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A dual compartment diffusion chamber for studying axonal chemotaxis in 3D collagen

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HIGHLIGHTS

- We present an inexpensive assay for studying axon and cell chemotaxis in 3D.
- The gradients formed in the assay are reproducible and long-lasting.
- We demonstrate the assay using DRGs/NGF and olfactory bulbs/Slit2.

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ABSTRACT

During nervous system development growing axons are often guided by diffusible chemical gradients. An important contribution to our understanding of the mechanisms involved in this process has been made by in vitro assays. However, an inexpensive and simple assay which allows the establishment of stable and reproducible gradients in a 3D collagen environment has been lacking. Here we present a simple two-compartment diffusion chamber for this purpose. We show that gradient steepnesses of up to 2% are achieved within 1 h post setup, and a gradient persists for at least 2 days. We demonstrate the assay by showing robust chemoattraction of dorsal root ganglion neurites by gradients of nerve growth factor (NGF), and chemorepulsion of olfactory bulb neurites by gradients of Slit2.

1. Introduction

During nervous system development, growing axons must navigate complex environments and integrate multiple guidance cues to reach their targets (reviewed in O'Donnell et al., 2009). A particularly important type of cue in vivo is chemical gradients (Mortimer et al., 2008). However, since the in vivo environment of the developing nervous system is poorly characterized and hard to manipulate, a crucial contribution to understanding the mechanisms of gradient-based axon guidance has come from in vitro studies, where the relevant variables can be more rigorously controlled.

The two most popular assays for studying diffusible gradients for axon guidance have been the 2D 'pipette' or growth cone turning assay, and the 3D collagen gel co-culture assay. In the former the chemotropic factor is ejected from a small pipette into liquid medium in a Petri dish (Gundersen and Barrett, 1979; Lohof et al., 1992; Pujic et al., 2008). This creates a gradient by diffusion over a range of about 100 μm, with a steepness at 100 μm of about 10% fractional change in concentration over 10 μm. The response of axons (normally from dissociated neurons) growing on the bottom of the Petri dish to the gradient can be observed over a period of 1–2 h, after which the gradient tends to lose stability and/or the pipette becomes clogged. Although very useful in many applications, this assay represents a poor model of the 3D in vivo environment, where axons are generally guided for longer periods of time over longer distances by gradients that are probably often substantially shallower than 10% (Goodhill, 1998).

In the 3D collagen gel co-culture assay a small piece of target tissue, or block of cells transfected with a chemotropic factor, is embedded in a 3D collagen matrix within a few hundred microns of a small piece of tissue within which the axons of interest are generated (Gil and del Rio, 2012; Lumsden and Davies, 1983). Although not actually observed, it is inferred that a gradient is produced by diffusion away from the target tissue. Biased growth of axons towards, compared to away from, the target can then be seen, usually on the timescale of days. Although axon turning is often not

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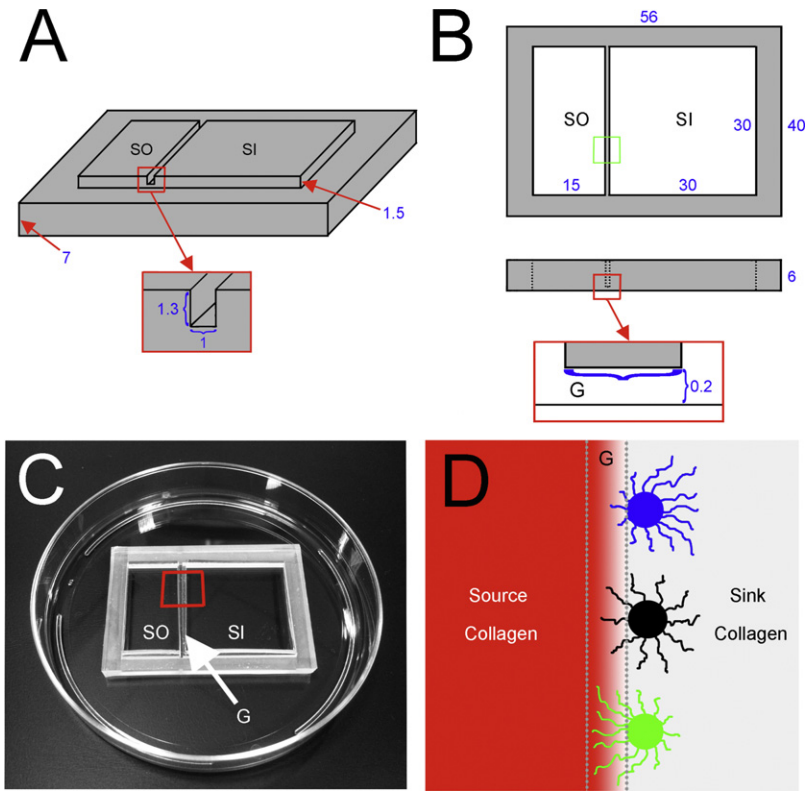

