

Analysis of the growth cone turning assay for studying axon guidance

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Abstract

The “pipette” or “growth cone turning” assay is widely used for studying how axons respond to diffusible guidance cues in their environment. However, little quantitative analysis has been presented of the gradient shapes produced by this assay, or how they depend on parameters of the assay. Here we used confocal microscopy of fluorescent gradients to characterize these shapes in 3 dimensions. We found that the shape, and more specifically the concentration at the position usually occupied by the growth cone in this assay, varied in sometimes unexpected ways with the molecular weight of the diffusible factor, charge, pulse duration and pulse frequency. These results suggest that direct observation of the gradient of the particular guidance factor under consideration may be necessary to quantitatively determine the signal to which the growth cone is responding. © 2008 Elsevier B.V. All rights reserved.

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1. Introduction

One of the principal methods used for studying how axons respond to diffusible guidance cues in vitro has been the “pipette” or “growth cone turning” assay (Fig. 1A) (Ming et al., 1997; Song et al., 1997, 1998; Campbell and Holt, 2001; Nishiyama et al., 2003; Pujic et al., in press). In Gundersen and Barrett’s early version (Gundersen and Barrett, 1979), gravity produced a steady outflow of Nerve Growth Factor from a micropipette with the tip placed close to the bottom of a fluid-filled culture dish. Neurites of chick dorsal root ganglion axons growing on the bottom of the dish could be guided towards the micropipette at close range (approximately 25 μm), presumably in response to a gradient of NGF produced by diffusion from the tip of the micropipette. Poo and colleagues subsequently improved upon this method by attaching the micropipette to a

picospritzer, and ejecting guidance factors using regular, short-duration pressure pulses (Lohof et al., 1992; Zheng et al., 1994; Dickson, 2002). The development of this version of the assay shortly preceded the discovery of several key families of axon guidance molecules (reviewed in Dickson, 2002). Since then, the assay has contributed greatly to our understanding of axon guidance, both in terms of identifying which types of axons respond to which types of cues, and dissecting the downstream signalling pathways which convert graded receptor binding of these cues into directed movement of the growth cone (Song and Poo, 2001; Zheng and Poo, 2007; Mortimer et al., 2008).

However, gradients of these guidance cues have not been directly observed in this assay. Instead, information about gradient shape has mostly been inferred from theoretical analysis and direct visualization of more accessible molecules. In particular, Lohof et al. (1992) and Zheng et al. (1994) quantitatively analysed the gradients produced in this assay by epifluorescence imaging of carboxyfluorescein (approx. 0.4 kDa) and fluorescein-dextran (9 kDa). This quantification showed that the fractional change in concentration across 10 μm at a distance of 100 μm from the tip of the pipette (the usual distance from

