layer of silicone grease between the Teflon divider and the substratum. The silicone grease is applied to the surface of the divider to be seated on the floor of the culture dish. For this purpose, we use a 1-ml syringe fitted with an 18-G hypodermic needle with the point ground off to make a squared-off, blunt opening (Fig. 5). The syringe/needle assembly is loaded with silicone grease and sterilized by autoclaving. Therefore, either a glass syringe or a custom made, all stainless steel syringe (Tyler Research Corporation) must be used, and the needle must be all steel. The syringe must have a Luer-Lock connector to make a squared-off, blunt opening. Using fine forceps, embed the 21 pins in the Parafilm, side by side in a row, oriented with the tip half of the pins embedded in the Parafilm and the back half extending beyond the edge of the aluminum sheet. (c) Align the tips evenly by placing the edge of a razor blade on the plate, parallel with the row of pin tips and pushing back on the tips until they are all flush with the blade edge. Turn off the hot plate and allow the aluminum plate to cool. (d) Join the pins together at their back ends with epoxy cement. After the epoxy cement has set, flip the aluminum plate over and apply epoxy cement to the other side of the back end of the pins. (e) Prepare a handle by sawing a slot, wide enough to accommodate the pin assembly and equal to or slightly shorter than the length of the pins, into a Phenolic or Plexiglas rod that is about 1.3 cm in diameter and 15 cm long. The end of the rod may be tapered as shown. (f) After the epoxy cement joining the pins has set, lift the pin assembly from the Parafilm. Coat the back end of the pin assembly and the inside of the slot with fresh epoxy cement. Insert the back end of the pin assembly into the slot with about 10 mm of the pin tips projecting from the handle. Build up the epoxy cement toward the pin tips, taking care that the cement does not extend into the last 3–5 mm. Allow the epoxy cement to cure.

**Wetting the substratum:** Before assembling the cultures, the scratched region of the collagen substratum is wet with culture solution, and the surface of the divider to be seated on the floor of the culture dish is wet with culture solution.

**Culture substratum:** The culture substratum is a film of dried collagen formed on the floor of a 35-mm tissue culture dish. Sterile collagen can be obtained commercially or extracted in acetic acid from rat tail tendons using a sterile procedure. Collagen solution is applied to the floors of tissue culture dishes and air-dried. Once the collagen has dried, a series of 21 parallel scratches are made in the substratum using a pin rake. The pin rake is composed of 21 insect pins attached together with epoxy cement and mounted in a handle (Fig. 4). The function of scratching the substratum is to remove the collagen and expose the bare plastic beneath the surface of the dish floor, which is a poor substratum for growth cone attachment. The result is that axon growth is confined to the collagen-coated tracks formed between the scratches. This directs the axons to grow to the left and right where they encounter the silicone grease barriers, grow under the silicone grease and emerge into the distal compartments. Omitting the scratching step results in cultures where the axons tend to be deflected away from the...
medium to prevent silicone grease from adhering directly to the substratum in this region when the Teflon divider is seated (Fig. 2a). Axons cannot penetrate barriers where the silicone grease is directly adhering to the collagen. Pre-wetting the collagen results in a ‘seal’ in which the silicone grease is pressed against the wet collagen but does not directly adhere to the substratum. The growth cones can grow along the wet collagen and cross under the silicone grease into the distal compartment. The silicone grease in the regions of contact between the divider and the dry collagen substratum adheres directly to the substratum, providing the mechanical stability needed to hold the Teflon divider in place and keep the silicone grease in the pre-wetted region tightly pressed against the substratum. As the medium in the droplet used to wet the collagen is present in all compartments, distal compartment medium is used because it does not contain rat serum and vitamin C, which are only supplied in proximal compartments.

It is important not to let the medium used for wetting the collagen run outside the area of the scratches before the divider is seated, as this can interfere with scaling of the perimeter of the divider to the dish. In practice, we generally leave one or two tracks at each edge dry, to reduce the risk of the droplet escaping beyond the scratches. The droplet of medium must not be allowed to significantly evaporate, as the droplet does not extend the full distance along the tracks, and evaporation can leave a deposit on the substratum coinciding with the edge of the droplet across the tracks. This deposit can form an invisible barrier to axon growth that remains after the distal compartments have been filled with medium. Therefore, it is best to complete the construction of each culture dish and add medium covering the full length of the tracks within 5 min after placing the droplet.

Assembling the cultures: The cultures are assembled after the floors of the dishes have been coated with collagen, scratched and the scratched region of the dish has been wet with culture medium. Working in a sterile environment outfitted with a binocular microscope, a sterile Teflon divider is clamped in a hemostatic forceps (Figs. 2b and 5) and is placed on the work surface so that the divider is held horizontally under the dissecting microscope. Cylinders of silicone grease ejected from the syringe are applied to the edge of the divider that will make contact with the culture dish (Fig. 2c). To ensure a continuous seal, the connecting points between cylinders of grease should be overlapping and touching. Grease must be applied as neatly as possible along the narrow regions of the septum that form the barriers under which the axons will cross between compartments. After the grease has been applied to the Teflon divider, the lid is removed from a prepared culture dish, the dish is picked up with fingers and inverted quickly along the axis parallel with the scratches so that the droplet does not run. Then, while viewing the divider under the binocular microscope, the dish is held in position over the divider, oriented so that the scratches will cross under the barriers between the proximal and distal compartments and placed so that the dish is resting gently on the silicone grease (Fig. 2d). Next, a pair of fine forceps (or other pointed implement) is used to gently press on the bottom of the culture dish around the perimeter of the divider and along the solid portion of the septum separating the left and right compartments, causing the silicone grease cylinders to flatten slightly and to adhere to the floor of the dish (Fig. 2e). Pressing down with the forceps in the regions where the scratches cross under the divider is avoided, as this may cause the cylinder of grease in these regions to flatten too much, making it more difficult for axons to grow across, and grease may squeeze into and obscure the center compartment. Too much pressure can also cause the grease to displace the medium under the barrier and adhere directly to the collagen, which will prevent axons from crossing. If grease adheres to the scratched region, grease residue will remain on the substratum after the divider has been removed. To release the dish, the hemostatic forceps holding the divider-dish assembly is picked up, turned over so that the dish is right-side-up, and the dish is hovered just above the work surface. Then the dish is released from the forceps, allowing it to drop gently to the work surface. Placing the dish on the work surface while the divider remains clamped in the forceps is avoided as this can cause the position of the divider to shift. Using the silicone grease syringe, a small mound of silicone grease is placed on the floor of the dish at the opening of the proximal compartment (Fig. 2f) to form a hydrophobic barrier on the dish floor. This will prevent cell suspension from spilling out of the center compartment when the neurons are plated and prevent the medium from spilling out when the center compartment is filled with medium but the dish perimeter is left empty. To prevent the original droplet from drying out, two drops of distal compartment medium are placed on the scratched region of the substratum in each distal compartment, ensuring that the scratched region is completely covered (Fig. 2f). This completes the dish assembly, and the cover is placed on the dish. The assembled dishes are placed in a 37 °C, 5% CO₂ incubator for a minimum of 3 h before filling the distal compartments with medium. During this time, the weight of the Teflon divider settling down on the silicone grease will cause the grease to spread slightly, improving the seal and allowing grease to flow into areas between the divider and the substratum that may otherwise leak. As the grease cannot spread onto collagen that has been wet, once medium has contacted and wet the substratum, the settling of the divider and spreading of the silicone grease will cease. After 3 h, the distal compartments are filled with the medium. Leaving the cultures for a longer period of time before filling the distal compartments is avoided as this can cause the dishes to dry out, because the volume of the medium is small and its surface area relatively large. Until the neurons are plated, the center compartment will have only the medium applied in the droplet used to wet the collagen tracks before dish assembly, and the dish perimeter will contain no medium.