Fig. 1. Dual-compartment diffusion chamber design. (A) Schematic diagram of the plastic mould used to generate the dual-compartment culture setup (i.e. the "negative image"). The inset in red shows a detail of region G, the 1-mm wide gap between the source and sink compartments which creates a dividing wall between the two compartments. All values are in millimetres. (B) Schematic of the PDMS chamber made using the mould in panel A (i.e. the "positive image", including a top view and a cross-section side view. A gradient of a guidance cue is formed across a 1-mm wide region (G) through diffusion from a source (SO) to sink (SI) compartments. Grey regions indicate cross-section through PDMS. Region G is 0.2 mm tall, measured from the substrate to the bottom of the dividing wall. The green box indicates the region shown in Fig. 2A and B. (C) Photograph of the final PDMS chamber bonded to a 100-mm diameter tissue-culture dish. The region shown in the red square includes the dividing wall, and is depicted in panel D as a top-down view. The dividing wall (G) separates the two compartments but allows diffusion through a 1-mm wide, 0.2-mm tall gap. (D) A top-down schematic of the region in the red box in panel C, indicating the positions of explants responding to a gradient formed in the 1 mm wide region G (between the dotted lines). If explants are grown with a chemoattractive cue only in the source collagen, neurites generally grow as shown in green. If a chemorepulsive cue is present only in the source collagen, neurites generally grow as shown in blue. If the concentration of a guidance cue is equal in both chambers (plateau), neurites generally grow as shown in black. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

present, biased outgrowth can still be a signature of a chemotropic response (Mortimer et al., 2010). This assay is relatively easy to set up, and simulates both the 3D environment and timescale of guidance of the in vivo environment much more closely than the growth cone turning assay. However, it offers little control over, or reproducibility in, the gradients established (Goodhill, 1998), probably contributing to the often wide variance in results produced.

The much more sophisticated 3D collagen printing assay (Rosoff et al., 2005, 2004) overcame many of these limitations, allowing precise control over gradient parameters in a 3D environment. However this control and flexibility came at the cost of high set-up expense, and restriction to gradient steepnesses of substantially less than 1%. Other assays that have frequently been applied to studying axon guidance by gradients in vitro include the Dunn chamber (Dudanova et al., 2010, 2012; Kent et al., 2010; Ruiz de Almodovar et al., 2011; Yam et al., 2009), and various microfluidics-based approaches (e.g. Wang et al., 2008). However, none of these have so far satisfied all the criteria of being simple to set up, and allowing guidance to be examined for long periods of time in 3D (see Section 4).

To overcome these limitations, we present a simple, low cost dual-compartment device that is easy to set up, and is capable of rapidly and reliably generating gradients with predictable values of gradient concentration and steepness. It can accommodate tissue explants (typically approximately 500 μm diameter) commonly used in axon guidance. To illustrate the use of the device for both attractive and repulsive factors we demonstrate attraction of neurites from dorsal root ganglia to NGF gradients, and repulsion of olfactory bulb neurites by Slit2 gradients.

2. Materials and methods

2.1. Generation of PDMS diffusion chambers

A diffusion chamber mould was prepared by machining Teflon to the dimensions shown in Fig. 1A. Polydimethylsiloxane (PDMS, Sylgard 184; Dow Corning) was prepared by mixing base polymer and curing agent in a 1:10 ratio. The PDMS was poured onto the plastic mould to a depth of 6 mm above the mould and allowed to set for 2 h at 65 °C. The PDMS chamber (Fig. 1B) was excised by cutting ca. 5 mm around the compartments of the mould. PDMS was removed from above the compartments to create openings through which collagen and explants can later be introduced. Free base polymer was extracted from the PDMS by 1 h washes (with stirring) in the following; 100% pentane, 100% acetone, 100% ethanol. Moulds were dried at 65 °C for 1 h and then bonded to 100 mm diameter Petri dishes using PDMS (Fig. 1C) applied to the 5 mm wide surface around the compartments. After curing again at 65 °C for 2 h, chambers were exposed to UV light in a tissue culture laminar hood for 1 h.

