Chapter 20

Axon Guidance Studies Using a Microfluidics-Based Chemotropic Gradient Generator

Zac Pujic, Huyen Nguyen, Nick Glass, Justin Cooper-White, and Geoffrey J. Goodhill

Abstract

Microfluidics can be used to generate flow-driven gradients of chemotropic guidance cues with precisely controlled steepnesses for indefinite lengths of time. Neuronal cells grown in the presence of these gradients can be studied for their response to the effects exerted by the cues. Here we describe a polydimethylsiloxane (PDMS) microfluidics chamber capable of producing linear gradients of soluble factors, stable for at least 18 h, suitable for axon guidance studies. Using this device we demonstrate turning of superior cervical ganglion axons by gradients of nerve growth factor (NGF). The chamber produces robust gradients, is inexpensive to mass produce, can be mounted on a tissue culture dish or glass coverslip for long term time-lapse microscopy imaging, and is suitable for immunostaining.

Key words Gradient, Chemotropic cue, Microfluidic, Axon guidance, Chemotaxis, Nerve growth factor, Superior cervical ganglion

1 Introduction

For the brain to develop correctly it must be wired up correctly. To achieve this, growing axons must navigate reliably to their targets and make synaptic connections. Understanding how this navigation occurs is important since axon miswiring may underlie many mental disorders [1], and it is also critical for axons to be able to reform appropriate connections after injury. Due to the enormous complexity of the developing brain, it is often desirable to study these mechanisms in vitro since it is possible to exclude confounding factors.

An important cellular process often required to accomplish correct guidance is the detection, by the growth cones at the tips of developing axons, of gradients of diffusible chemotropic cues within the developing tissues. The in vitro study of axon guidance by diffusible gradient cues ideally requires the generation of temporally and spatially stable gradients with precisely controllable characteristics, that can be applied to a large number of individual axons, and allow

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time-lapse imaging. However few assays used for axon guidance studies currently achieve these goals. Collagen gel explant coculture assays [2] produce gradients which are poorly characterized and decay with time, while more sophisticated efforts to produce gradients with known steepnesses in collagen gels [3] are expensive to set up and limited to shallow gradients. The widely used "growth cone turning" or "pipette" assay [4, 5] has a low throughput, limited gradient stability, and little control over gradient steepness. The Dunn chamber [6] can be effective for axon guidance studies, but as a passive device suffers from transients and gradient decay [6].

Many of these limitations can be overcome by using microfluidic technologies [7, 8]. Gradients of diffusible factors can be generated dynamically and therefore sustained at a particular steepness and concentration regime almost indefinitely. The number of isolated cells exposed to the gradient is generally greater than that for other assays, and the gradient can be defined with greater precision. Recent advances in microfluidics chamber design have employed innovative approaches which demonstrate that the approach is both powerful and versatile. For instance, microfluidically generated gradients of diffusible Slit-1 or Netrin-1 were able to elicit turning in hippocampal or dorsal root ganglion neurons [9]. Flow-based approaches can cause shear stresses which are damaging for growth cones, which are less robust than cell bodies. Various methods have been used to minimize this problem, such as culturing cells in a 3D hydrogel [9], using micro-well structures [10], or using a permeable membrane separated the fluid-flow driven gradient from the cells [11]. Here we describe a simple and easy to produce flow-based microfluidics chamber which can generate stable linear gradients despite using a flow rate low enough to be suitable for axon guidance studies.

2 Materials

To minimize the blockage of PDMS chambers by dust and other particulates, prepare all materials and solutions in a clean, dust-free environment. Where possible, work in laminar flow hoods to ensure that a minimal amount of dust and particulates are present. Use ultrapure water (deionized to attain 18 MΩ cm at 25 °C) and filter all aqueous solutions with 0.2 μm filters.

2.1 Microfluidics

1. AutoCad software (Autodesk, Australia).
2. Photoplate (Konica, Minolta, New South Wales, Australia).
3. Silicon wafers (M.M.R.C. Pty Ltd, Malvern, Vic, Australia).
4. Photolithography: A clean room with a spin coater, level hotplates, mask writer or photoplotter, mask aligner (EVG, St. Florian, Austria) or UV flood source and a fume hood.