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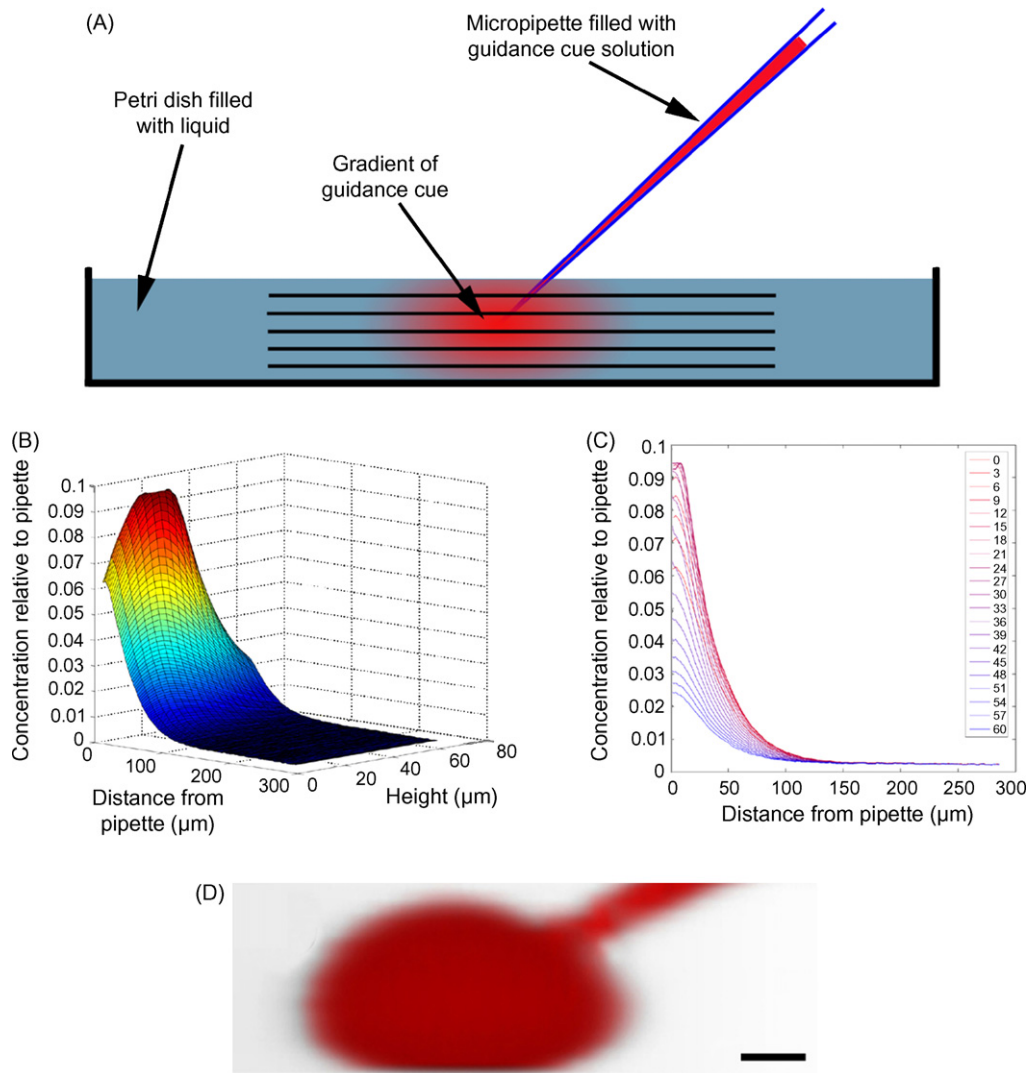


Fig. 1. Method of analysis of the pipette assay. Panel A depicts schematically a gradient produced in a 35 mm diameter Petri dish by a micropipette. Black horizontal lines represent the image planes produced by confocal imaging of a stable gradient in liquid (not to scale). Panel B shows a measured three-dimensional surface of concentration relative to the pipette at any point from the pipette (distance from pipette) and from the surface of the Petri dish floor (height). Panel C shows the same information but with the gradient profile at each z -plane colour-coded from red (images taken near the floor of the Petri dish) to blue. Panel D shows a three-dimensional reconstruction the same gradient. Gradients in panels B, C and D used 10 kDa dextran, 2 Hz picospritzing frequency and 20 ms pulse duration (scale bar in panel D = 20 μm). In panel D, the pipette can be seen entering the image at the upper right. The dextran fluorescence is visible extending roughly isotropically from the pipette tip.

the growth cone at which the pipette tip is positioned in the application of this assay) was about 10%. In subsequent papers it was also suggested that the concentration of guidance factor at the growth cone was approximately 1000 times less than the concentration of factor inside the pipette (Ming et al., 1997). This value is now widely quoted (Guan et al., 2007). However, epifluorescence imaging in two dimensions cannot accurately determine the concentration at the surface of the dish of a factor ejected by the pipette, since the measurement integrates over different heights in the dish which may have different local concentrations. Furthermore, the effects of several potential sources of variability on the gradients produced have not been directly addressed.