The total cost required to produce the chambers is very low. Generation of the mould by a machine shop is ~USD$100

(including parts and labour). The mould can be used repeatedly with no visible wear appearing after each chamber made from it. Chemical costs include PDMS, acetone and ethanol at ~$1 per chamber each, and pentane at $6 per chamber (all prices in USD). The total cost per chamber is approximately $10, and several explants of 600 μm diameter can be embedded per chamber.

2.2. Olfactory bulb and dorsal root ganglion dissection

All experiments were approved by The University of Queensland Animal Ethics Committee and were performed according to the National Health and Medical Research Council's animal code of practice. Olfactory bulbs (OBs) were excised from E15 Wistar rat pups into Leibovitz solution on ice. OBs were freed of meninges and then bisected along the antero-posterior axis. Dorsal root ganglia (DRGs, thoracic and lumbar) were excised from P0 to P3 rat pups into Leibovitz on ice and freed of axons. To loosen the connective tissue capsule surrounding the ganglia, the DRGs were digested in 0.25% trypsin for 10 min and then washed extensively with Leibovitz.

2.3. Setting up the assay

Collagen was prepared, on ice, with the following final concentrations; 0.2% rat tail collagen type 1 (BD Bioscience), 0.1% sodium bicarbonate, 1× Optimem-1, 1× penicillin–streptomycin, 1% foetal calf serum. For plateau experiments only, mouse submaxillary gland NGF (BD Biosciences), or Slit-2 (recombinant mouse Slit2, R&D Systems) was added to 0.3 nM. Enough collagen was added to fill both chambers (2.6 mL total) and explants were placed into the sink chamber and positioned next to the gradient region before the collagen had set. To ensure that the PDMS did not absorb moisture from the collagen, the Petri dish was filled with 10 mL of 1× optimem-1 with 1× penicillin–streptomycin so that the PDMS was in contact with growth medium. For gradient experiments, collagen without guidance cue was added to the sink chamber first (1.8 mL). Up to 8 explants were placed into the sink chamber and positioned next to the gradient region. The collagen was allowed to enter the gradient region but not to enter the source chamber. Once the sink collagen had set, collagen containing guidance cue (0.9 mL of 1 nM) was added to the source chamber and allowed to set. Optimem-1 containing penicillin/streptomycin was then added to the Petri dish as before. Plates with Slit-2 and OBs were incubated for 3 days at 5% CO_2/37 °C while plates with DRGs and NGF were incubated for 2 days. NGF and Slit2 gradient experiments were repeated three times. Plateau experiments were repeated twice.

To monitor the possible mixing of the source and sink collagen during setup, 2 μm fluorescence beads (Fluoresbrite YG Microspheres, Polysciences) were added to the sink collagen in control plates while 1 nM of 40 kDa-dextran tetramethylrhodamine (Molecular Probes, D1842, hereafter referred to as dextran) was added to the source collagen. A region (shown by the green box in Fig. 1B) was imaged using epifluorescence with a GFP emission filter to show the sink collagen/source collagen boundary, while the dextran was imaged with a DsRed emission filter.

2.4. Immunostaining

Explants embedded in collagen were fixed with an equal volume of 10% formalin/0.1% Triton X-100 in PBS overnight. Plates were washed for 5 × 1 h in PBS and then incubated overnight with 1 μg/mL of the antibody TuJ1 (specific for neuronal βIII tubulin, R&D Systems), followed by an additional 5 washes in PBS for 1 h each. The explants were then incubated overnight in the secondary antibody AlexaFluor 488-conjugated goat anti-mouse IgG (1:1000; Molecular Probes), washed 5 times in PBS for 1 h each, and photographed with an AxioCam HRm (Zeiss) camera on a Zeiss imager Z1 fluorescence microscope with a 2.5× objective.

2.5. Quantification of the guidance ratio and outgrowth

Fluorescence images of explants were imported into a custom written MatLab program which estimated the outgrowth (OG) and guidance ratio (GR) as described previously (Mortimer et al., 2009, 2010). GR is defined as (H − L)/(H + L) where H and L are the number of neurite pixels on the high- and low-ligand concentration sides of the explant respectively. Positive GR values denote attraction of neurites to a guidance cue. OG is calculated as the total number of neurite pixels (H + L) divided by E, the number of pixels in the explant.