5. Photolithography chemicals: Ti Prime (MicroChemicals, Ulm, Germany), SU-8 2050 and SU-8 2100 (MicroChem, Westborough, MA), Propylene glycol monomethyl ether acetate (PGMEA;Sigma-Aldrich,Australia)andTrichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl)silane (Sigma-Aldrich) (*see* **Note 1**).
6. Chamber testing: Epifluorescence microscope or confocal microscope and 10–63× objectives (*see* **Note 2**).
7. Optical profiler (Wyko NT1100, Veeco, Plainview, NY).
8. PDMS mixture: Combine polydimethylsiloxane (PDMS) base elastomer (Sylgard 184, Dow Corning, Midland, MI) and silicon elastomer curing agent in a 10:1 (m/m) ratio in a 50 mL plastic tube. Mix for 1 h either with a wooden tongue depressor or a rotary mixer.
9. Fluorescent dextran: Aqueous 10 mg/mL of 40 kDa dextran conjugated to tetramethylrhodamine (Life Technologies, Australia). Store 10 μL aliquots at −80 °C (*see* **Note 2**).
10. Tubing: Polyethylene tubing I.D. 0.58 mm, O.D. 0.965 mm (Intramedic Clay Adams Brand, Becton Dickinson Co.). Cut to length as required. Store in 70 % ethanol. Air-dry in laminar flow hood and rinse with filtered PBS prior to use.
11. Metal connectors: These can be made by cutting the metal needle from a 23 gauge syringe and smoothing down any rough metal burrs on a wetstone.
12. Syringe connectors: 23 Gauge syringes (Terumo Medical Corp., NSW Australia) with the bevelled-tip cut off with a pair of metal snips. The rough edges are smoothened on a wetstone.
13. Syringes: 100, 250, or 500 μL glass syringes (SGE Analytical Science, NSW Australia) or 1 mL plastic syringes (Terumo Medical Corp.).
14. Microfluidics pump (e.g., Harvard Apparatus Ultra, SGE Analytical Science).
15. 0.75 mm corer (Harris Uni-Core, Ted Pella, CA, USA).
16. Plasma cleaner (e.g., PDC-002, Harrick Plasma, NY, USA).
17. (3-Aminopropyl)triethoxysilane (APTES; Sigma-Aldrich).

2.2 Tissue Culture

1. Laminar flow hood and tissue culture incubator with 5 % CO_2 at 37 °C.
2. Leibovitz's L-15 medium: Add 5 mL of 45 % glucose to 500 mL L-15 (Life Technologies). Store at 4 °C.
3. Petri dishes: 35 mm wide, tissue-culture treated petri dishes (Sigma-Aldrich).

4. Trypsin solution: Add 0.5 mL 2.5 % trypsin to 2.5 mL calcium- and magnesium-free Hanks balanced salt solution. Prepare immediately before use.
5. Superior cervical ganglion (SCG) Growth Medium (SGM): 1× Opti-MEM-1 (Life Technologies) containing 1× penicillin/streptomycin, 10 μg/mL mouse laminin, 4 % (v/v) fetal calf serum, 2 % B-27 supplement (Life Technologies) (*see* **Note 3**).
6. Fixative: 4 % paraformaldehyde in 1× phosphate buffered saline (PBS) (*see* **Note 4**).
7. H Solution: 6 nM NGF in SGM, equivalent to $20 \times K_d$ (*see* **Note 5**).
8. Blocking solution: 4 % normal goat serum in PBS.
9. Primary antibody: 1:1000 mouse anti-neuron specific β-tubulin class III antibody (BD Biosciences, Australia) in blocking solution. Add Triton X-100 to 0.05 %.
10. Secondary antibody: 1:1000 goat anti-mouse Alexa 488 conjugated IgG (BD Biosciences, Australia) in blocking solution.