Culturing rat sympathetic neurons. Culture media: The culture media recipes we use follow the original formulation developed for culturing rat sympathetic neurons. Although this formulation yields excellent results, it uses rat serum, which is expensive to obtain commercially and expensive and labor-intensive to prepare in the laboratory. Some investigators have used other media formulations, but it must be kept in mind that some conditions may produce diminished axon growth and may reduce the ability of axons to cross into distal compartments. We use methylcellulose in all our culture medium. Methylcellulose thickens the medium, which reduces the shearing forces caused by fluid movements that can perturb the attachment of the neurons to the substratum. It facilitates the wetting of the collagen before seating the Teflon divider, and it prevents neurons from settling in the syringe during plating. There are variations in media and culture procedures used by other laboratories to produce compartmented cultures of sympathetic neurons. Because it is relatively rare for full photographs of the neurons to be included in published papers, it is difficult for us to assess the effect of these variations. In this protocol, we
Figure 6 | Dissection of superior cervical ganglia from a neonatal rat. (a) A head pinned out with the nose at the top. (b–g) Close-up view of the region boxed in a at progressive points in the dissection. The dissection field is shown with both (b) superior cervical ganglia in place (c) outlined with dashed lines. The dissection field is shown after the superior cervical ganglia on the left side has been removed showing the (d) bifurcating carotid artery outlined with (e) dashed lines. The dissection field is shown after the superior cervical ganglia on the right side has been removed showing the (f) bifurcating carotid artery outlined with (g) dashed lines. All experiments using live animals must conform to national and local regulations. Note that these photographs have been slightly retouched for clarity using Adobe Photoshop.

have adhered to formulations and procedures with which we have experience and know are effective.

For compartmented cultures of rat sympathetic neurons, we only provide the medium supplemented with rat serum in the proximal compartments that contain the cell bodies. Rat serum is omitted from the medium provided to the distal compartments containing the distal axons where its inclusion can promote increased cabling of the distal axons and detachment from the substratum. Vitamin C, which is included in the standard medium recipe, can cause visible damage when fresh medium containing it is supplied to the distal axons, and therefore vitamin C is included only in the medium supplied to cell bodies and proximal axons. Cytosine arabinoside is provided in proximal compartments containing the cell bodies for the initial 5–7 d after plating to kill non-neuronal cells. It is not included in the medium supplied to distal compartments, as these compartments are not exposed to cell suspension at plating.

NGF is also a variable in the culture medium. Most of the experiments that we perform use cultures that have been established 5–7 d before the start of the experiment. Rat sympathetic neurons are heavily dependent on NGF for survival for the first 2 weeks in culture, after which the survival requirement diminishes. Therefore, NGF must be supplied in the compartment in which the neurons are initially plated. For the initial culture conditions, we provide 10 ng ml⁻¹ NGF in the proximal compartments in which the neurons are initially plated and 50 ng ml⁻¹ NGF in the distal compartments into which the axons will grow. This results in cultures in which most of the axonal material is located in the distal compartments after 5–7 d (Fig. 1c), presumably because there is more branching of axons growing in compartments containing 50 ng ml⁻¹ NGF than in compartments containing 10 ng ml⁻¹ NGF, and axons may be less likely to grow from distal compartments containing the higher concentration of NGF back into the proximal compartments containing the lower concentration. However, a gradient of increasing NGF is not necessary for axons to cross into distal compartments; cultures supplied with 50 ng ml⁻¹ of NGF in the proximal compartments and the distal compartments still display substantial axonal growth into the distal compartments.

After 5–7 d, the distal axons of the neurons are sufficiently established in the distal compartments so that the NGF can be withdrawn from the proximal compartments containing the cell bodies and proximal axons, and neuronal survival is supported solely by the NGF supplied to the distal axons. The neurons will survive, and distal axons terminating in the proximal compartments will degenerate. This configuration is useful for studies of neurotrophin retrograde signaling mechanisms.

Preparing the neurons: Sympathetic neurons are obtained from superior cervical ganglia dissected from 0- to 2-d-old rats (Fig. 6).

We use Sprague–Dawley rats as they are readily available. There are two ganglia per rat, and we generally dissect ganglia from 10 rats to make 48 cultures. Superior cervical ganglia lie in the neck along the carotid artery and are easily located and removed. It is important to avoid tearing the sac surrounding the ganglia during dissection, as tears will allow neurons to escape, reducing the yield. Any attached carotid artery or other extraneous tissue should be removed from the dissected ganglia to reduce the number of non-neuronal cells plated in the cultures. Collagenase is used to weaken the collagen structure of the ganglia, and trypsin is used to weaken protein attachments of the neurons. As collagenase degrades quickly, the collagenase solution is thawed immediately before use. A plugged Pasteur pipette and bulb rather than aspiration is used when removing the medium as ganglia may float, and it is easy to inadvertently aspirate them. After these treatments, the tissue is mechanically dissociated into individual neurons. The neurons are spun down into a pellet that is resuspended in the culture medium. Collagenase is included only in the medium supplemented with rat serum in the proximal compartments. The neurons settle and attach to the substratum overnight, and then the perimeter of the dish is filled with the culture medium.

Changing the medium: Care must be taken not to disturb the neurons during medium changes. For routine medium changes, we aspirate the medium in the perimeter of the culture without disturbing the medium in the center compartment directly bathing the cell bodies and proximal axons. Then we add the fresh medium to the perimeter, ensuring it connects with the medium in the center compartment. The much smaller volume of medium remaining in the center compartment will equilibrate with the much larger volume of medium in the perimeter. In cases where it is important to completely remove the medium, such as when withdrawing NGF or drug treatments, the medium in the center compartment can be carefully aspirated using a gel-loading micropipette tip inserted near the back end of the center compartment. Care must be taken to avoid touching the substratum. Medium in the distal compartments is aspirated using a Pasteur pipette positioned at the back of the compartment away from the distal axons. Fresh medium is gently added in the same location.
Some experiments require axons to grow across a first barrier, enter an intermediate compartment, grow across the intermediate compartment and then grow across a second barrier to enter the distal compartment. This can be accomplished in two ways: A five-compartment Teflon divider can be used when assembling the culture dishes, with neurons plated as usual in the center compartments (Fig. 1d, right). The procedure is basically the same as described for three-compartment cultures. The intermediate compartment is filled with the medium containing 15 ng ml$^{-1}$ NGF so that the axons encounter an increase in NGF when they cross the first barrier. Alternatively, a modified three-compartment culture can be assembled, in which the neurons are plated in one of the side compartments rather than in the central compartment (Fig. 1d left and Fig. 7). The procedure for plating neurons in a side compartment is the same as the procedure for plating in the center compartment except:

1. Lay down a C-shaped cylinder of grease onto the dry, scratched region of the dish, as shown in Figure 7a. After laying down the grease, put the dish in a 37 °C, 5% CO$_2$ incubator to allow the grease to settle. After 1 h, apply a droplet of intermediate compartment medium within the C-shaped grease perimeter (Fig. 7b).
2. When seating the divider, place it so that the C-shaped grease perimeter forms a rectangular region, about 1 mm wide, where the neurons will be plated (Fig. 7c). The left compartment will be the proximal compartment, the center slot will be the intermediate compartment and the right compartment will be the distal compartment (Fig. 1d, left).
3. Once the divider is seated, add two drops of distal compartment medium (base medium supplied with 50 ng ml$^{-1}$ NGF) to the distal compartment and incubate the dish in the 37 °C, 5% CO$_2$ incubator for 3 h. Then, fill the distal compartment with the distal compartment medium and add 2.5 ml of the intermediate compartment medium (base medium supplied with 15 ng ml$^{-1}$ NGF) to the intermediate compartment and dish perimeter. Place the completed dish in the 37 °C, 5% CO$_2$ incubator until plating the neurons.
4. When plating the neurons, remove the medium from the rectangular grease perimeter in the proximal compartment using an empty syringe fitted with a needle. Inject the cell suspension (80 µl) within the grease perimeter until it forms a bulging droplet. The droplet can be spread up the wall of the divider with the needle tip so that it contacts and adheres with the Teflon wall of the divider. This will help support the droplet from spilling over the grease perimeter. Incubate the cultures overnight in the 37 °C, 5% CO$_2$ incubator.
5. The next day, fill the proximal compartment with ~2.5 ml of the proximal compartment medium.

Production of cultures with intermediate compartments. Compartmented cultures can be set up such that axons grow from a proximal compartment, across a first barrier, into an intermediate compartment, across a second barrier, and finally into the distal compartment (Fig. 1d and Box 1). Cultures with intermediate compartments are useful for many kinds of studies. For example, we have shown that the vast majority of [125I]NGF retrogradely transported after uptake by distal axons is not degraded during its passage through the axon segments in the intermediate compartments. Cultures with intermediate compartments are useful in studies on retrograde signaling as the transmission mechanism of the retrograde signal functions in the intermediate axons in relative isolation from the mechanisms that generate the retrograde signal in the distal axons and receive the retrograde signal in the cell bodies. This allows the transmission mechanism to be independently perturbed by applying reagents only in the intermediate compartments, and signaling molecules involved in transmission can be assayed by analyzing the intermediate axons.