2.6. Gradient dynamics

To determine the kinetics of gradient formation and stability in the chambers, we prepared source and sink collagen as in Section 2.3 but with the inclusion of 1 nM dextran in the source compartment. Standard control chambers consisted of a plateau of 0, 0.3, 0.6 or 1.2 nM dextran. Confocal images, using a 10× objective and excitation with a 532 nm laser using a Zeiss 5 Live confocal microscope, were taken of the G region (Fig. 1B) in gradient or standard control chambers. Images were taken at 1, 6, 24 and 48 h after setup of the chambers (where $t = 0$ h denotes setting of the source collagen). Standard control chambers were used to determine the relationship between fluorescence and concentration of the dextran. We excluded the first and last 60 μm of region G in each image due to light scattering from the source and sink wells. A custom-written MATLAB program was used to derive gradient steepness from the fluorescence images of the gradients. We define a gradient of p% as meaning a fractional change of $p/100$ in concentration of guidance cue across 10 μm, the typical length scale for a growth cone.

3. Results

3.1. Gradients form within 1 h

To determine if significant mixing of the collagen in the two compartments occurs postsetup, we included 2 μm green fluorescent beads in the sink collagen in some experiments. At 1 h postsetup, we imaged an area (shown in the green box in Fig. 1B) for dextran or for beads. Fig. 2A is an epifluorescence image of the dextran in the two compartments as well as the gradient formed in region G, and shows that a gradient has formed in region G. Fig. 2B is an epifluorescence image taken with a GFP filter and shows that the sink collagen remains separated from the source collagen.

To determine the steepness of gradients formed using the diffusion chamber, we prepared gradients using collagen containing 1 nM dextran in the source well of the diffusion chamber. We obtained time-lapse images of the gradient region (region G in Fig. 1B) at 1, 6, 24 and 48 h post-setting of the source collagen using a confocal microscope and determined the concentration of the dextran in region G, as well as the steepness of the gradient in region G.

At all times examined, the dextran exhibited a decrease in concentration in region G as a function of distance from the source well. The absolute concentration of dextran in region G generally increased between 1 and 48 h postsetup. At 1 h following setup (red line, Fig. 2C), the concentration of dextran varied from 0.5 to 0.6 nM. There was a progressive increase in concentration thereafter to 48 h postsetup where the concentration varied between about 0.8 and 1 nM. Analysis of gradient steepness showed that the gradient forms within 1 h after the source and sink collagens