3 Methods

3.1 Shear Stress Determination

An important consideration when designing any microfluidics chamber with liquid flow is the potential for significant shear stress on the cells in the growing chamber. Various methods have been used to minimize this problem, such as culturing cells in a 3D hydrogel [9], using micro-well structures [10], or using a permeable membrane separating the fluid-flow driven gradient from the cells [11]. Using the chamber design in Fig. 1a, we found no correlation between the final direction of axons and the fluid flow direction (i.e., no bias in neurite growth) due to liquid flow rates up to 200 μL/h (data not shown). Assuming the Poiseuille model [12], the shear stress τ is calculated as follows:

$$\tau = -\frac{12\mu Q}{wh^2} \quad \text{for} \quad w \gg h$$

where Q is the flow rate (m^3/s), μ is the fluid viscosity (Pa s), and h (m) and w (m) are the channel height and width, respectively. According to this equation, a flow rate of 200 μL/h caused shear of 1.7 N/m^2. Since shear is not dependent on position within the growth chamber, and since the cells are all in contact with the growth chamber floor, the shear is uniform across all cells. Little is currently known about how much shear stress can be tolerated by neurons of different type or on different substrates. Morel et al. [11] found that at 5×10^{-2} N/m^2, the growth cones of rat DRG neurons displayed damage, but that at 5×10^{-4} N/m^2, damage due to the shear stress was undetectable. Wang et al. [10] found that at

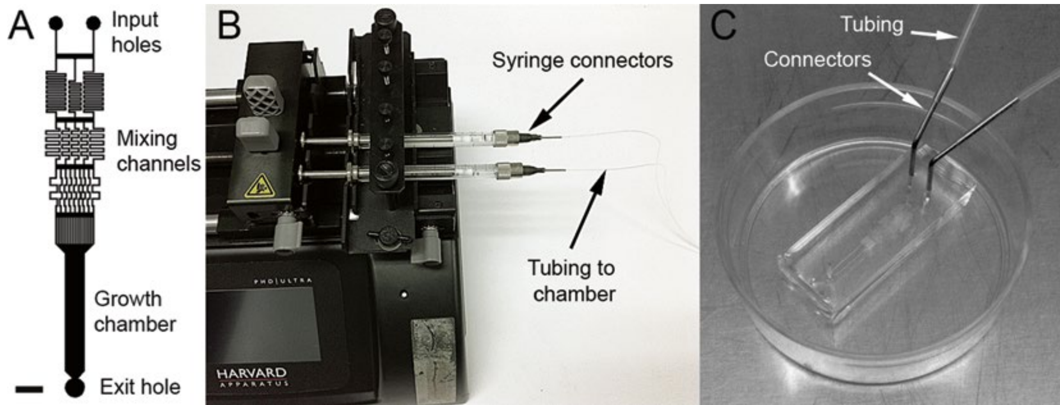

Fig. 1 **(a)** A schematic of the microfluidics chamber channels. The two input holes separately receive SGM containing NGF (H solution) or SGM only (L solution). The media are combined in the mixing channels and enter the growth chamber and eventually leave through the exit hole. The heights of the growth chamber and exit hole are 150 μm, while all other regions of the device are 50 μm. Scale bar = 1 mm. **(b)** Two syringes mounted on a Harvard Apparatus infusion pump via syringe connectors. The polyethylene tubing enters the PDMS chamber (see panel c). **(c)** A completed PDMS chamber bonded to a 35 mm tissue culture grade petri dish. Metal connector tubes are used to connect the polyethylene tubing to the chamber

0.72×10^{-2} N/m^2, *Xenopus* spinal neurons undergo collapse, but that at 4.5×10^{-4} N/m^2, they did not. Consequently, the shear stress in our device at 200 μL/h falls well below that of the detrimental value of 5×10^{-2} N/m^2 used by Morel et al., and is only about twofold above that found to be damaging for *Xenopus* spinal neurons by Wang et al. This suggests that the shear stress in our device is in a domain which, at least for rat SCG cells grown on plastic, is still conducive to growth. However, we note that the no-slip boundary condition for viscous fluid flow states that the velocity profile in a rectangular channel is parabolic [13], implying that the shear stress in the thin layer in which axons are growing may be significantly less than the value calculated above.

3.2 Microfluidics Chambers

3.2.1 Microfluidics Chamber Design

Draft the chamber design using AutoCad software and plot it onto the photoplate. Figure 1a illustrates the design used for the photomask for the first layer of lithography. In order to reduce the shear stress on neurons growing in the growth chamber, the design shown in Fig. 1a is two-layered. The first layer consists of the entire pattern shown in Fig. 1a with a height of 50 μm. The second layer is 100 μm thick and deposited over only the growing region. This means that although the fluid mixing occurs in channel heights of 50 μm, the cells are growing in a region with a 150 μm height. Since shear goes as $1/h^2$, the reduction in shear stress to the cells is considerable. Therefore, a relatively fast flow rate in the mixing channels using only a small expenditure of fluid can be used to establish the gradient quickly.