This uncertainty in knowledge of the gradient is potentially significant because recent work has shown that (similarly to

neutrophils and *Dictyostelium* (Zigmond, 1981; Fisher et al., 1989)) axons are only sensitive to gradients over a relatively narrow range of concentration (Rosoff et al., 2004). While one can simply vary the concentration in the pipette until a level that produces guidance is found, this does not allow a more quantitative analysis of how axonal response varies with gradient parameters. When no guidance is seen, it is also difficult to exclude the possibility that the lack of response is simply due to a lack of precise control over the gradient in the assay.

Here we explore these issues by using confocal microscopy of fluorescent gradients to characterize gradient shapes produced in 3 dimensions by the pipette assay. We found that the shape, and more specifically the concentration at the position usually occupied by the growth cone in this assay, varied with the molecular weight of the diffusible factor, its charge, the pulse duration and

pulse frequency. We also found that there could be substantial variability in gradient shape between different runs, even when all parameters were apparently constant. These results suggest that it is difficult to quantitatively predict the gradient shape of a specific factor unless it is directly observed during the actual experiment.

2. Methods

2.1. Micropipettes

Micropipettes were pulled from BK-7 glass (GC150F-7.5, Harvard Apparatus) with a multistage puller (Sutter P-97) to produce an average tip inner diameter of 1.3 μm . The bore of each micropipette was photographed under brightfield microscopy and its diameter was measured. The average internal bore diameter of pipettes was $1.3 \pm 0.13 \mu\text{m}$. Pipettes with internal bore diameters of 1.0–1.5 μm were used for assays. The generation of a gradient of a guidance cue in the pipette assay requires its ejection from a micropipette. However the narrowing of the bore of the tip, and the use of positive pressure for ejection can lead to the obstruction of the bore at the tip by insoluble particulates present in the solution being ejected. The source of the particulates can be in the solution loaded into the tip, or glass particles present in the capillary. The ends of capillaries are prone to shatter while present in the holder prior to being pulled. We found that it is crucial that such particulates be removed from the capillaries by flushing with compressed filtered gas such as nitrogen or air. We performed a flush of capillaries for 5 s using compressed air prior to pulling the capillaries. After flushing with gas, the capillaries were stored by adhesion to BlueTack™ which prevented their ends from being damaged (and thereby producing fresh glass particles within the bore).

2.2. Gradient generation

We generated gradients using the pulsatile ejection method reported previously (Lohof et al., 1992). The pipettes were filled with several microlitres of 1 μM dextran solution, and connected to a World Precision Instruments picospritzer II. The angle between the pipette and the Petri dish floor was always maintained at 45° in all experiments. Pulse duration was controlled using the picospritzer which produced pulsatile ejection of the solution using nitrogen gas (~3 psi). Ejection rate was controlled using a crystal clock-driven pulse generator with a variable ejection rate. To visualize the gradient, we used 10, 40 or 70 kDa dextran-TMR (dextran conjugated to tetramethylrhodamine, Molecular Probes) dissolved in water (1 mM). Micropipettes were loaded with several microlitres of dextran diluted to 1 μM with water. To determine the effect of charge on gradient shape, we used anionic 10 kDa dextran-TMR. We used fluorophore-conjugated dextrans to visualize gradients since they are commercially available in a range of molecular weights, and in anionic or neutral forms.

Unless otherwise stated, dishes were coated with poly-D-lysine and laminin. Dextran solutions were centrifuged for 10 min in a microfuge at 14,000 rpm to remove particulates, and

several microlitres of dextran solution was loaded into the pipette using a Hamilton syringe. Prior to use, the Hamilton syringe was flushed with several mL of 100% ethanol. The ethanol itself was centrifuged for 10 min at 3000 rpm to pellet any particulates. The Hamilton was allowed to air dry for 1 h prior to use. Loaded micropipettes were positioned with their tips above the bottom of a 35-mm diameter coverslip-bottom dish which had been filled with 3 mL of water. The micropipette position was adjusted using a micromanipulator (Narishige) on a Zeiss 5 Live confocal microscope. By imaging the micropipette with a 543 nm laser, it was possible to determine the height of the tip from the coverslip to within 1–2 μm . Unless otherwise stated, we used a height of 25 μm . Pulsatile ejection of the dextran from the micropipette was then sustained for 10 min in order to allow the gradient to form and stabilize.