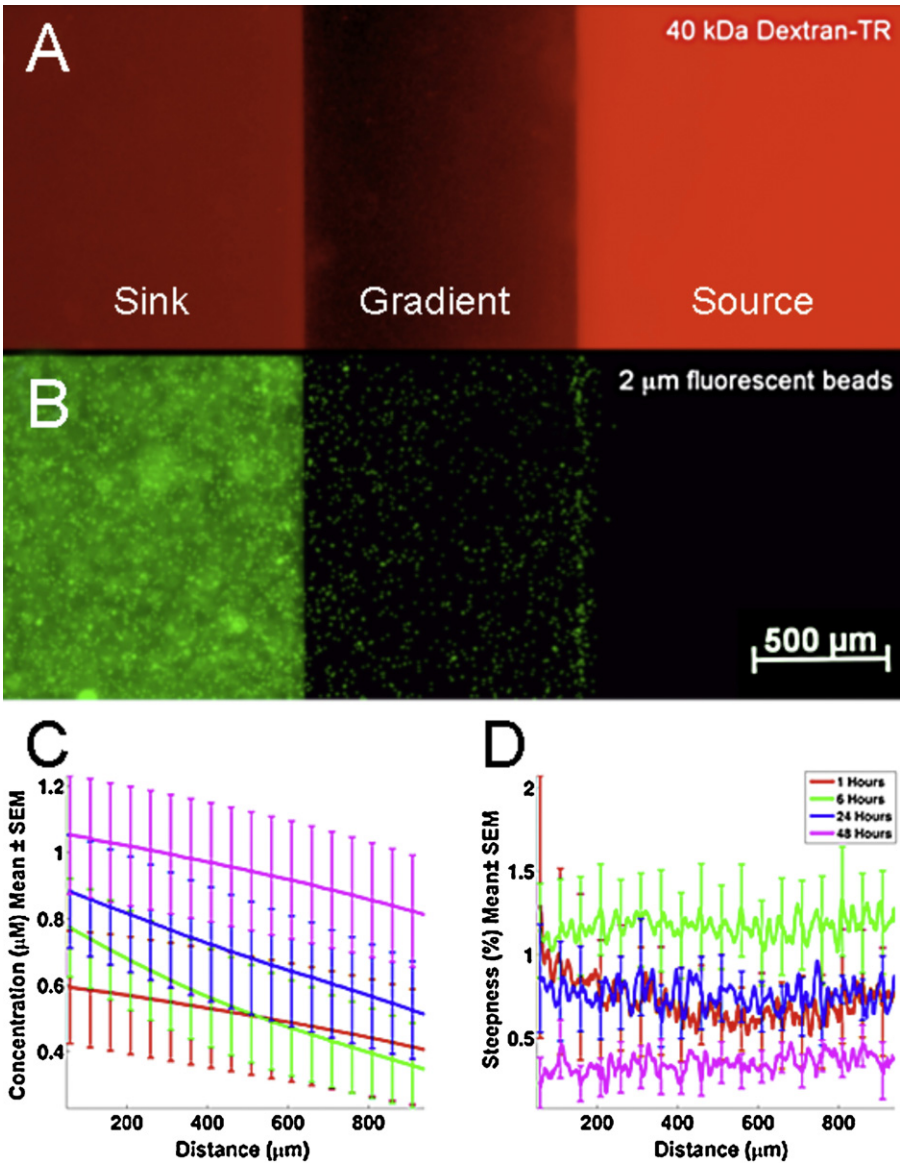

Fig. 2. Gradient steepness and concentration profiles. (A) Gradient visualized using 40 kDa dextran tetramethylrhodamine at 3 h post setup. Gradients are formed across a 1-mm wide region, in a 3D collagen matrix, by diffusion from source compartment (right) to sink compartment (left). (B) Source and sink collagen boundary visualized with 2 μm fluorescent beads in the sink collagen. The boundary remains sharp, indicating minimal mixing of the collagen solutions during setup. (C and D) Gradient concentration and steepness determined from the profile of dextran fluorescence across the 1-mm wide gradient region, measured as a function of distance from the source well. Gradient concentration at any point in region G increases between 1 and 48 h postsetup. Gradient steepness increases between 1 and 6 h postsetup to reach a value of ~1.2% across region G, and then declines by 48 h to ~0.4% (steepness is fractional change in concentration across 10 μm).

are generated within the chamber, with a steepness between 0.6% and 1% across region G (red line, Fig. 1D). At 6 h following setup, the gradient has reached an almost uniform value of 1.25% across region G. At 24 and 48 h following setup, the gradient decays to approximately 0.8% and 0.4% respectively (Fig. 2D).

3.2. Guidance of DRG neurites to NGF

To determine if the diffusion chamber could elicit chemotropism of neurites to a known chemoattractant, we cultured dorsal root ganglia from P2 Wistar rats in the sink chamber adjacent to the gradient region, with either a 0–1 nM NGF gradient, or a plateau of 0.5 nM NGF (Fig. 3A and B). DRGs grown in the plateau had neurites which grew out evenly from the explant. The guidance ratio (GR), a measure of outgrowth asymmetry (see Section 2) was 0.00 ± 0.01, while the normalized total outgrowth OG (see Section 2) was 1.61 ± 0.30 (2 chambers, 7 explants, mean ± SEM). However, DRGs grown in the gradient chambers had neurites which grew preferentially into the direction of the gradient. Although the OG of these explants ($OG = 1.03 \pm 0.17$, 6 chambers, 42 explants) was similar to that of the DRGs grown in the plateau ($p = 0.09$), their GR was much higher and statistically different ($GR = 0.22 \pm 0.05$, $p = 0.04$ t-test, compared to plateau).

3.3. Guidance of OB neurites to Slit2

To confirm that the diffusion chamber could also elicit chemotropism of neurites to a known chemorepellent, and works for tissue other than DRGs, we cultured OB explants from E15 Wistar rats in the sink chamber adjacent to the gradient region, with either a 0–1 nM Slit2-N gradient, or a plateau of 0.5 nM Slit2-N (Fig. 3C and D). Due to the slower growth of neurites from OBs compared to those from DRGs, we incubated the OBs for 3 days rather than 2 days. OBs grown in the plateau had neurites