3.2.2 Microfluidics Chamber Fabrication

Fabricate chamber molds using standard SU-8 multilayer photolithography techniques as follows:

1. Clean silicon wafers using a plasma cleaner with a power of 200 W at a pressure of 200 mTorr for 5 min.
2. Immediately after cleaning the wafers, coat them with Ti Prime by spin-coating at 3000 rpm for 30 s, followed by a 2 min bake at 110 °C. Then allow the wafers to cool to room temperature.
3. Next, deposit a layer of SU-8 2050 on the wafers by spin-coating, according to the manufacturer's recommendations. To achieve an approximately 50 μm thickness, spin the SU-8 at 500 rpm followed by 1500 rpm for 10 and 30 s, respectively.
4. The wafers are then soft baked at 65 °C and 95 °C for 3 min and 9 min, respectively.
5. After allowing the wafers to cool to room temperature, expose them in the mask aligner to a dose of 175 mJ/cm^2. Figure 1a shows the pattern that was used.
6. Perform a post-exposure bake at 65 °C and 95 °C for 2 min and 7 min, respectively, and then allow the wafers to cool to room temperature.
7. Apply a second layer of photoresist, this time SU-8 2100, by spin-coating at 500 rpm followed by 3000 rpm for 10 and 30 s, respectively. This allows for an approximate additional height of 100 μm.
8. The wafers are then soft baked at 65 °C and 95 °C for 5 min and 30 min, respectively.
9. Next align and expose a second mask using the mask aligner at a dose of 250 mJ/cm^2. The second mask is designed to increase the height of the growth chamber and exit hole regions of the devices.
10. When the wafers return to room temperature, develop them in PEGMA for approximately 15 min.
11. Verify the height of the mold using the optical profiler. The lowest and highest features should measure around 50 μm and 150 μm, respectively.
12. Next silanize the molds to prevent PDMS adhesion. Clean the freshly processed masters using an oxygen plasma cleaner and then place them in a vacuum desiccator with several drops of Trichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl)silane for approximately 20 min.
13. Pour enough PDMS mixture onto the silicon master to cover it to a depth of about 4 mm.
14. Place the mold with PDMS into a vacuum chamber and apply vacuum for 2 h to degas the PDMS (*see* **Note 6**). During degassing, minute bubbles will form in the low air pressure and rise to the top of the liquid PDMS. After 2–3 h, the PDMS will appear completely transparent.

15. Bake (cure) the mold for at least 2 h at 80 °C. Baking at higher temperatures for slightly shorter times will also lead to curing.
16. Using a scalpel, carefully cut around the chamber, taking care to avoid damaging the lithographed pattern on the silicon wafer. Gently pull the chamber out of the mold.
17. Cover the channel side of the chamber with Scotch magic tape to protect the channels from airborne dust which can lead to clogging during later stages.
18. Using a 0.75 mm corer, core holes into the PDMS where fluid or cells can be introduced. The corer should have an external diameter slightly smaller than that of the metal connecting tubes. Make sure the PDMS "noodle" is pushed out of the holes and the corer goes all the way through the PDMS.
19. To bond the PDMS chamber to a plastic tissue-culture petri dish, plasma treat the petri dish (using 100 W at a pressure of 380 mTorr for 30 s) and then pour enough APTES solution (5 % APTES in 70 % ethanol) into the dish to cover the bottom surface and leave for 5 min. Meanwhile, plasma treat the PDMS chamber with high power for 40 s. Make sure the PDMS chamber is placed into the plasma cleaner so the channels side is face-up, otherwise the plasma will not properly treat the surface. Discard the APTES solution from the petri dish, wash thoroughly with water, and allow it to air-dry or blow-dry. Press the PDMS chamber onto the APTES-treated petri dish. Make sure to press the channel-side surface of the PDMS onto the dish. If bonding of PDMS chambers onto glass is required, plasma treat the glass and the chamber at the same time and gently press the chamber on glass (*see* **Note 7**).
20. Bake the dish for 30 min at 65 °C. Although the bond forms within a few seconds, baking will increase bond strength. After baking, the chamber is ready to be used for tissue culture, otherwise chambers can be prepared ahead of time and stored at room temperature (*see* **Note 8**).
21. Fill the plate with filtered PBS and penicillin/streptomycin and degas in the vacuum chamber for 5 min. Take out the chambers at least 15 min before injecting cells into them to allow the solution to fill up all the channels to avoid air bubbles.