One method for constructing cultures with intermediate compartments involves constructing culture dishes using the same Teflon dividers as commonly used for standard three-compartment cultures, but plating the neurons in the left (or right) distal compartment instead of in the center compartment (Fig. 1d, left). This involves the additional step of laying down a cylinder of silicone grease to form a perimeter in the left compartment to locate the neurons near the barrier (Fig. 7). The axons will continue to grow across the center compartment, which functions as the intermediate compartment, and then cross into the right, distal compartment. This method has the advantage of not requiring the purchase of five-compartment dividers, but has the disadvantage that axons that grow to the left (when neurons are plated in the left compartment) will remain in the left compartment. Alternatively, dividers can be used which divide the culture dish into five compartments (Fig. 1d, right) so that the neurons are plated in the center compartment, and axons growing to the left and right first cross into intermediate compartments and then into distal compartments. The construction of five-compartment cultures is essentially the
same as constructing three-compartment cultures, in which the neurons are plated in the center compartment. The scratches forming the tracks for axon growth must be long enough to span across all five compartments.

Assessing cultures for leakage between compartments. Leaking of medium between compartments is rare but easy to detect. Cultures are ordinarily maintained with higher levels of medium in the left and right compartments than in the dish perimeter. Therefore, culture dishes in which there is a leak between compartments display a decrease in the level of culture medium in the distal compartment that is leaking and an increase in culture medium in the dish perimeter. As the dishes are usually constructed a day in advance, leaking dishes are normally identified before plating the neurons by the appearance of culture medium in the dish perimeter. After plating the neurons, leaking cultures are identified by a decrease in fluid level in the left or right compartment. A culture with a low level of medium in one distal compartment is obvious, making leaks easy to detect. Some experiments require a small amount of medium in the left and right compartments, such as when applying $^{125}$I NGF labeling medium to distal axons. Although leaking is more difficult to detect by changes in fluid levels in this instance, most leaks are apparent within 24 h of dish construction, and the development of leaks at a later time is extremely rare.

One can also check for leaks by omitting phenol red from the distal compartment medium when assembling cultures and checking for the appearance of phenol red in distal compartments. It should be kept in mind that assays for leakage must be done in culture dishes containing neurons with axons that are crossing the barriers because the penetration of the axons through the grease could, hypothetically, cause a leak. Consequently, tracers used to measure leakage must not be taken up by the neurons and transported through the axons across the barrier. There are also functional assays for leakage. Neurons plated on collagen tracks that were not wet before seating the divider and whose axons do not cross into distal compartments die when NGF is supplied only in the distal compartments. In contrast, neurons on adjacent tracks that were wet and whose axons cross into distal compartments survive because their distal axons are bathed in NGF-containing medium. This indicates that biologically effective concentrations of NGF do not leak across the barriers. This bioassay is confirmed by the failure of $^{125}$I NGF to move intact from the medium supplied with $^{125}$I NGF into the medium in adjacent compartments not supplied with $^{125}$I NGF. Also, it is possible to test whether the effects of membrane-permeant drugs are localized to the compartments in which they are applied, and do not spread to adjacent compartments.

Axotomy. Studies of the neurons after axonal injury and during axonal regeneration can be accomplished in compartmented cultures. Once distal axons have grown a substantial amount within distal compartments, which occurs within 5–7 d in culture, they can be readily removed with a jet of distilled water delivered with a syringe. If medium containing NGF is supplied in the distal compartments after axotomy, axons will regenerate from the proximal stumps of the severed axons. This allows studies of axonal regeneration to be carried out. In addition, it is easy to measure how far the regenerating axons extend along the collagen tracks over time, which is a good measure of the rate of axonal elongation (see Box 2). This technique can be used to test the effect of treatments on axonal elongation. Do not use isotonic solutions such as phosphate-buffered saline (PBS) to remove axons. The osmotic pressure of the water lyses the axons and makes it easier to sever the distal axons completely. Using isotonic solutions will result in bundles of axons that are detached from the substratum but still attached at the compartment barrier.

Analysis of neurons. Cultured neurons can be harvested for downstream analysis. Cell bodies/proximal axons can be harvested separately from distal axons. Appropriate wash buffers for these procedures can consist of ice-cold PBS or Tris-buffered saline (TBS) supplemented with protease and phosphatase inhibitors. Appropriate harvesting buffers include SDS sample buffer, immunoprecipitation lysis buffer, homogenization buffer and RNA extraction buffer. The volume of buffer required for harvesting depends on the analysis. For extracts collected for immunoblotting, small volumes are required so that extracts from ≥ 3 cultures can be pooled and loaded in their entirety on a single gel lane, allowing comparison of the abundance of proteins and phosphoproteins in the cell bodies/proximal axons versus the distal axons. For immunoblotting, we use 8 µl of double-strength SDS sample buffer for harvesting the cell extract from the center compartment of a culture and 15 µl for sequentially harvesting the two distal compartments, i.e., using the entire 15 µl first in one distal compartment and then transferring it to the other distal compartment. For collecting extracts for procedures such as immunoprecipitation, which are not as volume sensitive, we use 20–40 µl of lysis buffer in the center compartment and 20–60 µl in each of the distal compartments. We use gel-loading, 200 µl micro-pipette tips for harvesting center compartments (half the length of the tip can be snapped off with scissors to produce a stiffer tip for scraping) and ordinary 200 µl micropipette tips for harvesting distal compartments. Harvesting with SDS sample buffer is performed at room temperature as the proteins are quickly denatured, and all the other harvesting is done with ice-cold buffers maintained in an ice bucket and with the cultures placed on ice.

MATERIALS

REAGENTS

Animals
• 0–2-d-old Sprague–Dawley rats (Biological Sciences, University of Alberta) | **CAUTION** All experiments using live animals must conform to national and local regulations.

Culture substratum
• Collagen prepared from rat tails in the laboratory or purchased (Sigma, cat. no. C7661)
• Tissue culture grade, double distilled water (ddH₂O)

Enzymes for cell dissociation
• Collagenase type 1 from Clostridium histolyticum (Sigma, cat. no. C0130)
• Trypsin (Sigma, cat. no. T4665)

Standard base medium components
• L-15 medium powder with glutamine and without sodium bicarbonate (Invitrogen, cat. no. 41300-070)

**CAUTION** It is flammable and corrosive; wear eye protection and protective clothing.

Glacial acetic acid (Fisher Scientific, cat. no. A38P212)
**PROTOCOL**

## BOX 2 | MEASUREMENT OF AXON EXTENSION

As axons extend greater distances into the distal compartments than the diameter of the microscopic field, measuring axonal extension requires the capability of measuring the distance the microscope stage moves between the point the axons emerge into the distal compartments and the distance they extend along the tracks. The simplest way to accomplish this is to mount a machinist’s electronic digital indicator (Starrett electronic digital indicator) on the microscope stage so that left and right movements of the stage move the plunger on the digital indicator, giving a reading of the distance the plunger is depressed or extended relative to a zero point established by pressing a button on the digital indicator. Mounting usually involves a small amount of machining. To measure the extension of the axons:

1. Place the culture on the microscope stage insert that fits the 35-mm culture dish.
2. Rotate the dish itself or rotate the dish by rotating the insert in the microscope stage to align the culture so that the tracks extend to the left and right, parallel with the left–right movement of the stage. This can be accomplished by moving the stage so that the crosshairs in the center of the microscopic field align on the end of one of the scratches in the left compartment. Then, without adjusting the up-down axis, move the stage to the right so that the crosshairs are as close as possible to the end of the same scratch in the right compartment. If the end of the scratch is above or below the crosshairs, rotate the dish to bring the crosshairs about half the distance toward the end of the scratch. Then move the stage along the up-down axis to bring the crosshairs into alignment with the end of the scratch. Then, without adjusting the up-down axis, move the stage back to the end of the scratch in the left compartment, placing the crosshairs as close as possible to the left end of the scratch. If the crosshairs align with the left end of the scratch, then the culture is oriented properly. If not, repeat the process of rotating the dish, and checking the alignment until the crosshairs can be brought into alignment with both ends of the scratch without moving the up-down axis of the stage. Although the scratches are never perfectly straight, this procedure produces a repeatable alignment of the culture, and any deviations of a few degrees from straightness along the track have an inconsequential effect on the measurement.
3. Once the alignment is established, the distance the axons extend along each track, e.g., in the right compartment, can be determined by setting the crosshairs on the base of each track where the axons first emerge into the right compartment, zeroing the electronic digital indicator and moving the stage to place the crosshairs on the tip of the farthest extending axon on each track. Alternatively, a zero point can be established at some landmark in the center compartment, and the distance to the base of each track can be measured and recorded. Then the distances to the farthest axon on each track from the same zero point in the center compartment are measured. The distance the axons extend on each track is obtained by subtracting the position of the base of each track from the distance extended by the farthest axon on each track. This second method is particularly convenient when axon extension in a culture is measured several times, as the base measurements do not change, so only a single measurement per track is required at each time point.