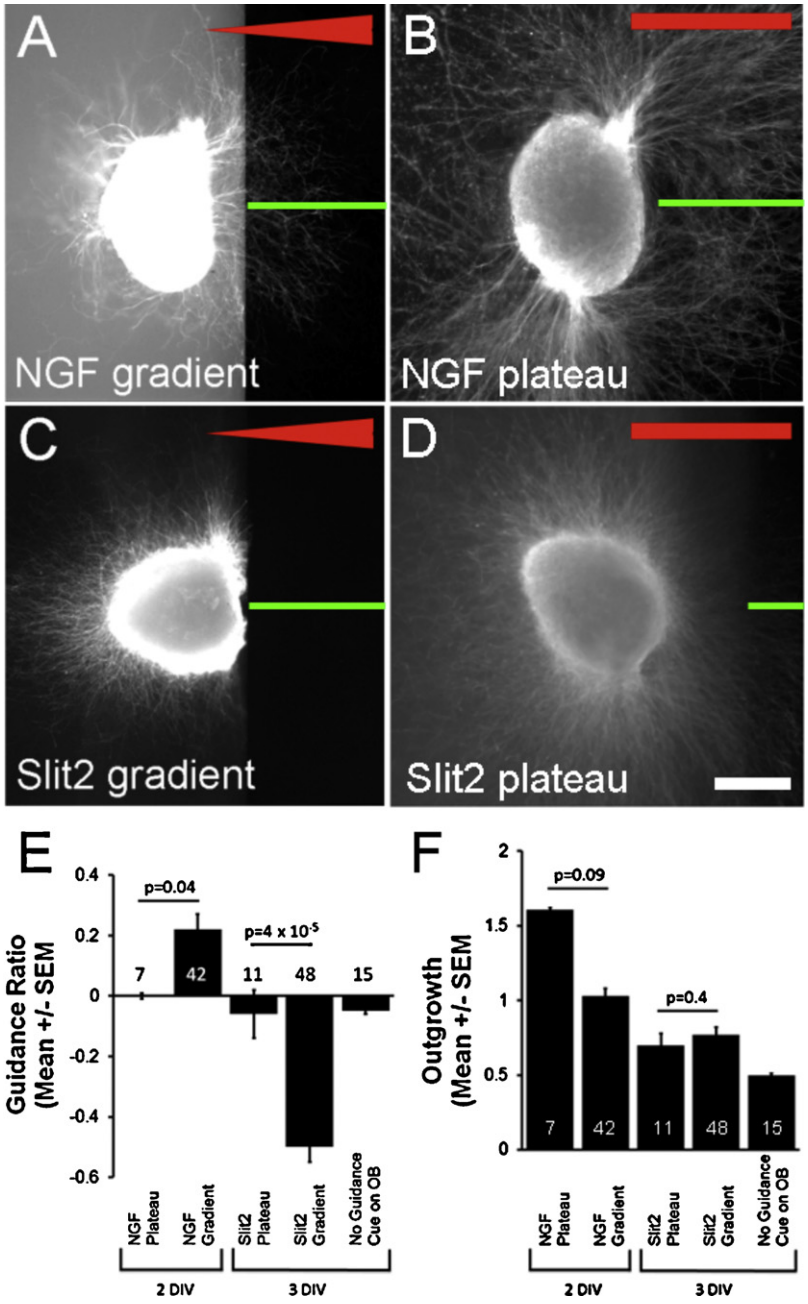

Fig. 3. Guidance of DRG and OB neurites. (A) A DRG grown for 2 days in vitro (DIV) in a 0 to >1 nM NGF gradient. Neurites grow preferentially towards the source chamber (right). (B) Neurites of DRGs grown in an NGF plateau (0.5 nM) grow evenly towards source and sink chambers. (C) Similarly, neurites of OBs grown for 3 days in vitro (DIV) in a 0 to >1 nM Slit2 gradient grow preferentially away from the source of Slit2. (D) Neurites of OBs grown in a 0.5 nM Slit2 plateau grow evenly towards both chambers. Red triangles indicate gradient direction. Red rectangles indicate plateau. Green bars indicate the extent of region G visible in the image. Scale bar = 300 μm. (E) Guidance ratios of DRGs or OBs grown in gradients are significantly different to those grown in plateaus (*t*-test, $p = 0.04$ and $p = 4 \times 10^{-5}$ respectively) or in a no-guidance cue chamber ($p = 2 \times 10^{-5}$, for Slit2 only). (F) Outgrowth from DRGs and OBs is similar in gradient or plateau conditions ($p = 0.09$ and 0.4 respectively) or in a no-guidance cue chamber ($p = 0.4$, for Slit2 only). The number of explants for each group is shown in each column in panels E and F. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

which grew out evenly from the explant, with a GR of -0.06 ± 0.08 and OG of 0.67 ± 0.07 (2 chambers, 11 explants). However OBs grown in the gradient chambers had neurites which grew preferentially away from the gradient. Although the OG of these explants (OG = 0.77 ± 0.17, 6 chambers, 48 explants) was similar to that of the OBs grown in the plateau ($p = 0.4$), their GR was much lower and statistically different (GR = -0.50 ± 0.05, $p = 4 \times 10^{-5}$ *t*-test, compared to plateau).

