Chapter 16

Quantitative Studies of Neuronal Chemotaxis in 3D

William J. Rosoff, Ryan G. McAllister, Geoffrey J. Goodhill, and Jeffrey S. Urbach

Summary

During development a variety of cell types are guided by molecular concentration gradients to form tissues and organ systems. In the nervous system, the migration and neuronal pathfinding that occurs during development is organized and driven by "guidance cues." Some of these cues are substrate bound or nondiffusible, while many are diffusible and form gradients within the developing embryo to guide neurons and neurites to their appropriate destination. There have been many approaches used to discover and characterize the multitude of guidance cues, their cognate receptors, and how these cues and receptors are regulated to achieve the highly detailed connections found in the nervous system.

Here we present a method for creating precisely controlled gradients of molecular factors within a three-dimensional culture environment. The method is based on a non contact mediated delivery of biomolecules to the surface of a collagen gel. The factors are printed in a pattern on the top of a gel containing the tissue or cell type of interest embedded in the gel. The formation of the gradient is dependent upon the diffusion of the printed molecule in the gel. The concentration of the factor within the gel becomes independent of depth rapidly, and the gradient becomes smooth on a similar time scale. The gradients formed can remain relatively stable for a day or more. Moreover, the steepness and molar concentration of tropic or trophic factors within the gradient can be controlled.

Key words: Chemotaxis, Molecular gradients, Collagen gel, Axon guidance, Diffusible factors, Guidance cues, Cell motility

1. Introduction

Understanding how the nervous system is wired up during development is important for understanding both how neurological disorders based on wiring defects can be avoided, and how axons can be made to regenerate appropriately after injury. In the past 15 years or so several families of guidance molecules

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have been shown to be important for guiding axons in vivo, including the Netrins, Semaphorins, Slits, Ephrins, Wnts, and some morphogens such as Shh *(1–4)*. Much of our current knowledge about the mechanisms by which such cues guide axons comes from a small number of in vitro assays *(2)*. The most influential of these are the collagen gel coculture assay *(5)* and the "pipette" or "growth cone turning" assay *(6)*. In the former, neurons are embedded in a three-dimensional collagen gel close (a few hundred microns) to a source of the putative chemotropic factor, such as a tissue explant or a block of transfected cells. The chemotropic factor diffuses from the source to create a gradient in the gel, and the trajectories of axons emerging from the neurons are examined after 1–2 days in this environment. This assay recapitulates the timescale and 3D environment of many axon guidance events in vivo, but does not offer any knowledge, control, or stability of the gradient steepness and absolute concentration *(7)*. In the pipette assay the chemotropic factor is expelled into a fluid environment from a narrow pipette about 100 μm from the tip of an individual axon growing on the surface of a culture dish. Providing convection currents are carefully controlled a relatively stable gradient can be established by diffusion from the pipette. The response of the axon to this gradient is then examined over timescales of about 1 h. Although this assay offers more control over gradient conditions than the collagen gel coculture assay, steepness and absolute concentration are still variable and hard to predict *(8)*, and the timescale and dimensionality are less like the in vivo situation. Motivated by these limitations, we investigated whether it was possible to design a long-term collagen gel-type assay which would allow precise knowledge and control of the gradient conditions.

A variety of previous techniques have been developed for producing controlled gradients of chemical factors in three-dimensional gels. A simple approach is to place the gel in contact with a chamber of solution or gel containing the factor of interest. Diffusion of the factor creates a continuously evolving gradient *(9–11)*. A more controlled gradient can be produced by placing a gel between two chambers with differing concentrations of factor. After an initial transient, a linear gradient is established between the two chambers *(12–15)*. The main drawbacks of this method are that the gradient is established only after an initial transient that can be fairly long for large proteins and other slowly diffusing molecules, the reservoirs require a large excess of chemical factor, only linear gradients can be produced, and a separate apparatus is required for each culture dish. Protein gradients have also been incapsulated in a microporous gel by creating a linear gradient in a mold before gelation *(16)*. There are many means of generating gradients of guidance cues, permissive, and

nonpermissive substrates in two-dimensional cultures which will not be discussed here.