### EQUIPMENT

- **General equipment requirements** are that of a laboratory setup for the preparation of primary neuronal cultures. Specifically, our procedures use the following:
  - Stereomicroscope with a long working distance (Zeiss Stemi 2000, Zeiss)
  - Touch-O-Matic Bunsen Burner (Fisher Scientific)
  - Nikon Diaphot Inverted Microscope (Nikon Canada). A modification to the condenser and light source of the microscope has been provided by Tyler Research Corporation
  - Water bath (Versa Bath, Fisher Scientific)

### Standard base medium additives

- L-Glutamine (Sigma, cat. no. G7513)
- Glucose (Sigma, cat. no. G8207)
- Penicillin–Streptomycin solution (10,000 U ml⁻¹; Sigma, cat. no. P0781)
- 6,7-Dimethyl-3,6,7,8-tetrahydropterine hydrochloride (Sigma, cat. no. D0387)
- Glutathione (Sigma, cat. no. G6013)

### Variable base medium additives

- 2.5S Nerve growth factor (NGF; Alamone, cat. no. N-100)
- Anti-NGF (Cedarlane, cat. no. CLMCNET-041.5)
- Rat serum (see MEDIA AND SOLUTIONS SETUP)
- L-Ascorbic acid (Sigma, cat. no. A4544)
- Cytosine 3-D-arabino-furanoside (cytosine arabinoside) (Sigma, cat. no. B4639)

### Protocol

1. Place the culture on the microscope stage insert that fits the 35-mm culture dish.
2. Rotate the dish itself or rotate the dish by rotating the insert in the microscope stage to align the culture so that the tracks extend to the left and right, parallel with the left–right movement of the stage. This can be accomplished by moving the stage so that the crosshairs in the center of the microscopic field align on the end of one of the scratches in the left compartment. Then, without adjusting the up-down axis, move the stage to the right so that the crosshairs are as close as possible to the end of the same scratch in the right compartment. If the end of the scratch is above or below the crosshairs, rotate the dish to bring the crosshairs about half the distance toward the end of the scratch. Then move the stage along the up-down axis to bring the crosshairs into alignment with the end of the scratch. Then, without adjusting the up-down axis, move the stage back to the end of the scratch in the left compartment, placing the crosshairs as close as possible to the left end of the scratch. If the crosshairs align with the left end of the scratch, then the culture is oriented properly. If not, repeat the process of rotating the dish, and checking the alignment until the crosshairs can be brought into alignment with both ends of the scratch without moving the up-down axis of the stage. Although the scratches are never perfectly straight, this procedure produces a repeatable alignment of the culture, and any deviations of a few degrees from straightness along the track have an inconsequential effect on the measurement.
3. Once the alignment is established, the distance the axons extend along each track, e.g., in the right compartment, can be determined by setting the crosshairs on the base of each track where the axons first emerge into the right compartment, zeroing the electronic digital indicator and moving the stage to place the crosshairs on the tip of the farthest extending axon on each track. Alternatively, a zero point can be established at some landmark in the center compartment, and the distance to the base of each track can be measured and recorded. Then the distances to the farthest axon on each track from the same zero point in the center compartment are measured. The distance the axons extend on each track is obtained by subtracting the position of the base of each track from the distance extended by the farthest axon on each track. This second method is particularly convenient when axon extension in a culture is measured several times, as the base measurements do not change, so only a single measurement per track is required at each time point.
PROTOCOL

Silicone grease syringe preparation materials list
- Dow Corning High-Vacuum Grease packaged in a 150-g tube (Fisher Scientific, cat. no. 146355D)
- 1-ml Glass Luer-Lock syringe (Popper & Sons, cat. no. 5101) ▲ CRITICAL Glass Luer-Lock syringes have become difficult to acquire and may no longer be manufactured. Alternatively, Tyler Research Corporation manufactures a stainless steel syringe designed for applying silicone grease to Teflon dividers, which has the advantage that the plunger will not break under the pressure of applying silicone grease.
- All metal hypodermic needle (18 G, BD Scientific, cat. no. 511097)
- Short piece of vacuum tubing that will slip over the glass syringe barrel
- Short piece of rubber tubing to make a cushion for the syringe plunger
- Wire, single strand, uninsulated, 25 cm
- Disposable 10 ml syringe (BD Scientific, cat. no. 309604)

Compartmented culture construction and dissection materials list
- Sterile Teflon dividers (Tyler Research Corporation, cat. nos. Camp 10 and Camp 13, Fig. 1a, b, and d)
- Glass Petri dishes (10 cm) for autoclaving and storing Teflon dividers
- Bent (90°) hemostatic forceps (Fine Science Tools, cat. no. 13013-14)

(Files 2 and 5)
- Dumont 3 mm Fine Straight Mirror Finish Forceps, two pairs (Fine Science Tools, cat. no. 11252-23)
- Ordinary forceps (Fine forceps with damaged tips can be used.)
- 35-mm Falcon Plastic Tissue Culture Dishes, sterile (VWR, cat. no. 353001)
- 60-mm plastic Petri dishes (not tissue culture treated) (Fisher Scientific, cat. no. 08-757-13A)
- Pin rake, constructed in the laboratory (Fig. 4) or purchased from Tyler Research Corporation.

Culturing materials list
- 15 ml Falcon centrifuge tubes (VWR, cat. no. 352096)
- 50 ml Falcon centrifuge tubes (VWR, cat. no. 352098)
- Falcon Cell strainer (VWR, cat. no. 352340)
- 0.22-µm vacuum-driven, bottle-top filter unit (Millipore, cat. no. SCGFT05RE)
- 0.22-mm syringe-driven filter unit (Millipore, cat. no. SLG033RS)
- One-liter glass autoclavable medium bottles (Fisher Scientific, cat. no. 13-700-404)
- Disposable 5 ml syringe (BD Scientific, cat. no. 309603) equipped with a 22-G, 1.5-inches needle; BD Scientific, cat. no. 305156)

Pin rake materials list (for optional laboratory construction)
- 21 insect pins, size 00 (Carolina Biological Supply, cat. no. 65-4331) ▲ CRITICAL The diameter of the pins determines the width between collagen scratches, which is critical for directing axon growth toward and across barriers.
- Thermix Hot plate (Fisher Scientific, model 210T)
- Stereo microscope (Zeiss, Stemi 2000)
- Aluminum sheet (about 8 × 8 × 1 cm)
- Parafilm (VWR, cat. no. 52858-000)
- Fine forceps
- Phenolic (ACME Plastics) or acrylic (Professional Plastics) rod 1.3 cm diameter × 15 cm.
- Starrett electronic digital indicator, KBC Tools & Machinery, cat. no. 2600-0, part no. 1-856-65593

REAGENT SETUP
▲ CRITICAL Cell culture grade distilled water should be used throughout the protocol. All glassware, plasticware and pipette tips that contact the media and solutions should be maintained clean for cell culture. Media and solutions are either prepared sterile or filter-sterilized after they are mixed.

Stock solutions
PBS ddH₂O to a final volume of 2 liters, 16 g NaCl, 0.4 g KCl, 0.48 g KH₂PO₄, and 2.88 g Na₂HPO₄. Adjust pH to 7.4. Filter-sterilize and store at 4 °C for up to 6 months.

TBS ddH₂O to a final volume of 2 L, 16 g NaCl and 4.84 g UltraPure Tris. Adjust pH to 7.4. Filter-sterilize and store at 4 °C for up to 6 months.

Stable vitamin mix ddH₂O to a final volume of 100 ml, 300 mg L-aspartic acid, 300 mg L-glutamic acid, 300 mg L-proline, 300 mg L-cystine, 100 mg p-aminobenzoic acid, 100 mg β-alanine, 40 mg vitamin B12, 200 mg myo-inositol, 200 mg choline chloride, 500 mg fumaric acid, 8 mg coenzyme A, 4 mg D-biotin and 100 mg DL-6,8-thiocolic acid. ▲ CRITICAL Stable vitamin mix is prepared non-sterile and is added to the medium before the medium is filter-sterilized. The components will not completely dissolve in the solution at the specified concentrations. Divide into 1 ml aliquots while stirring vigorously to maintain a uniform suspension. Store at −20 °C for up to 1 year.