In a separate experiment, we examined the GR and OG of neurites of OBs grown in the chamber with no Slit2 ($n = 15$ explants). Neither the GR (-0.05 ± 0.01) and OG (0.50 ± 0.10) were statistically different to that of OB neurites grown in a Slit2 plateau ($p = 1.0$ and 0.2 respectively). However when compared to the GR and OG of OB neurites grown in a Slit2 gradient, the GR was significantly different ($p = 2 \times 10^{-5}$) but the OG was not ($p = 0.4$).

4. Discussion

A significant contribution to understanding gradient-based axon guidance has come from in vitro experiments which

generated gradients of diffusible cues in 2D or 3D environments for the study of axons from single cells or tissue explants (Gallo et al., 1997; Gundersen and Barrett, 1979; Li et al., 1999; Lohof et al., 1992; Lumsden and Davies, 1983; Mortimer et al., 2009; Pujic et al., 2009; Song et al., 1997; Tessier-Lavigne et al., 1988). Although assays which use 2D environments are easier to generate, 3D environments possess the advantage of being more synonymous with in vivo tissue, and growth cones display altered morphology in 2D compared to 3D (Balgude et al., 2001).

Gradients of chemotropic cues can be generated in 3D through the diffusion of the cues from a point source into an effectively infinite sink, such as in explant co-culture assays (Gil and del Rio, 2012; Lumsden and Davies, 1983; Tessier-Lavigne et al., 1988). Co-culture assays investigate the effect of a diffusing compound (released by a source) on a target tissue explant. The source and target are, conventionally, held in close proximity in a 3D matrix conducive to cell growth. The source can be another explant, dissociated cells embedded in an agarose cube, or an agarose cube impregnated with the compound being studied. In the context of neurobiology, the effects on the target cells can include, but not be limited to, outgrowth of neurites or directional growth of the neurites. Although easy and rapid to set up, co-culture assays provide gradients with poorly defined values of guidance cue concentrations or gradient steepness, as well as a poor ability to control either of these. Gradients in 3D environments can also be formed via patterning mechanisms such as the "printing assay" which uses a commercially available nanoplotter capable of generating a gradient over a relatively large area (up to 20 mm × 20 mm) by ejecting guidance cue into 3D collagen in which explants are grown (Rosoff et al., 2005, 2004). This system has proven to be capable of precisely generating shallow gradients with known values of gradient steepness and guidance cue concentration, and has provided data for computational modelling of axonal responses (Mortimer et al., 2009; Xu et al., 2005). However this assay is costly in terms of equipment, and steepnesses are limited to <0.4%.

As another alternative, gradients can be actively generated using a microfluidics-based approach which rely on the mixing of different concentrations of cue in a controlled manner to form a continuous concentration profile (Cimetta et al., 2010; Wang et al., 2008) Microfluidic devices which employ diffusion have also successfully generated gradients in 3D (Kothapalli et al., 2011). While microfluidics is poised to fulfil the promise of providing precise control over gradient characteristics, suitable for a wide range of cell and explant types, it comes at a high cost. Manufacturing equipment and complexities are beyond the scope of many labs, and with increasing microfluidics design comes decreased reliability.

To overcome these limitations, we have presented a simple dual-compartment device capable of rapidly generating gradients with known values of gradient concentration and steepness, and capable of accommodating up to 600 μm diameter explants. Our design differs from other dual-compartment chambers such as the Dunn, Zigmond and Boyden chambers because it can reproducibly produce a gradient in a 3D collagen environment. To improve reproducibility in gradient characteristics, our chamber constrains the distance across which the gradient forms (region G in our design). This ensures a relatively consistent set of gradient parameters including steepness and concentration. A knowledge of, and control over, gradient steepness are critical for an understanding of axon guidance because the response of growth cones in shallow gradients is different to that in steep gradients (Thompson et al., 2011).