We have developed a technique for gradient generation that relies on a computer-controlled micropump to create patterns of chemical factors on the surface of a relatively thin gel *(17,18)*. As detailed later, this approach offers several significant advantages over most existing methods: There is no contact between the micropump and the gel, gradients can easily be reproduced in multiple experimental chambers, a variety of gradient shapes can be generated with the same hardware, no excess factor is required, and the gradients are established quickly. The shape of the gradients evolves in time in a way that can be accurately modeled by the diffusion equation. This evolution is the main drawback of this method compared to those described above, which can produce nearly stable gradients after sufficient time. However, the slow diffusion of many large proteins results in gradients which are stable for a day or more, which is adequate time for many in vitro studies. This technique can be used to investigate the role of gradients of diffusible substances in processes such as chemotaxis, morphogenesis, and pattern formation, as well as for high-throughput screening of system responses to a wide range of chemical concentrations. Moreover, multiple gradients of arbitrary spatial orientation can be applied, to examine the interaction and effect of a number of different biomolecules.

2. Materials

2.1. Tissue Preparation

1. P2 Sprague-Dawley Rat pups.
2. Dulbecco Modified Eagle's Medium (DMEM), high glucose, no glutamine, no pyruvate (Cambrex).
3. Ca^{2+}–Mg^{2+} free Hanks Balanced Salt Solution (Cambrex).
4. 2.5% trypsin solution (Cambrex) diluted 1:10 in HBSS for 0.25% final, aliquot and store at –20°C, stable for 1 year.
5. DNAse I (Boehringer Mannheim) prepare 1 mg/mL stock in PBS aliquot store at –20°C stable for 1 year.
6. Heat-inactivated FBS (Cambrex) aliquot and store at –20°C.

2.2. Collagen Preparation

1. Tissue-culture-grade water (Cambrex).
2. Powder OptiMEM made to 10× OptiMEM (Invitrogen).
3. Tissue-culture-grade sterile 7.5% sodium bicarbonate solution (Sigma).

4. Penicillin/Streptomycin/Fungizone solution (100×) (Bio-Whittaker).
5. Type I rat tail collagen, 3–4 mg/ml solution (Becton-Dickinson).

2.3. Tissue Embedding in Collagen Gel

1. Dissection microscope (Nikon).
2. 35-mm tissue culture dishes (Corning).
3. Custom acrylic dish holder.
4. Printed gradient template.

2.4. Gradient Printing

1. Pump head microdispenser (Gesim #A010–001).
2. Multidose pump head controller (Gesim).
3. x-y translational stage (Velmex Inc., Bloomfield, NY).
4. Labview software (National Instruments, Austin, TX).
5. Custom-machined culture dish holder.
6. High-resolution video camera with zoom.
7. Video camera monitor.
8. Murine 2.5S NGF (Roche Diagnostics).
9. Capillary tubing 1 mm inner diameter with a luer lock end (Fisher).
10. 10-cc luer lock syringes (Becton-Dickinson).
11. Nanoplotter 2.0 (Gesim) (Optional, replaces items 1–7).
12. Standard 5% CO_2 tissue culture incubator with high humidity.

3. Methods

3.1. Tissue Preparation

Follow protocols for tissue preparation appropriate for culturing in collagen. Protocols for rat DRG harvesting and digestion have previously been described *(17)* (*see* **Note 1**).

1. Remove the DRGs from the lumbar region of P2 rat pups and place them in DMEM on ice.
2. Trim the DRGs of any nerve bundles or extraneous material.
3. Enzymatically digest the DRGs in 0.25% trypsin/10ug/mL DNAseI/Ca^{2+}- and $Mg2^{+}$-free HBSS for 12 min.
4. Stop the digest with addition of an equal volume of heat-inactivated FBS.
5. Pellet and resuspend the DRGs 3 times in DMEM, then place on ice.