2.3. Gradient imaging

z-Stack imaging of the gradient was performed by imaging at 3 μm intervals from the surface of the coverslip up to 60 μm above using a 20 \times objective (Fig. 1A). Pulsatile ejection was maintained during imaging. 12 bit images, 336 μm square, at a resolution of 256 \times 256 pixels were obtained at 2 fps, and 20–100 complete *z*-stacks were acquired for each gradient. An average image (at each *z*-level) was then obtained by averaging over the 20–100 stacks using Matlab (Mathworks) to give a 3D image of each gradient composed of 21 slices at 3 μm intervals.

2.4. Quantitation

To characterize the gradient, a line intensity profile was obtained from the tip of the micropipette in line with the pipette, extending up to 250 μm from the tip for each of the 21 confocal slice images. This line intensity profile was then calibrated for concentration using standard solutions of dextran-TMR of varying concentrations (from 1 to 500 nM) obtained using the same imaging conditions as those employed to image the gradient (Fig. 1B–D). The resulting 3D image of each gradient was then assessed for the gradient slope and dextran concentration at 100 μm from the tip. Confocal image stacks were resectioned in the *z*-*x* plane using Zeiss LSM510 software. An example of a dextran gradient is shown in Fig. 1D. For panels 2E–H, 4E, F and 5A, B, only the image planes adjacent to the Petri dish floor were analysed, since this is where the growth cone is located.

3. Results

3.1. Height of pipette from floor

The height of the pipette above the bottom of the dish during the assay has generally not been reported in previous studies. We measured the concentration of dextran at 100 μm from pipettes placed with their tips either 20 or 40 μm above the dish and found that this 2-fold change in pipette height did not significantly change the dextran concentration at 100 μm from the tip (data not shown).

3.2. Guidance cue molecular weight

To determine the effect of molecular weight of the guidance cue on its concentration and gradient slope at the growth cone, we produced gradients using 10 (Fig. 2A and B), 40, or 70 kDa (Fig. 2C and D) dextran under similar picospritzing conditions. Our results (Fig. 2E) show that 40 kDa dextran was present at a 1.4-fold higher level at 100 μm from the pipette tip compared to 10 kDa dextran, which is close to the expected 1.6-fold increase assuming that the diffusion constant scales as roughly the cube root of the molecular weight. However, the concentration of 70 kDa dextran was much higher than expected theoretically, about 15-fold higher than the 10 kDa dextran and 10-fold higher than the 40 kDa dextran (Fig. 2E). The reason for this is unclear. It is significant because most of the factors implicated in axon guidance *in vivo* (e.g. Netrin) have molecular weights larger than this. Since the ratio of 70 kDa dextran concentration at 100 μm was approximately 1:10, this suggests that the commonly quoted value of 1:1000 may be a significant underestimate.

3.3. Effect of distance from pipette

Fig. 2G and H shows the effect of distance from the pipette on concentration (relative to the pipette) and gradient steepness for 10 and 40 kDa dextrans. For distances from the pipette of 50 to 130 μm , the concentration of dextran decreases. For the same distance range, gradient steepness of the dextran remains either stable (for 40 kDa) or decreases slightly. Gradient steepness is more variable at larger distances from the pipette tip.

3.4. Guidance cue charge

To determine if the charge on the guidance cue significantly affects the concentration and slope of the gradient at the growth cone, we produced gradients using either neutral 10 kDa dextran (Fig. 1B–D) or anionic 10 kDa dextran (Fig. 3A–C) under similar picospritzing conditions. The neutral dextran was in the zwitterion form (i.e. containing a single positive and single negative charge), while the anionic form contains a net negative charge. Fig. 3 shows that the charge on the guidance cue is an important determinant of how it interacts even with a coated glass surface. Gradients of zwitterionic (but neutral) dextran produced in Petri dishes with coated glass floors show a predominantly diffusion-driven distribution (Figs. 1D and 2A–D). However, although anionic dextran diffuses out from the tip in a similar manner, it tends to then accumulate in the vicinity of the floor, leading to elevated levels of dextran (Fig. 3A–C). This is important because the charge distribution of factors important for axon guidance is generally unknown. We also found that zwitterionic dextran interacts strongly with uncoated glass, leading to a layer of bright fluorescence at the surface of the dish (data not shown).