Our assay overcomes many sources of variability in explant co-culture assays. One such source of variability is the lack of spatial precision in the placement of tissue and guidance source, which affects the gradient experienced by the tissue (Goodhill, 1998). Another source of variability in explant co-culture assays is that of the quantity of guidance cue being released from a source explant, or agarose cube impregnated with the cue. Due to the difficulty in generating cubes of reproducible volume, the quantity of guidance cue being released in explant co-culture assays is variable. For assays where a purified source of the guidance cue is available, our chamber eliminates this variability since all explants in our chamber will be subject to a common source of guidance cue (i.e. that within the source chamber). This is important because the release of guidance cue from agarose cubes is a function of volume and surface area. For smaller cubes, the release of guidance cue is faster, leading to a faster decay in the gradient.

We therefore expect that the variance in guidance response in our assay should be less than that in standard collagen gel co-culture assays. However it is difficult to compare these variances directly: because of the large number of factors that are difficult to tightly control in co-culture assays any individual experiment will not necessarily be representative of the variance across co-culture assays in general.

Another potential advantage of our dual-compartment chamber is the possibility of time-lapse imaging of both growth cone filopodial activity as well as axon growth in the presence of a well-defined guidance cue gradient in a 3D environment. Our cultures can be bonded to a coverslip substrate which should permit examination of growth cones at high magnifications in the collagen environment. Although several dual-compartment devices such as Dunn, Zigmond, Campenot and the newer PDMS-based chambers do permit live imaging of growth cones and axons (Campenot, 1977; Liu et al., 2008; Zicha et al., 1991; Zigmond, 1977) these devices either do not produce gradients (e.g. Campenot chamber) or only provide 2D surfaces for the growth of neuronal cells, and are more difficult to set up. Furthermore, using our chamber, it should be possible to perform cell migration assays in a similar manner.

The gradient formed in region G of our diffusion chamber reached a steepness of ∼1.2% at 6 h following postsetup. The steepness decreased to approximately 0.4% by 48 h postsetup which suggests that for a 48 h assay, neurites will be subjected to a gradient between ∼0.4% and ∼1.2% steepness. Since the chamber uses passive diffusion from a fixed reservoir to create a gradient of the guidance cue, the absolute concentration and steepness of the gradient inevitably change over time. For our chamber these changes are in the range of 2–5-fold. However, these changes do not detract from the value of our chamber as an assay for axon guidance by gradients that is fast, easy-to-assemble and easy-to-analyse.

A recent microfluidics-based gradient-generating chamber design by Kothapalli et al. (2011) achieved gradients of steepness 3–4% of Slit2 and Netrin in a 3D collagen environment within 30 min after setup, which were stable for up to 48 h. Guidance of neurites from dissociated DRG (Slit2) or hippocampal neurons (Netrin) was demonstrated. This chamber is conceptually similar to our dual-compartment chamber, and is able to achieve similar guidance responses. However our design is able to accommodate tissue explants, which present a more in vivo-like environment for the cells within them, particularly since many cell–cell interaction pathways are retained in the explants. Furthermore, our chamber can be produced without any photolithography or soft-lithography, procedures which may require a dedicated cleanroom. Our plastic mould is generated using techniques common to any machine shop and can be used to produce a large number of PDMS chambers.

We quantified the degree of chemoattraction of DRG neurites using the guidance ratio (GR) and found that in the diffusion chamber, the DRGs display a GR of 0.22 when grown in an NGF gradient (compared to a GR of 0 for those grown in an NGF plateau). This GR was similar to a value of ∼0.17 we found for NGF gradients of 0.3% using a printing assay (Mortimer et al., 2009, 2010). The slightly higher GR we observed using our diffusion chamber may be due to the steeper NGF gradient it produces (∼1%) compared to that

generated by the printing assay. OB neurites growing in a Slit2 gradient in the diffusion chamber displayed a GR of −0.5 indicating strong repulsion to the gradient. Neurites of OBs grown in a Slit2 plateau, or with no Slit2, showed no net guidance, suggesting that the chamber design itself did not impart any guidance to the axons. This suggests that it should be possible to use a diverse range of guidance cues, including multiple guidance cues in the same chamber.

The dual compartment diffusion chamber we have described here can be generated using commonly available laboratory techniques. The chambers can be mass-produced for high throughput analysis of a large number of explants, and the gradients made by them are more reproducible compared to those found in explant co-culture assays. These advantages, together with the three-dimensional matrix in which the neurites are growing make this chamber a valuable research tool for the study of axonal chemotaxis in a biologically relevant environment.

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