3.2. Collagen Gel Preparation

1. Prepare 10× OptiMEM from powder according to the manufacturer's instructions except use one-tenth of the specified final volume (*see* **Note 2**).
2. Based on the concentration and volume of type I rat tail collagen stock to be used, calculate the volumes of 10× OptiMEM, 100× Pen./Strep./Fung., 7.5% sodium bicarbonate solution, and water needed to formulate collagen gel solution (2 mg/mL collagen, 1× OptiMEM, 1× Pen./Strep./Fung.), factoring in that 27 μL of 7.5% sodium bicarbonate must be included for every mL of collagen stock used.
3. Prepare the collagen gel solution on ice (*see* **Note 3**), adding its components in order as follows (1) water, (2) 10× OptiMEM, (3) 7.5% sodium bicarbonate solution, 100× Pen./Strep./Fung., then the collagen stock, making the solution homogenous by pipetting up and down.
4. Using a 1,000-μL micropipettor, pipette 0.75 mL of the collagen solution into the bottom of the 35-mm culture dishes. Swirl and tilt the dish to make sure there is even coverage. The resulting "bottom collagen" gel will be about 0.5 mm thick. Plate the bottom collagen for all required experimental dishes and allow to gel for ~20 min at 21°C or in the tissue culture incubator.

3.3. Tissue Embedding in the Collagen Gel

1. Make a custom acrylic dish holder that snugly fits the culture dishes. The same brand and exact tissue culture dishes are used for all experiments. The bottom of the acrylic dish holder should be no more than 4 mm thick, and the holder must be shallow enough to allow the edges of the dish to be handled.
2. Create a template as shown in **Fig. 1** in a graphics program. In this instance the circular area is exactly the size of the bottom of the 35-mm dishes. The various lines of the gradient to be printed as well as the pregradient are denoted and are 1 mm apart.
3. Tape a printout of this template to the bottom of the acrylic dish holder. The printing template is used to ensure consistent explant placement and gradient production.
4. Wash the explants to be embedded in cold collagen solution. First remove the majority of the DMEM from the explant containing dishes leaving just enough to keep the explants moist. Place a second 35-mm culture dish on ice and pipette 250 μL of collagen solution into the dish. Using forceps transfer six explants into the collagen solution while trying to minimize transfer of DMEM.
5. Embed the explants in the collagen gel. Pipette 0.75 mL of collagen solution (this will be the "top collagen") into one of the dishes containing the already gelled "bottom collagen."

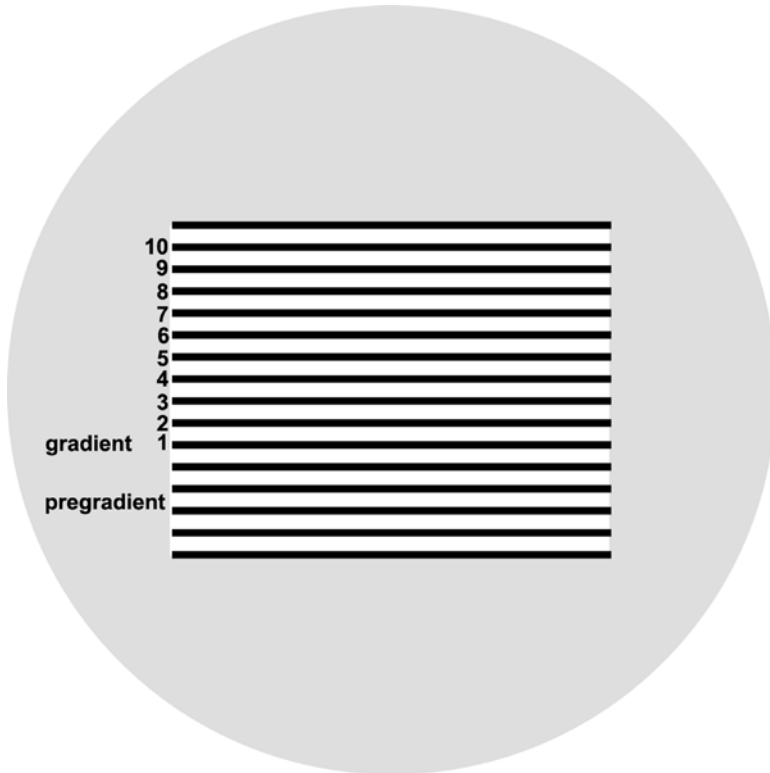

Fig. 1. A template created in a graphics software application to be used for embedding tissue in the collagen gel. This same template can be used to ascertain that the lines or spots of the gradient array are printed at the proper location within the culture dish. The template should be sized to exactly match the printed pattern.