3.3 Tissue Dissociation

1. Cut out the superior cervical ganglia (SCG) from P0–P3 rat pups into about 2 mL Leibovitz medium in a petri dish kept on ice. Leave in trypsin for 30 min at 37 °C in a 15 mL tube. Use a sterilized flame polished glass Pasteur pipette to gently triturate the cells by aspirating up and down slowly (*see* **Note 9**).
2. Stop the trypsin by filling the tube with Leibovitz medium to 15 mL. Centrifuge at 190 rcf for 5 min at 4 °C. Discard the supernatant carefully and then repeat with 15 mL Leibovitz, then 15 mL Opti-MEM and then with 0.5 mL filtered SGM

containing 0.3 nM NGF. Suck out most of the solution to leave 50 μL of solution per SCG. Very gently resuspend the cells. Using the microfluidics pump or pumping by hand with a short length of polyethylene tubing connected to a metal connector, aspirate the solution at 1 μL/s and then inject into a test chamber through the outlet with the chambers fully immersed in PBS/PS. Make sure there is no air bubble in the injected solution or in the chambers. Adjust the cell density so that 20–100 cells enter the growth chamber. Very low density often leads to poor growth and very high density makes imaging difficult.

3.4 Growth in the Gradient

1. Leave the cells in the incubator for at least 1 h to allow cells to adhere to the substrate before setting up the flow.
2. Cut two lengths of polyethylene tubing approximately 60 cm (*see* **Note 10**). Connect a metal connector to one end of each tube (Fig. 1b). Connect the other end of each tube to a syringe connector and connect, via a Luer lock, to a 250, 500, or 1000 μL glass syringe or a plastic 1 mL syringe (the size of the syringe limits the duration of the experiment). Insert the syringes into the microfluidics pump. The syringes contain H solution (SGM with a high concentration of guidance cue (in our case NGF), equivalent to $20 \times K_d$) and L solution (SGM with no guidance cue). Make sure there are no air bubbles in the tubing and that there is no air in the syringes. To generate a linear gradient, the flow rates of both syringes have to be the same (5 μL/h each). To increase the throughput, four chambers can be run in parallel, with eight syringes on two PhD Harvard pumps.
3. Pump out a small amount of solution then quickly put the metal pins of the H and L tubes into the appropriate inlets (Fig. 1c; *see* **Note 11**). Turn on the flow to 5 μL/h. Gradients should establish within 5 min.
4. Place the chambers into a tissue culture incubator (with the pump outside). After 2–4 h, neurites will start to grow from the cell bodies. At this point, the cells may be used for live imaging by moving the chambers and pump(s) to an incubated inverted microscope. The tubing can be attached to the incubator door higher than the plate so that air bubbles will rise and not enter the chambers. Otherwise incubate the chambers overnight. Make sure there is enough solution in the syringes for the desired duration of the experiment.

3.5 Quantification of Guidance

1. The chambers can be used for in vitro imaging of growth cone guidance using time-lapse microscopy. The chambers should be housed in an inverted microscope with environmental con-

trol including temperature (37 °C) and a 5 % CO_2 atmosphere. Images can be obtained using phase contrast microscopy at 1 min intervals for many hours. The degree of guidance can be estimated for each growth cone from the time-lapse data using the definition of the turning angle shown in Fig. 3b [6]; however, other definitions of turning can also be used [10, 14].