Glucose (30% wt/vol) stock solution Add glucose to warm (37 °C) ddH₂O; filter-sterilize; store in 10 ml aliquots at −20 °C for up to 3 months.

Fresh vitamin mix Add 25 mg of glutathione and 50 mg of 6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride; add ddH₂O to a final volume of 100 ml; filter-sterilize; and store in 10 ml aliquots at −20 °C for up to 3 months.

Vitamin C stock solution 30 mM L-ascorbic acid in ddH₂O; adjust pH to 5.5; filter-sterilize; store in 1 ml aliquots at −20 °C for up to 1 month.

Rat serum Obtain the blood from a minimum of 30 male rats. We obtained the blood of anesthetized animals collected by cardiac puncture performed by the University of Alberta Health Sciences Laboratory Animal Services. Allow the blood to clot overnight at 4 °C. Remove the unclotted solution using a pipette and discard the clots. Spin the unclotted solution for 30 min at 12,000g, 4 °C. Collect and filter-sterilize the supernatant. Store in 2.5 ml aliquots at −20 °C for up to 3 months or at −80 °C for up to 1 year. ▲ CAUTION Ensure the methods of euthanasia and the collection of blood conforms to the national and local regulations. ▲ CRITICAL We have not found a commercial source of rat serum that consistently produces high-quality cultures.

Cytosine arabinoside stock solution 1 mM cytosine arabinoside in ddH₂O; filter-sterilize; store in 1 ml aliquots at −20 °C for up to 2 months.

NGF stock solution 0.250 mg NGF in 12.5 ml PBS (20 µg ml⁻¹ anti-NGF); filter-sterilize; store in 250 µl aliquots at −20 °C for up to 6 months or at −80 °C for up to 1 year.

Anti-NGF stock solution 0.5 mg anti-NGF in 0.5 ml sterile PBS (1 mg ml⁻¹ anti-NGF); Store in 20 µl aliquots at −20 °C for up to 6 months or at −80 °C for up to 1 year.

L-15 air and L-15 CO₂ stock medium L-15 air stock medium is used to prepare dissecting medium for the isolation of neurons. L-15 CO₂ stock medium is used to prepare culture medium for maintaining the cultures inside the 5% CO₂, 37 °C incubator. Production of 48 cultures requires about 21 ml of dissecting medium and 175 ml of culture medium, and the maintenance of 48 cultures requires about 175 ml of culture medium per week.

Anti-NGF stock solution 0.250 mg NGF in 12.5 ml PBS (20 µg ml⁻¹ anti-NGF); Store in 250 µl aliquots at −20 °C for up to 6 months or at −80 °C for up to 1 year.

Fresh vitamin mix ddH₂O, 1.2% (wt/vol) L-15 powder, 0.1% (vol/vol) stable vitamin mix, 0.69 mM idamazole and 0.024 mM phenol red. Adjust the pH to 7.35. Set aside the desired amount for preparing L-15 CO₂ stock medium. Filter-sterilize the remainder and store at −20 °C for up to 6 months.

L-15 L-15 CO₂ stock medium To the L-15 air stock medium, add sodium bicarbonate to a final concentration of 2.16 mg ml⁻¹. Filter-sterilize. For each liter of L-15 CO₂ stock medium desired, autoclave 4 g of methylcellulose powder in a 1-liter glass medium bottle containing a magnetic stir bar. Add 1 liter of medium to each sterile, methylcellulose-containing medium bottle and stir overnight at 4 °C until dissolved. Store at −20 °C for up to 6 months.

Dissecting medium To the L-15 air stock medium, add glucose stock solution (final concentration 2%, vol/vol), 1-glutamine (final concentration 1%, vol/vol), penicillin-streptomycin (final concentration 1%, vol/vol) and fresh vitamin mix (final concentration 1%, vol/vol). Store in 7 ml aliquots at −20 °C for up to 6 months. ▲ CRITICAL Thawed dissecting medium will contain a precipitate that will dissolve at 37 °C.

Base medium To the L-15 CO₂ stock medium, add glucose stock solution (final concentration 2% (vol/vol)), L-glutamine (final concentration 1% (vol/vol)), penicillin-streptomycin (final concentration 1% (vol/vol)) and fresh vitamin mix (final concentration 1% (vol/vol)). Store in 100 ml aliquots at −20 °C for up to 6 months.

Proximal compartment medium To the base medium, add rat serum (final concentration 2.5% (vol/vol)) and vitamin C stock solution (final concentration 1% (vol/vol)). Store at −20 °C for up to 3 months.

Proximal compartment medium for freshly plated neurons To the base medium, add rat serum (final concentration 2.5% (vol/vol)) and vitamin C stock solution (final concentration 1% (vol/vol)) and NGF (final concentration 10 ng ml⁻¹) and cytosine arabinoside stock solution (final concentration 1% (vol/vol)). Store at −20 °C for up to 3 months.

Proximal compartment medium for depriving cell bodies and proximal axons of NGF To the base medium, add rat serum (final concentration 2.5% (vol/vol)), vitamin C stock solution (final concentration 1% (vol/vol)) and anti-NGF stock solution (final concentration 0.2% (vol/vol)). Store at −20 °C for up to 3 months.
Distal compartment medium

To the base medium, add NGF to a final concentration of 50 ng ml⁻¹. Store at −20 °C for up to 3 months.

Collagen solution

Collagen is prepared as previously described, except using sterile technique. Dissect the tendons from one large rat tail (rat tails can be stored 1 year at −80 °C). Mince the tendons and then stir overnight at 4 °C in 250 ml of 0.1% (vol/vol) glacial acetic acid. Centrifuge for 30 min at 11,000g at 4 °C. Discard the debris. The prepared collagen solution can be stored for 2 months at 4 °C.

Alternatively, we have found that sterile rat tail collagen from Sigma (cat. no. C7661) prepared as follows produces satisfactory results. Mix 1 ml of ddH₂O and 40 μl of glacial acetic acid. Filter-sterilize, and then add 5 mg of Sigma rat tail collagen. Once the collagen has dissolved, dilute the collagen solution by adding 49 ml of sterile ddH₂O. Store at 4 °C for up to 3 weeks.

Collagenase solution

0.1% (wt/vol) collagenase in PBS; filter-sterilize; and store in 1 ml aliquots at −20 °C for up to 2 weeks.

Trypsin solution

1% (wt/vol) trypsin in PBS; filter-sterilize; and store in 90 μl aliquots at −20 °C for up to 2 weeks.

2x SDS sample buffer

Add a pinch (~40 mg) of bromophenol blue, 15 ml of glycerol, 4 g of SDS, 16 ml of Tris-HCl (pH 6.8) and ddH₂O to a final volume of 100 ml. Store at room temperature (18–22 °C) for up to 6 months. Add beta-mercaptoethanol to a final concentration of 0.05% (vol/vol) before using.

Immunoprecipitation buffer

Add 1 ml of glycerol and 100 μl of IGFPAL CA-630 to 10 ml of TBS. Store at 4 °C for up to 6 months. Add one Complete Protease Inhibitor Cocktail tablet before using.

Phosphatase inhibitors

Sodium fluoride stock solution

Prepare a 4.2 mg ml⁻¹ sodium fluoride solution in ddH₂O and store in 1 ml aliquots at −20 °C indefinitely. Add the solution to the appropriate buffer at a final concentration of 0.042 mg ml⁻¹ (1 μM).

Activated sodium orthovanadate stock solution

Prepare 36.8 mg ml⁻¹ sodium orthovanadate in ddH₂O. Adjust the pH to 10 using 1 N HCl or 1 N NaOH. At pH 10, the solution will be yellow. Boil the solution until it becomes colorless. Cool the solution to room temperature. Store in 1 ml aliquots at −20 °C indefinitely. Add to the appropriate buffer at a final concentration of 0.368 mg ml⁻¹ (2 μM).

EQUIPMENT SETUP

Preparation of silicone grease syringes

The silicone grease syringe consists of a 1-ml glass syringe fitted with an all steel, 18-G needle with the point filed off to make a flat opening. After filing off the point, it may be necessary to restore the opening in the needle by inserting a pin. Some pressure is required on the syringe plunger to squeeze the grease through the 18-G needle.

To aid in this, slip a 1/2-inch length of rubber tubing (see Fig. 4). To dispense the grease, place the first and second finger around the syringe barrel in front of the needle and depress the plunger with the thumb. To refill the syringe, remove the needle by twisting with pliers and depress the plunger fully to squeeze out any remaining grease. Then slowly and carefully withdraw the plunger.