Transfer the six explants into the top collagen and place the dish in the acrylic holder with the gradient template on the stage of the dissection microscope.

6. Position the explants in the appropriate coordinates within the collagen gel. Using forceps gently position the explants within the gradient. Mark the culture dish with a permanent marker to denote the gradient orientation. For the 10 line gradients used in *(17)*, explants are placed between the third and fourth lines of the gradient, the region that is most stable in steepness and molar concentration (*see* **Note 4)**. The explants should be placed such that they are evenly spaced, well separated, and not within 2 mm of the sides of the gradient (*see* **Note 5**). A gradient that is 20 mm wide can easily accommodate 6 DRG explants in this manner (*see* **Note 6**).
7. Allow the top collagen containing the explants to fully gel (usually 5–10 min) before moving the culture dish to the tissue culture incubator. Failure to allow collagen to fully gel

will result in disturbing the collagen matrix when the plate is moved, which can cause improper placement of the explants and spurious neurite guidance through the areas of disturbed collagen matrix.

8. Continue embedding the explants in this manner, changing the collagen solution in which the explants are washed every three dishes prepared (*see* **Note 7**).

3.4. Gradient Printing

1. Choose one of the following approaches to providing the required increase in guidance cue from one line to the next:

   Approach a) Different stock solutions can be loaded into the pump head for each line printed while keeping the amount of solution printed constant from line to line. This method has the advantage that a constant amount of solution is printed, so a countergradient of vehicle is not required. However the pump head must be loaded a number of times, which requires washing and drying between each loading of a new solution. This approach is most suited to gradient production using the Gesim Nanoplotter (see alternative method later).

   Approach b) The amount of guidance cue solution printed can be varied from line to line. The advantage of this approach is that typically only two solutions need be loaded into the pump head: the guidance cue and the vehicle for printing the countergradient and pregradient, as discussed later.
2. Adjust the microdispenser parameters to ensure droplet ejection. There are a number of settings, such as the voltage, pulse duration, and pulse frequency that affect the output of the microdispenser. The values of each of these settings must be tailored to ensure proper droplet ejection. A stroboscope is available from Gesim that enables visualization of ejected droplets, and the fine tuning of these settings to ensure a reliable droplet ejection.
3. Calibrate the microdispenser output. Each pump head is individually manufactured and calibration data are provided with each pump. The typical output for one pulse is on the order of 1 nl, depending on the voltage and pulse duration. We have found that with a pulse duration of 100 μs and a voltage of 60 V most pump heads provide a droplet size of 1.4 nl. The output can be measured by using a Metler balance to measure the change in mass of a microcentrifuge tube caused by injection of 1×10^6 water droplets from the pump. We have also found that the pump heads have a reliable output independent of viscosity, by measuring the output of a BSA PBS solution ranging over three orders of magnitude, from 0.001 to 1% BSA.
4. Calculate the number of droplets and concentration of stock solution in order to achieve the desired molar concentration of the guidance cue at the location of the explants. The gradients

are created by printing ten lines of solution each 1 mm apart onto the surface of the collagen gel. Gradients 20 mm wide accommodate 6 DRGs without the neurites from the separate DRGs encountering one another. Because the collagen gel is 1 mm thick and the lines are spaced 1 mm apart, there is 20 μL of gel per line. Therefore the molar quantity of guidance cue applied will diffuse into the 20 μL bed volume to result in the final concentration required at that point in the gradient. An example of the necessary calculation is displayed in **Table 1**.