2. If, following growth of neurites in the growing chamber, the axons need to be immunostained, then gently remove the tubing from the microfluidics chamber and examine the growing chamber. Neurites should be visible under phase contrast microscopy (*see* **Note 12** and Fig. 3a). Fill a 1 mL plastic syringe with fixative and, with a short length of polyethylene tubing (and using the appropriate connectors) pump through the fixative into the exit hole at 1 μL/s for 60 s. Replace the fixative in the syringe with PBS and pump through at the same flow rate for 5 min to remove all fixative. Pump through blocking solution and then primary antibody at the same flow rate for 60 s. Leave the primary antibody solution in for 15 min without flow. Pump through the appropriate fluorophore labeled secondary antibody at the same flow rate for 60 s and leave without flow for 15 min. Finally, wash with PBS for 5 min (*see* **Notes 13** and **14**).
3. Photograph neurites at 20× using fluorescence microscopy for the appropriate fluorophore.

4 Notes

1. Chamber design: A comprehensive description of the concepts used in the design of our chamber can be found in Campbell et al. [15]. The dimensions of the design were modified to suit the diffusion constant of nerve growth factor.
2. To test whether the chamber is capable of generating a gradient, a fluorescently labeled dextran, of a molecular weight roughly equivalent to the guidance cue being studied, can be used to visualize the gradient. 40 kDa-dextran fluorescently labeled with tetramethylrhodamine has a molecular weight similar to that of nerve growth factor. To visualize the gradient, set up a gradient as in Subheading 3.4, **step 2**; however, exclude the guidance cue and instead, include the fluorophore in the H solution. L solution should contain no fluorophore. The exact concentration of the fluorophore in the H solution is not important as long as it is high enough to provide an image of the gradient using a relatively short exposure (e.g., 1 s) (Fig. 2a, b). Time-lapse imaging of the chamber can be used to assess the stability of the gradient.

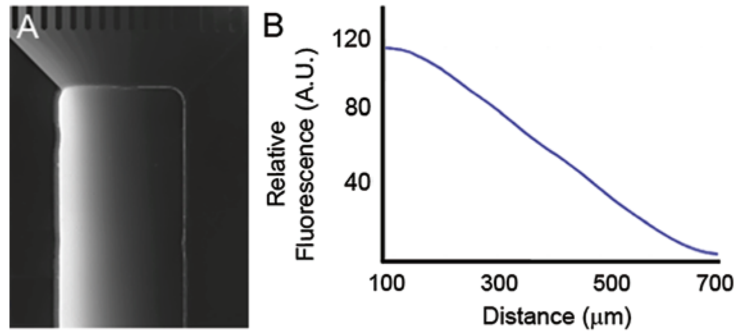

Fig. 2 **(a)** A gradient of 40 kDa dextran-tetramethylrhodamine in the growth chamber of the PDMS chamber shown in **(b)**. **(b)** The relative fluorescence of 40 kDa dextran-tetramethylrhodamine in a transverse section through the gradient in **(a)**. (A.U. = arbitrary units)

3. This is also suitable for the growth of dorsal root ganglion neurons from newborn rat pups up to about postnatal day 7 of age.
4. 4 % paraformaldehyde in PBS can be stored at −20 °C for several months and should be thawed to room temperature before use. It produces sufficient fixation for the visualization of axons, however we find it unsuitable for the fixation of finer cellular detail such as filopodia.
5. The dissociation constant (K_d) of NGF for its high affinity receptor, TrkA, is about 0.3 nM [16].
6. When PDMS is mixed or poured, tiny bubbles will be generated which, if not removed, will become incorporated into the microfluidics chamber during curing.
7. If PDMS chambers do not adhere to glass or plastic substrates, the plasma oxidation conditions should be optimized. Determine optimal oxidation time, ionization strength and O_2 pressure within the plasma cleaner. For our Harrick Expanded Plasma Cleaner, typical values are 380–410 mTorr O_2 pressure, 30 W power, and 30–50 s ionization time.
8. Contamination by bacterial and fungal cells may be reduced by spraying the chambers with 70 % ethanol following by irradiation with short-wavelength UV for several hours. Once dried, the chambers may be used for tissue culture.
9. Poor cell growth can also be due to poor trituration. Optimize the trypsin concentration and incubation time. Optimize the size of the flame-polished pipette bore. Holes which are too large will result in poor dissociation. Holes which are too small will result in high cell death. Ganglia should dissociate into cells within 2 min of trituration.