CAUTION Owing to the resistance caused by grease between the syringe barrel and plunger, the barrel can shatter if the plunger is removed too quickly.

It is difficult to fill the 1-ml syringe directly from the tube of silicone grease, so fill a 10-ml syringe from the tube and fill the 1-ml syringe from the 10-ml syringe. First, remove the plunger from a 10-ml disposable syringe with no needle attached and squeeze the grease from the tube into the syringe barrel. Replace the plunger and press it into the syringe until the grease is about to emerge from the tip. Then insert the tip of the 10-ml syringe into the back end of the barrel of the 1-ml syringe. Press tightly to form a seal and push the grease from the 10-ml syringe into the 1-ml syringe until the barrel of the 1-ml syringe is ¾ full. Insert the plunger into the 1-ml syringe and depress the plunger until the grease begins to eject out of the tip of the syringe. Then replace the needle and press the plunger until grease is ejected from the tip of the needle. Wipe off any grease that has emerged. Wind a piece of stiff, bendable wire around the base of the needle and over the end of the plunger (Fig. 5). CRITICAL If the plunger is not secured in this way, any air bubbles trapped in the syringe can expand during autoclaving and blow out the plunger.

Wrap the syringe in foil or seal it in an autoclave bag and autoclave. Sterile grease-filled syringes are generally stored wrapped and can be kept indefinitely until use stored at room temperature (18–22 °C). To re-sterilize a grease-filled syringe that has been exposed to a non-sterile environment, eject a small amount of grease from the needle, wipe it off, immerse the needle and front half of the syringe in 70% ethanol (vol/vol) and allow it to air dry in a sterile environment.

Cleaning Teflon dividers

To clean Teflon dividers for re-use, remove the dividers from the culture dishes with ordinary forceps. Rinse the dividers with tap water and wipe off as much grease as possible with a paper towel. Put the dividers in an Erlenmeyer flask and place in a fume hood. Dissolve one package of Nochrimix into a 2.5-liter jug of sulfuric acid. Add sufficient sulfuric acid—Nochrimix solution to the flask to cover the dividers. New dividers should also be prepared starting at this step. Soak the dividers for at least 1 h. CAUTION Sulfuric acid is highly corrosive. Wear eye protection, protective clothing and handle with caution in a fume hood.

Apparatus for changing the medium in center compartments

We use a Pasteur pipette connected to a vacuum to remove the medium from the distal compartments and the dish perimeter. When the medium must be removed from the center compartment, we use a Pasteur pipette with the narrow tip broken off and a gel-loading micropipette tip placed over the broken end. The vacuum creates sufficient force to hold the micropipette tip in place while it is used to aspirate the medium from the center compartment. (If about half the length of the micropipette tip is snipped off, this will increase the aperture and facilitate the aspiration of the medium.) We use an ordinary 200 μl micropipette tip to add medium back into the center compartment.

PROCEDURE

Assembling three-compartment culture dishes

TIMING 3 h for 48 culture dishes

To coat the culture dishes with collagen, dilute lab-prepared collagen solution to 20% (vol/vol) or use undiluted commercial collagen. In a laminar flow hood, remove the lid from a sterile, 35-mm culture dish and pour or pipette the solution from the first dish into it. The collagen solution left behind will form the coating. Replace the lid of the first culture dish. Continue this process until all the dishes are coated, replenishing the solution as necessary. Allow the coated dishes to air dry in the laminar flow hood for 3–6 h or overnight. Ensure the dishes are completely dry before proceeding to the next step.
**Critical step** Do not sterilize the collagen-coated dishes with UV light as we have found that collagen-coated dishes exposed to UV light are not as favorable a substratum for axon growth.

2] Clean and sterilize the pin rake with 70% ethanol and allow it to air dry in a laminar flow hood. Place a 35-mm collagen-coated tissue culture dish on a black acrylic work surface to aid in viewing the scratches (optional). Remove the lid. Hold the dish flat against the work surface with the thumb and index finger of one hand. Hold the pin-rake in the other hand with the handle angled away from you. Apply firm pressure on the substratum and draw the rake away from you, creating the scratches positioned as in Figure 2a. Replace the lid. Apply sufficient pressure to create scratches that look similar to those in Figure 1c when viewed under an inverted, phase-contrast microscope.

**Critical step** Prepare a surplus of scratched dishes to allow for discarding substandard dishes at a later point.

**Pause point** Scratched dishes can be repackaged and stored at −20 °C for up to 1 month.

**Troubleshooting**

3] While viewing the culture dish with a stereo microscope, use a Pasteur pipette to place a droplet of distal compartment medium onto the scratched region of the dish. Spread the medium over the central region of the tracks, ensuring the region where the barriers will be located is covered (Fig. 2a). Replace the dish lid.

**Critical step** Avoid touching the dish floor with the Pasteur pipette, which may scratch the collagen coating.

**Critical step** Do not let the medium run outside the area of the scratches. In practice, we usually leave one or two tracks at each edge dry, to reduce the risk of the droplet escaping.

**Critical step** Once the droplet has been placed, complete the dish assembly without delay.

4] In a laminar flow hood, flame sterilize a pair of hemostatic forceps and a pair of ordinary forceps. Remove a sterile Teflon divider from the Petri dish by gripping the rim of the divider with the ordinary forceps. Transfer the divider to the hemostatic forceps, clamping the divider by the solid portion of the septum so that the divider is held horizontally when the hemostatic forceps is placed on its back on the work surface (Fig. 2b). Place the hemostatic forceps on the work surface so that the divider is viewed under the stereo microscope. Using the grease syringe, apply cylinders of silicone grease to the divider in the pattern and direction (arrows) indicated in Figure 2c. Make the ends of the cylinders of grease overlap and touch one another. Lay the grease as neatly as possible along the narrow regions of the septum separating proximal and distal compartments under which the axons will cross.

**Critical step** Use even pressure when depressing the syringe plunger and even movements of the syringe so that the cylinders of grease lay down with uniform thickness.

5] Remove the lid of the culture dish prepared in Step 3. Invert the culture dish quickly, flipping it in the direction at a right angle to the orientation of the scratches. Position the upside-down dish over the divider as in Figure 2d and gently release the dish onto the grease. Use the tips of a pair of ordinary, non-sterile forceps (or other pointed implement) to gently press down on the dish bottom around the outer perimeter of the divider and over the solid region of the septum. The elimination of the air interface between the grease and the dish floor indicates that the grease is sealed (Fig. 2e).

**Critical step** Use the minimum pressure required to seal the grease. Gaps in the seal, if they are along the perimeter of the Teflon divider (as indicated with a red X in Fig. 2e), can be repaired during the next step.

6] Pick up the hemostatic forceps holding the divider–dish assembly, flip the forceps over and hover the right-side-up dish just above the work surface. Unclamp the forceps and allow the dish to drop gently to the work surface. Using the silicone grease syringe, place a small mound of silicone grease on the floor of the dish at the opening of the proximal compartment (Fig. 2f). Patch any gaps in the grease that were identified along the perimeter of the divider in the previous step with additional grease applied adjacent to the outer perimeter of the divider. Place two drops of the distal compartment medium in each distal compartment, making sure the scratched region of the substratum is completely covered (Fig. 2f). Place the lid on the dish and place it in the 37 °C, 5% CO₂ incubator for 3 h. Then fill the distal compartments with the distal compartment medium (~ 0.75 ml) and return the dish to the 37 °C, 5% CO₂ incubator until plating the neurons.

**Critical step** Ensure that contact between the grease and the dish is continuous around each individual compartment, as well as between compartments, so that each compartment is sealed from adjacent compartments.

**Pause point** Assembled culture dishes can be stored in a 37 °C, 5% CO₂ incubator for up to 30 h before plating, provided that the small amount of medium covering the substratum in the proximal compartment does not evaporate.
**PROTOCOL**

Obtaining dissociated neurons from 20 superior cervical ganglia and plating them into 48 compartmented culture dishes ● **TIMING 3 h**

7] Dissect superior cervical ganglia from 0- to 2-d-old rats. Decapitate the rat pups with scissors just above the shoulders. Pin the head out under a dissecting microscope (Fig. 6). Using fine forceps (small scissors such as iridectomy scissors are also helpful), free and peel back the trachea and the esophagus to expose the carotid bifurcation on each side, along which lie the superior cervical ganglia. The ganglia are recognizable as spindle-shaped translucent structures (Fig. 6), with nerve trunks extending from each end. Using fine forceps, peel a ganglion away from the carotid artery, severing the nerve trunks. Place the ganglion in a sterile, disposable, non-tissue culture-treated Petri dish containing 7 ml of dissecting medium at room temperature. Repeat the procedure for the contralateral ganglion. Once ganglia are obtained from all 10 rats, transfer them along with the dissecting medium into a 15-ml centrifuge tube.