5. Determine the change in factor from one line to the next to attain the desired gradient shape. To form a linear gradient the quantity is increased by a constant amount from one line to the next. For exponential gradients the quantity is increased by a constant ratio. For a concentration change of *x*% across 10 μm (the typical size of a axonal growth cone), the ratio is $(1 + x/100)^{(100)}$. (The factor of 100 is the spacing between lines divided by 10 μm.). *See* **Table 1**.

Table 1
The parameters required to create a 10-line exponential gradient of 0.2%, 1 nM NGF at the explant using incremental deposition of the volume of chemotropic factor onto the collagen gel, when the concentration of the stock remains constant in the microdispenser

Line #	Pump stock (nM)	Droplets	Final concentration (nM)	Countergradient droplets
1	117	82	0.67	491
2	117	100	0.82	473
3	117	122	1	451
4	117	148	1.22	424
5	117	181	1.48	391
6	117	221	1.81	351
7	117	270	2.21	303
8	117	329	2.70	243
9	117	402	3.29	171
10	117	490	4.01	82

The explants are placed between the third and fourth lines, the exponential factor = 1.22, droplet size = 1.4 nL, the final bed volume/line is 20 μL. The total aqueous volume applied including countergradient ~573 drops; therefore, a pregradient of 5 lines of 573 drops of PBS is applied

6. Calculate the number of droplets required to form a countergradient (in the case of approach b). Because the addition of different numbers of droplets in different gradient lines may change the local collagen concentration or have other more subtle symmetry breaking effects, we also apply a countergradient of vehicle (PBS) so that the number of drops printed on each line is the same. Specifically, the countergradient is chosen such that the number of gradient drops printed on each line plus the number of countergradient drops printed on each line is the equal to the number of drops printed on the line with the largest number of drops.
7. Calculate the number of droplets required to form a pregradient (in the case of approach b). We have also found that application of a 5-line pregradient of vehicle containing the same number of droplets present in the gradient and countergradient helps to reduce artifacts. In particular, we have found that the gradient of collagen density that can be present when the tissue is close to the edge of the printed region can sometimes produce a weak guidance effect by itself, confounding the effect of the chemotropic factor gradient.
8. Confirm that the lines of the printed gradient will be in the desired coordinates of the culture dish. The gradient pump system is controlled by a custom Labview program (available upon request). The program consists of a subroutine that ejects droplets from the pump head at a specified rate, a routine that moves along each gradient line at a specified rate, a routine that moves between lines, and a shell that accepts user inputs and performs calculations to direct the subroutines. Apply a gradient template **(Fig. 1)** to the bottom of a culture dish, and place the culture dish in the *x*–*y* translation stage holder. Load the pump head with vehicle (see below for loading procedure). Print a gradient or line and adjust the initial *x*–*y* coordinates until the printed gradient matches the lines on the template.
9. Load the stock solution of guidance cue into the pump head. If the pump head is not clean and dry, wash and dry the pump head as described later. Use a short (~1 cm) length of the capillary tubing that has been attached to 200-μL micropipettor tip the very end of which has been removed to allow easier flow, attached to a 20- or 200-μL micropipettor (*see* **Note 8**). Take the solution up into the capillary tubing and gently attach the capillary tubing to the pump head inlet **(Figs. 2** and **3)**. Tilt the micropipettor upward and allow the solution to flow into the pump by gravity. If the pump head does not fill by gravity, a small amount of pressure can be used by depressing

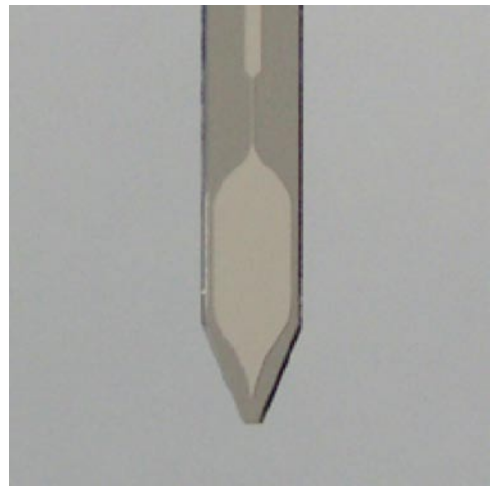

Fig. 2. A magnified view of the Gesim pump head (microdispenser), showing the piezo-activated reservoir.