3.5. Pulse duration

We investigated whether we could control the concentration of guidance cue at the axon by varying the quantity ejected from the pipette via changing the pulse duration. We performed a

series of experiments in which the concentration of dextran at 100 μm from the pipette was measured in gradients produced with pulse durations from 10 to 200 ms using the same tip in three independent experiments. This range encompasses the pulse duration of 20 ms used in most previous studies. Fig. 4A–C and E shows that between 10 and 50 ms pulse duration, the level of guidance cue at the growth cone rises roughly proportionally to pulse duration. However for 200 ms pulse duration (Figs. 4D and E), the movement of the guidance cue away from the micropipette tip appeared to be no longer dependent only on diffusion, but also partly on jetting of the material out of the micropipette tip. Furthermore, the direction of the jetting is subject to currents in the liquid into which the dextran is being ejected. This had the effect of producing artefactually low values of concentration of dextran (relative to inside the pipette) (Fig. 4E). As predicted from theory, the slope of the gradient was less susceptible to the quantity of material ejected from the pipette and slope at the position of the axon (Fig. 4F) in one of three experiments. Thus, while pulse duration could potentially be used to effect changes in concentration of factors at the growth cone on a fine scale, it is not a reliable means of effecting controllable changes at a gross scale.

3.6. Pulsing frequency

We also investigated whether we could control the concentration of guidance cue at the axon by varying the quantity ejected via changing the pulsing frequency. We performed a series of experiments in which the concentration of 70 kDa dextran at 100 μm from pipettes was measured using pulsing frequencies of either 0.5, 1, 2 or 4 Hz (Fig. 5). Although there is an increase in the concentration of dextran at 100 μm from the pipette for pulsing frequencies between approximately 0.5 and 2 Hz, it did not scale as might be expected. In addition, no increase at all was observed between 2 and 4 Hz (Fig. 5A). The slope of the gradient was less affected by pulsing frequency, in agreement with theory (Fig. 5B).

4. Discussion

Our results show that several parameters are critical in determining the concentration of guidance cue presented at the growth cone while performing a ‘turning assay’. We found values for the concentration at 100 μm compared to the intrapipette concentration of 1:10 to 1:140, for varying conditions, in contrast to the commonly quoted value of 1:1000. An important parameter affecting this ratio is the molecular weight: increasing this increases the concentration at the growth cone, but not in an obviously predictable way. Charge on the guidance cue also affects its attraction to the substrate, potentially causing changes in this ratio. Furthermore, poor reproducibility prevents precise control of guidance cue concentration for longer pulse durations. The assay in its current form is thus suited for determining qualitatively the response of axons to guidance cues, but is of limited use for probing quantitatively how axonal response depends on gradient parameters. In addition, while a definite turning response of a growth cone in the assay provides evi-