\* CAUTION All experiments using live animals must conform to the institutional regulations.

▲ CRITICAL STEP Avoid tearing the sac surrounding the ganglia, which will allow neurons to escape during the dissection.

▲ CRITICAL STEP Clean the dissected ganglia of any attached carotid artery or other extraneous tissue.

8] Warm 20 ml of PBS and two 7 ml aliquots of dissecting medium in a 37 °C water bath. Immediately before use, thaw a tube containing a 1-ml aliquot of collagenase solution in the 37 °C water bath. Allow the ganglia to settle to the bottom of the 15-ml centrifuge tube. Remove the dissecting medium using a plugged Pasteur pipette. Wash once with 10 ml of sterile PBS. Remove the PBS and replace with 1 ml of collagenase solution and incubate at 37 °C for 25 min.

▲ CRITICAL STEP Warm all solutions that are to be used during the preparation of dissociated neurons to 37 °C.

▲ CRITICAL STEP Thaw collagenase immediately before use, as it degrades quickly.

9] Add 90 µl of trypsin solution to the 15-ml tube and incubate in the 37 °C water bath. During the incubation, prepare 8 ml of 10% (vol/vol) rat serum in dissecting medium. After 5 min (no longer), remove the trypsin solution, wash the ganglia once with 10 ml of sterile PBS and then replace the PBS with 8 ml of 10% (vol/vol) rat serum. Allow the ganglia to settle, but if necessary they can be centrifuged for 1 min at 200g, at room temperature, in a bench-top centrifuge.

10] Remove the 10% (vol/vol) rat serum solution from the ganglia and replace with 1 ml dissection medium. Modify a 2-ml disposable pipette by inserting the tip into a 200-µl micropipette tip. Triturate by drawing the ganglia into the pipette tip and ejecting the ganglia out of the pipette tip three times. (If the ganglia pieces are too large, the trituration can be performed with a plugged Pasteur pipette instead.) Avoid making bubbles during trituration. Allow the ganglia fragments to settle, and transfer the medium containing the dissociated cells into a 15-ml centrifuge tube. Add 1 ml of fresh dissecting medium to the remaining ganglia fragments, triturate by passing the ganglia in and out of a 200-µl micropipette tip three times. Let any fragments still remaining settle, collect the cell suspension and add it to the 15-ml collection tube. Repeat this process until ganglia fragments are no longer visible.

▲ CRITICAL STEP Always collect and replace the dissecting medium after passing the ganglia fragments in and out of the pipette tip three times, as excessive trituration will lyse the dissociated cells in the medium.

11] Remove the debris by passing the cell suspension through a sterile cell strainer. Centrifuge the cell suspension for 3 min at 200g, room temperature, in a bench-top centrifuge. Carefully remove the supernatant without disturbing the cell pellet. Resuspend the pellet in the proximal compartment medium by vortexing in short bursts. Avoid generating bubbles. Neurons from 20 ganglia should be resuspended in 4 ml of medium and is sufficient to make 48 cultures. (Adjust the volume proportionately for fewer ganglia.)

12] Draw the cell suspension into a sterile, disposable syringe fitted with a 22-G, 1.5-inch needle. Insert the tip of the needle into the proximal compartment of a culture dish, bringing the tip close to, but not in contact with, the dish surface. Dispense ~80 µl of cell suspension into the proximal compartment, filling the slot. Repeat this procedure with each culture dish. Place the cultures in an incubator at 37 °C, 5% CO₂, overnight to allow the neurons to settle and adhere to the substratum.

▲ CRITICAL STEP When injecting the cell suspension into the proximal compartment, ensure there are no air spaces persisting between the cell suspension and the substratum.

Addition of medium the day after plating ● **TIMING 15 min**

13] The next day, fill the dish perimeter with ~2.5 ml of the proximal compartment medium containing cytosine arabinoside (see REAGENT SETUP). The appearance of neurons 1 and 5 d after plating is shown in Figure 3.
Ensure that the medium in the proximal compartment connects with the medium in the dish perimeter.

**TROUBLESHOOTING**

Change the medium every 5–7 d in culture ● **TIMING 1 h**

14 | After 5–7 d in culture, replace the culture medium in the proximal compartments with proximal compartment medium containing rat serum and vitamin C, but without cytosine arabinoside. As neuronal survival can now be supported by NGF supplied in the distal compartments, NGF in the proximal compartment is optional and can be dictated by the experiment. Replace the culture medium in the distal compartments with distal compartment medium. Change the medium at least every 5–7 d as long as the cultures are maintained. Generally, cultures are used in experiments between 5 and 14 d after plating, but they can be maintained for several months.

**TROUBLESHOOTING**

Axotomy of distal axons for studies of axon regeneration ● **TIMING 3–5 min per culture**

15 | Warm the distal compartment medium and proximal compartment medium in a 37 °C water bath.

16 | Load a 3-ml syringe fitted with a 22-G needle with sterile ddH$_2$O.

17 | Aspirate the medium from the distal compartments.

18 | Hold the syringe at a 45° angle with the needle tip 3 mm above the dish surface and parallel with the Teflon barrier. Target the scratched region of the dish floor just beside the Teflon barrier, and vigorously inject a jet of water, about 0.3 ml, into the distal compartment. Repeat the injection in the other distal compartment. Then aspirate the water from both compartments and repeat the injection-aspiration twice. Check with an inverted microscope to determine whether the axons have been completely removed. If there is still cellular material attached to the substratum or barrier, repeat the injections-aspirations until it is removed.

**CRITICAL STEP** Do not aim the jet of water directly at the barrier, as this can disrupt the seal.

**CRITICAL STEP** Do not inject the water so vigorously that it splashes into the proximal compartment. If a significant amount of water splashes into the proximal compartment, quickly replace the medium to avoid osmotic stress to the neurons.

19 | Replace the ddH$_2$O from the last injection with distal compartment medium (130–200 µl per compartment) appropriate for the experiment.

**TIMING**

Harvesting of cell extracts ● **TIMING 20 min for 12 cultures with 3 cultures per treatment group**

20 | Aspirate the medium from the distal compartment of a culture, and gently add 500 µl of wash buffer (e.g., ice-cold PBS or TBS supplemented with protease and phosphatase inhibitors) to each distal compartment. Aspirate the medium from the dish perimeter and use a 200-µl gel-loading micropipette tip to aspirate the medium from the center compartment. Gently rinse the center compartment with 100 µl of wash buffer.

21 | Aspirate the wash buffer from the center compartment and replace it with the appropriate volume (see Experimental design) of harvesting buffer (e.g., SDS sample buffer and immunoprecipitation buffer) using a 200-µl gel-loading micropipette tip. Use the tip to scrape the surface of the center compartment several times up and down the length of the entire compartment to dislodge the cell bodies and proximal axons. Take up the cell extract into the micropipette tip and transfer it to an appropriate tube.

**CRITICAL STEP** Harvesting with SDS sample buffer is performed at room temperature, as the proteins are quickly denatured, and all other harvesting is done with ice-cold buffers maintained in an ice bucket and with the cultures placed on ice.

22 | Aspirate the wash buffer from the distal compartments and replace it with the appropriate volume (see Experimental design) of harvesting buffer. Use the micropipette tip to scrape the surface of the distal compartments, take up the extract into the micropipette tip and gently eject it over the substratum in the compartment two times. Then, take up the cell extract into the micropipette tip and transfer it to an appropriate tube.

**CRITICAL STEP** When scraping the compartments, take care not to push the Teflon divider from its original position. This will cause the cellular material and buffer to mix between compartments, spoiling the sample.

**CRITICAL STEP** Do not scrape directly over grease areas. This can cause leakage across the barriers and can dislodge pieces of grease into the buffer, making it more difficult to collect the extract.

23 | Use an inverted microscope to check if the material in the compartments has been completely detached from the dish surface and grease barriers.
24] Repeat Steps 21 and 22 for each culture within a treatment group to produce a pooled sample of extracts from the cell bodies/proximal axons and distal axons from all of the cultures in the group.