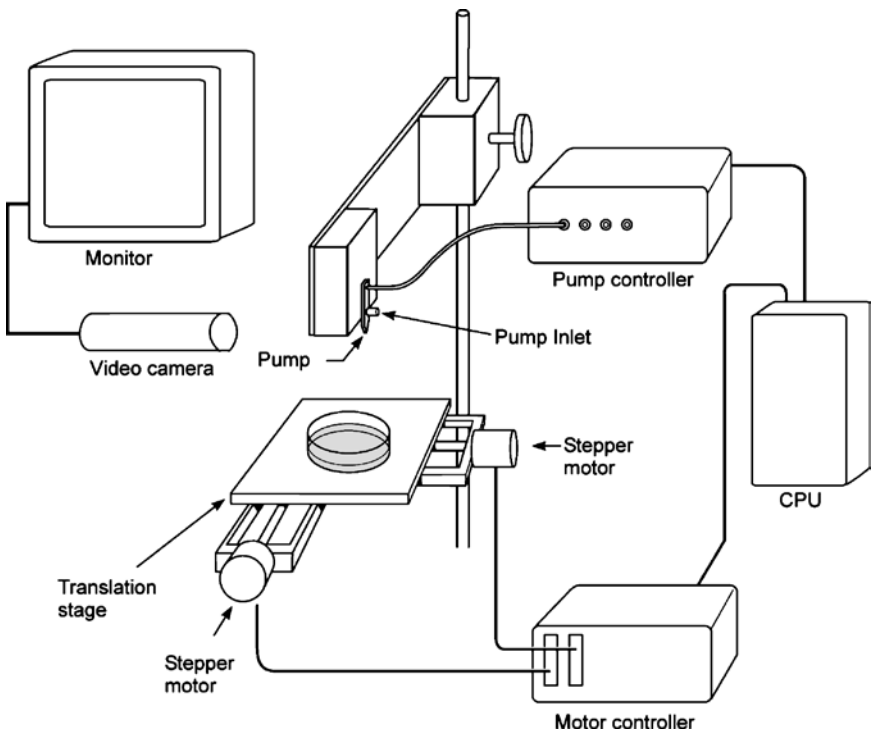

Fig. 3. A schematic representation of the original pump applied gradient generating apparatus. The CPU or computer runs the required program to control the microdispenser control unit, and the motorized translational stage. The video camera and monitor are used to ensure proper filling, washing, and drying of the microdispenser (*17*, *18*).

the micropipettor actuator. Once the pump head is filled, gently eject the tip from the micropipettor.

10. Confirm that the pump head is properly loaded and ejecting droplets prior to printing a gradient. Examine the pump head for air bubbles on the video monitor **(Fig. 3)**. If air bubbles are present, droplets will not eject properly, and the pump head must be washed and dried thoroughly (see later) and then reloaded with solution. Hold a piece of paper under the pump head while the dispenser is activated to check for droplet ejection.
11. Print the gradient (in the case of approach b), or individual lines of the gradient (in the case of approach a). While printing the gradient, or lines of the gradient, closely examine the surface of the collagen gel to ensure that droplets are properly deposited on the gel.
12. Wash the pump head. Take 2–3 mL of H_2O into a 10-cc luer lock syringe that has ~ 6 cm of capillary tubing attached to the luer lock. Attach the end of the capillary tubing to the pump head inlet and gently apply pressure to flow 2 mL of H_2O through the pump head. The tubing and the syringe are then removed and the pump head is dried externally with a Kimwipe.
13. Remove all remaining H_2O and dry the internal chamber of the pump head. Another such 10-cc syringe with capillary tubing is attached to pump inlet. Then a gentle vacuum is applied by pulling out the plunger of the syringe out. Repeat the application of the vacuum three times while examining the pump head on the video monitor **Fig. 3**. There should be no H_2O remaining. The microdispenser pump head must be washed and dried between each change of solution in the pump head, and after use for storage.
14. Print all remaining lines of the gradient (in the case of approach a). Reload the pump head with desired stock and print each successive line of the gradient. Wash and dry the pump head between loadings of each new stock solution, and application of each new line of the gradient.
15. Print the countergradient (in the case of approach b). Load the pump head with vehicle and print the countergradient as described earlier for the gradient. Wash and dry the pump head.
16. Print the pregradient. Load the pump head with vehicle and print the pregradient as described earlier for the gradient. Wash and dry the pump head.
17. Alternative method for approach a. An alternative method, which allows for greater automation of and control over some of the steps described earlier, is to use a device such as the