10. The length of both tubes has to be enough so that the microfluidics chamber can be placed in a tissue culture incubator with the microfluidics pump outside the incubator.
11. A major problem which will be encountered when using microfluidics is the accidental introduction of air bubbles into the chamber. This can occur mostly as a result of poor connections where the metal connector tubes are inserted into the chambers. The metal connectors can be sealed by making a small amount of PDMS with curing agent and spreading about 10 μL around the insertion site followed by curing at 65 °C. Then fill the plate with filtered PBS to immerse both the chambers and connector pins in solution and degas. Use the tubing to suck solution through the connector pin to fill up all the channels and pins with PBS. However we find that the best solution is to ensure that coring achieves clean defect-free holes which are less likely to leak and inject into the chambers while they are immersed in solution.
12. If neuronal cell growth is poor, it may be necessary to perform PDMS extraction prior to bonding to the substrate. This is done in order to remove unpolymerized PDMS monomers from the PDMS chamber which may cause cell toxicity. Numerous PDMS extraction techniques exist. We have found the following to improve neuronal cell growth: Following removal of the PDMS from the mold, immerse chambers for ~200 mL for 1 h into each of the following; 100 % pentane, 100 % acetone and then 100 % ethanol. PDMS will swell significantly while in the pentane and acetone, and care should be taken to ensure the liquid volume is significantly larger than the PDMS volume. Do not delay in transferring chambers from one solution to the next otherwise cracking of the PDMS will occur. After PDMS have soaked in the ethanol, bake at 65 °C for 2 h and proceed with bonding to the substrate. Discard the used solutions according to institutional guidelines.
13. Image quality of the immunostained cells will be better with a glass substrate.
14. We find that a high solution (H) of 20–40 × K_d and a low solution (L) of 0 × K_d elicited the strongest turning responses in SCG axons whereas a very high solution of H = 200 × K_d abolishes the turning (Fig. 3c shows turning of growth cones in a gradient using nerve growth factor with L = 0 nM, H = 20 × K_d nM). If no turning is observed, these parameters should be optimized.

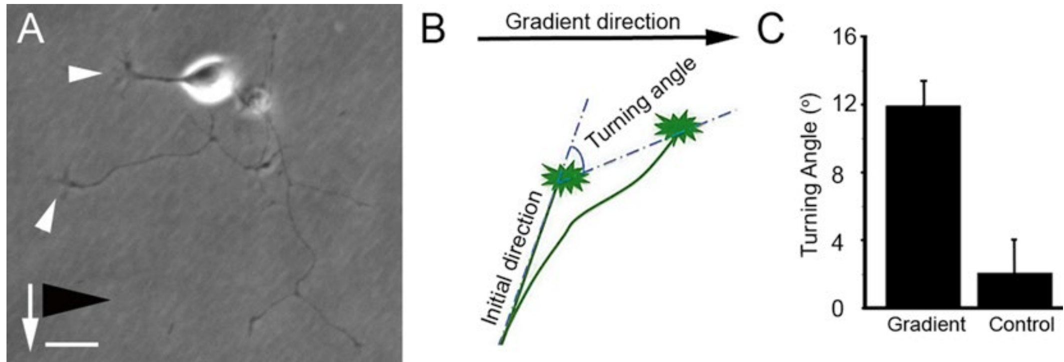

Fig. 3 (a) Phase contrast image, obtained with a 20× objective, of an SCG neuron grown in a microfluidics chamber with an NGF gradient (gradient direction is shown by the *black triangle*). The *white arrow* indicates direction of fluid flow. *White arrowheads* indicate growth cones with filopodia. Scale bar = 10 μm. **(b)** Definition of initial angle and turning angle in short-term turning assay. The reference axis is perpendicular to the direction of flow, which is the gradient direction. The initial direction is defined as the tangential direction of the 15 μm neurite segment. The initial angle is defined as the angle between initial direction and the reference axis. The turning angle is defined as the angle between the line connecting the growth cone before and after the assay and the initial direction. **(c)** Turning angles of axons grown either in a gradient of NGF ($n = 190$ axons) compared to those grown in an NGF plateau control ($n = 110$ axons) using a flow rate of 10 μL/h. Axons grown in the gradient display a positive turning response of 12° ± 1.4° compared to 2.1° ± 2.0° (mean ± S.E.M.) for those grown in a plateau of NGF ($p = 0.033$, *t*-test)

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