**TIMING**
Preparing compartmented cultures of primary neurons generally takes place over several days:
- Steps 1–6, assemble 48 compartmented cultures: 3 h
- Steps 7–12, obtain dissociated neurons from 20 ganglia and plate into 48 cultures: 3 h
- Step 13, add the medium to the dish perimeter of 48 cultures: 15 min
- Step 14, change the medium in all compartments of 48 cultures: 1 h
- Steps 15–19, axotomize distal axons of a single culture: 3–5 min
- Steps 20–24, harvest cell extracts of four groups of three cultures each: 20 min

? TROUBLESHOOTING
Troubleshooting advice can be found in Table 1.

<table>
<thead>
<tr>
<th>Step(s)</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Scratching the substratum with the pin rake produces white powder</td>
<td>Collagen is peeling from the tracks between the scratches</td>
<td>Replace the batch of collagen used with fresh collagen solution</td>
</tr>
<tr>
<td>13, 14</td>
<td>Neurons appear healthy, but few or no axons cross the barriers, resulting in excessive axon growth in the proximal compartment</td>
<td>Substratum was not wetted and/or too much pressure was used when seating the divider. Collagen substratum may be suboptimal</td>
<td>Carefully remove the Teflon divider from the dish with forceps and determine whether islands of grease adhere to the collagen tracks that were under the barrier. Avoid this by applying less force when seating the divider. Check that the collagen solution was correctly prepared and possibly change to a different batch of collagen</td>
</tr>
<tr>
<td></td>
<td>Axons grow across the scratches</td>
<td>The scratches may be too shallow and narrow, or residual collagen solution present during scratching may have coated the plastic in the scratches</td>
<td>When making the scratches, make sure the dishes are completely dry and apply more pressure on the pin rake</td>
</tr>
<tr>
<td></td>
<td>Cell bodies and axons look unhealthy, axons may be disintegrating and the neurons may be dying</td>
<td>Contamination or suboptimal medium or substratum may be indicated. Undefined problems with the batch of rat serum are a common cause</td>
<td>First try a different batch of rat serum, then try different batches of medium and collagen. Make sure the medium in the dish perimeter connects to the medium in the center compartment, as failure to connect can cause the death of neurons over time</td>
</tr>
<tr>
<td></td>
<td>Axons cross into the distal compartments, but their growth is obstructed along a transverse line across the scratches</td>
<td>The droplet placed on the collagen tracks during dish construction may have partially evaporated</td>
<td>Make sure the entire length of the collagen tracks are covered with medium within 5 min of placing the droplet on the collagen</td>
</tr>
</tbody>
</table>
ANTICIPATED RESULTS
Proximal compartments always contain proximal axons

The day after plating, neurons have settled onto the collagen substratum in the center compartment and produced a dense outgrowth of fibers (Fig. 3a,b). On days 5–7 after plating, neuronal cell bodies are clustered together along dense cables of axons (Figs. 1c and 3c). The cell body of a rat sympathetic neuron can be approximated as a 35-µm diameter sphere (ignoring dendrites), and the axon can be approximated as a 1-µm diameter cylinder. Comparison of the relative surface area and volume reveals that in a proximal compartment containing a 1-mm length of axon for every cell body, 45% of the total surface membrane and 3% of the total cytoplasm would be axonal. It is difficult to know the actual length of the axons present in proximal compartments with the cell bodies, but it is certainly >1 mm per neuron, as 1 mm would only amount to a single axon spanning the proximal compartment per neuron. Axons in the proximal compartment branch considerably while directly exposed to NGF during the initial 5–7 d, so it is likely that there are several millimeters of axon per neuron in the proximal compartment. Therefore, the cellular material collected from proximal compartments likely contains several times more axonal surface membrane than cell body surface membrane, and a significant proportion of the cytoplasm likely belongs to the axons. This is why we specify that experimental treatments applied in proximal compartments are applied to cell bodies and proximal axons and that cell extracts taken from proximal compartments represent the constituents of the cell bodies and proximal axons. In some published reports it is evident that most of the axonal material resides within the proximal compartments with the cell bodies; hence, the proportion of axonal material harvested with the cell bodies likely makes up the majority of surface membrane and the cytoplasm in the sample. This must always be considered when interpreting results. Because of the presence of proximal axons in the cell body–containing compartment, it cannot be assumed that effects of reagents applied to the cell body–containing compartments must act exclusively by affecting the mechanisms in the cell bodies. Nor can it be assumed that molecules detected in cell extracts harvested from both cell body–containing compartments and distal axon-containing compartments are necessarily present and functioning in the cell bodies.

Location of distal axons and axon terminals

Axonal elongation, while confined along linear collagen tracks, is not necessarily directed exclusively away from the cell bodies. U-turns can occur within the tracks, especially when a growth cone divides, sending off two growth cones in diverging directions. This leads to situations where the branch of an elongating axon within the distal compartment can grow back to reenter the proximal compartment. Thus, there can be distal axons in the proximal compartment connected to more proximal axon segments in the distal compartment.

In the case of sympathetic neurons, the NGF distribution can be manipulated to create cultures where axon branching and growth within the proximal compartments are reduced and where distal axon segments are localized to distal compartments. First, we plate the neurons with 10 ng ml⁻¹ NGF in the proximal compartments and with 50–200 ng ml⁻¹ NGF in the distal compartments. Although 10 ng ml⁻¹ NGF supports neuronal survival and axonal growth, axonal branching is reduced at this concentration. Once the axons cross the barrier and encounter the higher concentration of NGF, they branch more profusely. Crossing of axons into distal compartments takes place within 2 d in culture and is well established within the first week. After 5–7 d in culture, NGF from the proximal compartments is removed. If the experiment requires rigorous removal, the NGF-containing medium is replaced with medium containing function-blocking NGF antibodies. The removal of NGF from the proximal compartments produces cultures that are dependent on retrograde survival signals produced by NGF at the distal axons. Axonal survival is also dependent on retrograde survival signals, and consequently distal axons that terminate in the proximal compartment from which NGF has been withdrawn will degenerate. The end result is that several days after NGF withdrawal from the proximal compartments, the cultures consist of proximal axon segments and cell bodies in the proximal compartments and distal axons and axon terminals largely confined to the distal compartments.

Measurement of axonal ‘growth’

The imprecise term ‘growth’ applied to axons can be a source of confusion. We most commonly use ‘axon extension’ as a measure of axonal growth. We define axonal extension as the distance between the silicone grease where the axons emerge into the distal compartment and the end of the farthest axon on each collagen track (see Box 2). The average rate of axonal extension in each culture is calculated from axons on about 16 tracks, and combining the data from three cultures gives a sample size of about 48 tracks. Axonal extension approximates the maximum rate of axonal elongation, but is somewhat less as the axons do not elongate in perfectly straight lines. We most commonly use this measure to assess axonal growth in cultures where the distal axons are regenerating after axotomy. The regenerating axons are generally present on every track the day after axotomy, and they advance along the collagen tracks as a front of growth cones extending at rates ranging about 1–1.2 mm d⁻¹ (refs. 3 and 7). Treatments that have a major effect on axonal elongation produce easily detectable results by this measure. For example, both withdrawal of NGF from the distal compartments and the blockage of TrkA phosphorylation in distal axons with the kinase inhibitor, K252a, arrest the extension of the regenerating distal axons. We prefer this measure to the alternative method of determining how many axons cross lines that are established at certain
AKNOWLEDGMENTS We thank the researchers who have worked in the laboratory and contributed improvements to our techniques over the years: Dr. Daren Ure, Dr. Donna Senger, Grace Martin, Russell Watts, Dr. Bronwyn MacInnis, Norma Jean Valli and Dr. Barbara Karten. Special thanks to Dr. Edwin Furshpan and Dr. David Potter in whose laboratory R.B.C. developed the compartmented culture during a postdoctoral fellowship in 1976–1978. Work contributing to the development and refinement of the techniques described here has been funded by The National Institutes of Health (USA), The Canadian Institutes of Health Research, The Alberta Heritage Foundation for Medical Research, The Alberta Paraplegic Foundation and the Rick Hansen Man in Motion Foundation.

AUTHOR CONTRIBUTIONS All authors contributed extensively to the development of this protocol and preparation of the paper. K.L. developed several of the procedures and prepared a laboratory manual upon which much of the detail in the protocol is based; S.A.M. developed several of the procedures, created the graphic images and prepared part of the initial draft of the paper; R.B.C. originally developed the compartmented culture system, produced most of the photographs and wrote the final paper.

Published online at http://www.natureprotocols.com/. Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.