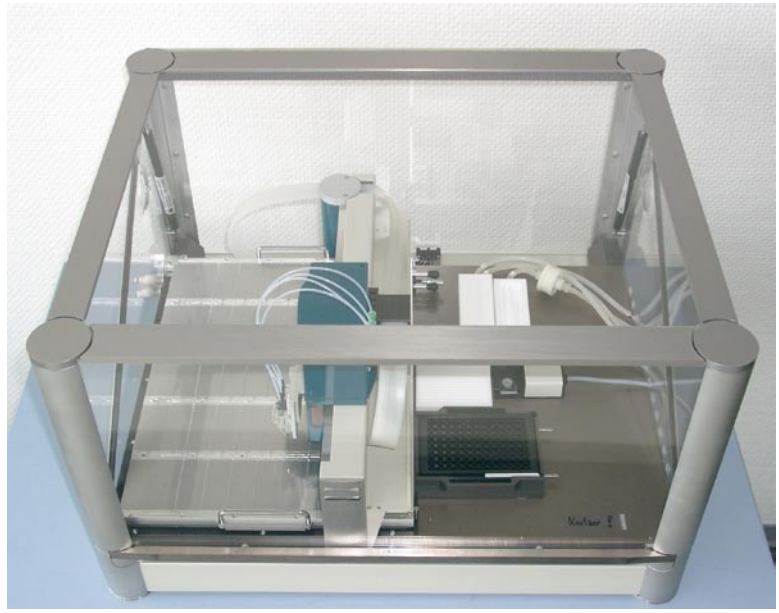

Fig. 4. The Gesim nanoplotter, a robotic fluid delivery system that facilitates the production of chemotropic gradients.

Gesim Nanoplotter **(Fig. 4)** (see www.gesim.de), which is a commercially available high-precision liquid delivery system. Although this device was originally designed to create proteomic and DNA arrays, it can be easily programmed to provide gradient patterns. The nanoplotter is robotic, and can contain a number of picoliter delivery piezo microdispensers, automated wash system, drying pad, and stroboscope with pattern recognition software. Chemotropic solution is taken up by the microdispensers from multiwell microtiter plates, and then is deposited on the collagen gel in an array. Instead of printing lines, in our current usage each line in the gradient is now made up of 20 spots 1 mm apart *(19)*. Because the nanoplotter has an automated wash system to clean the microdispensers every time sample is taken up and printed, a constant volume of different concentrations of chemotropic factor can easily be placed in each line, and no countergradient is required. Moreover, the stroboscope and the pattern recognition software ensure that the microdispenser is ejecting droplets. If there is a failure in droplet ejection the nanoplotter will wash the microdispenser and repeat the sample pick up. If droplet ejection fails a second time, the nanoplotter notes this and moves on to the next sample while creating a program that can be ran after printing to correct for any errors in sample delivery. Steps for using the nanoplotter are as follows:

a) Calculate the number of droplets and concentration of stock solution in order to achieve the desired steepness of the gradient and the molar concentration of the guidance cue at the location of the explants. An example of the stock solutions and calculations to create a gradient in this fashion is displayed in **Table** 2.

b) Program the nanoplotter to deliver the required number of droplets from each well of the microtiter plate to the lines of the gradient. The nanoplotter has a number of transfer programs that will deposit solutions from the microtiter plate to the slide target area. Create the program as per the Gesim Nanoplotter manual.

c) Confirm that the spots of the printed gradient will be in the desired coordinates of the culture dish. Apply a gradient template **Fig.** 1 to the bottom of a culture

Table 2
The parameters required to create a 12-line exponential gradient of 0.2%, 1 nM NGF at the explant using a constant volume of chemotropic factor deposited onto then collagen gel, when varying the concentration of the stock in the microdispenser

Line #	Pump stock (nM)	Final concentration (nM)
1	110	0.55
2	134	0.67
3	164	0.82
4	200	1.0
5	243	1.22
6	297	1.48
7	362	1.81
8	442	2.21
9	539	2.70
10	657	3.29
11	802	4.01
12	978	4.89

The explants are placed at fourth line, the droplet volume is 0.5 nL for picodispenser. The final bed volume/line is 20 μL. With 20 spots/line, 10 droplets/spot ~0.1 μL volume is applied/line. The exponential factor = 1.22. A pregradient of 4 lines, 20 spots/line, 10 droplets/spot of PBS is applied

dish, and place the culture dish on the desired location of the slide tray of the nanoplotter. Pipette vehicle into the wells of the microtiter plate and run the transfer program. Adjust the placement of the culture dish until the spots delivered by the nanoplotter coalign with the lines of the gradient template.

d) Load the microtiter plate with the necessary stock solutions. Include the pregradient vehicle in the appropriate wells.

e) Run the nanoplotter program. If errors in microdispenser loading and ejection occur, "repair" programs will be generated. Run the repair programs as necessary.

18. Once the gradients have been printed, incubate the culture dishes containing the explants in a 37°C 5% CO_2 tissue culture incubator for 36–40 hs (*see* **Note 9**).

4. Notes

1. All efforts should be made to maintain the DRGs on ice to increase viability. Leibovitz L-15 medium can be substituted for the DMEM.
2. The 10X OptiMEM is the most critical component of the mixture. As the collagen gel will be moist with no extra liquid media overlaying the gel, the only initial source of nutrients will be the OptiMEM included in the collagen mixture. The final volume of OptiMEM should be made in a volumetric flask. All glassware and stir bars for media preparation are only used for this purpose and never exposed to detergents. A 10X concentration of other media can be substituted depending on the cell type or tissue used. Note this is a serum-free culture system.
3. Because collagen stocks vary in concentration, the volume of each component of the collagen gel solution will have to be calculated for each stock. Prepare Collagen solution in ice with all cold components, just prior to the DRG digestion. If air bubbles are introduced the solution can be centrifuged at 1,000 x g, 4° C for 5 minutes to remove the bubbles. The solution once made will eventually gel even if left on ice after about 3 hrs. Each 35-mm culture dish will require approximately 1.8 mL of collagen solution. The minimum collagen stock concentration that can be used to achieve a 0.2% gel is 2.32 mg/mL. A low concentration of NGF (0.1nM), N2 supplement (Gibco) or other neurotrophic factor can be

included in the collagen to help promote survival of the explants or cells. We have found this to not be necessary for DRGs if the gradient is applied within a few hours of embedding.

4. Larger gradients that contain more lines can be more stable. If a gradient with more lines is desired adjust the placement of the explants to be ~ 1/3 up the gradient from the bottom of the gradient.
5. We have found through finite element analysis that the concentration of guidance cue likely diminishes within 2 mm of the sides of the gradient.
6. When using dissociated cells, the cells are plated onto the "bottom" collagen in a 50-μL volume of 1X OptiMEM and allowed to adhere for 1 hr. The excess media is then gently removed with a 200-μL micropipettor and the "top" collagen is applied.
7. The collagen in which the explants are washed will eventually accumulate DMEM and will start to gel if not replaced every three dishes or 18 explants.
8. As little as 10 uL and as much as 200 uL can be loaded onto the pump head with the micropipettor tip acting as a reservoir of solution.
9. There is no liquid media on top of the gels, because that would disrupt the stability of the gradient. Thus the incubator must have high humidity, which can be achieved by creating a gas-permeable humid chamber for the culture dishes within the incubator.

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