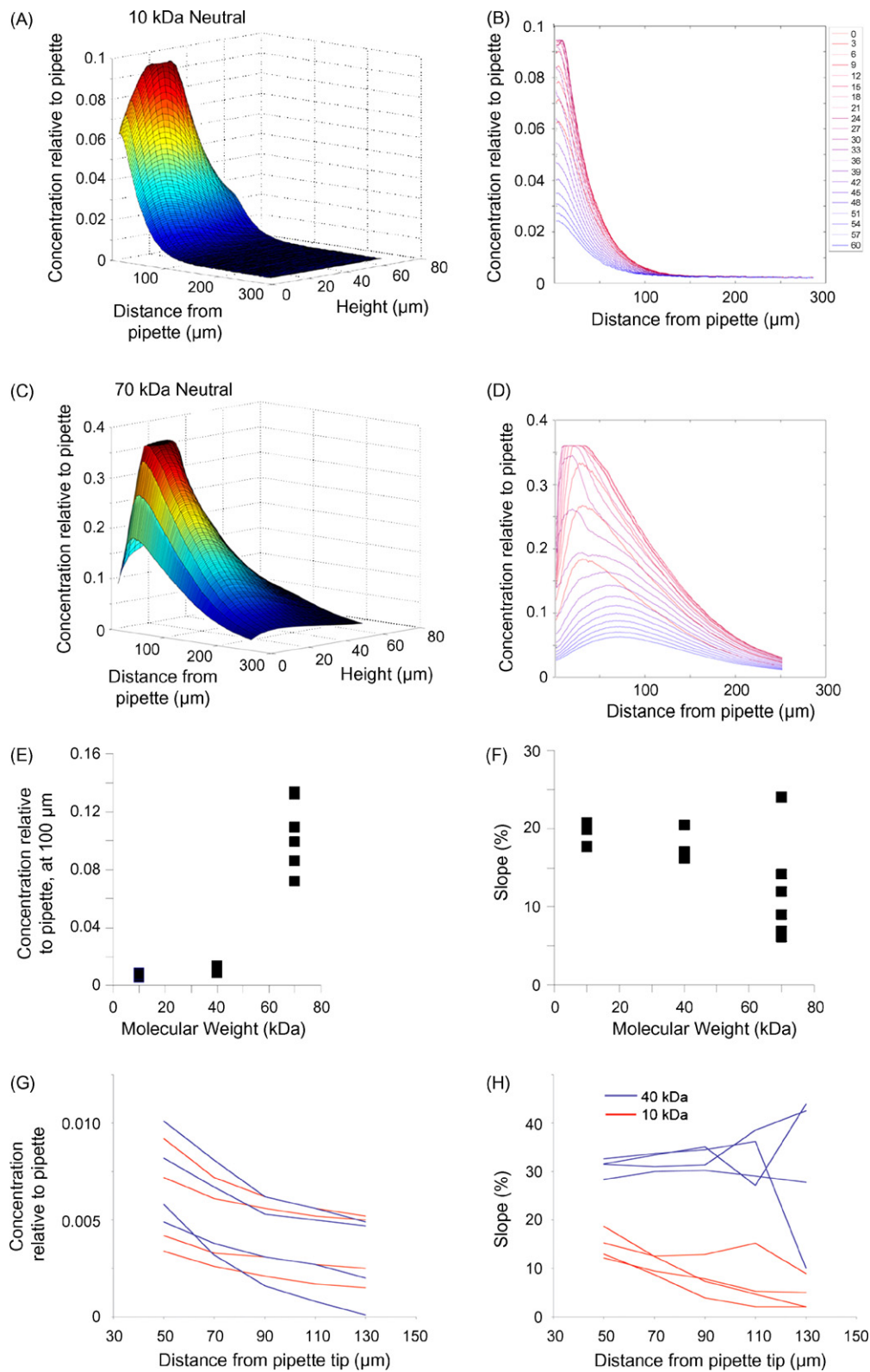


Fig. 2. Effect of molecular weight on gradient shape. Gradients of 10 kDa (panels A and B) or 70 kDa (panels C and D) TMR dextran were produced using pulse durations of 20 ms and 2 Hz picospritzing frequency. Horizontal axes (in panels A and C) show distance from the micropipette (μm) and height of micropipette from the floor of the Petri dish (μm). The vertical axis shows the concentration of dextran relative to the concentration in the micropipette. Panels B and D show the concentration relative to the pipette as a function of distance at each of the 21 z-slices. Panels E and F show the concentration of dextran, relative to that in the pipette, at 100 μm from the pipette tip, and the slope at the same location for neutral 10 ($n=3$), 40 ($n=3$) or 70 ($n=6$) kDa dextran. Panels G and H show the values of gradient concentration (relative to the pipette) and gradient steepness of 10 ($n=4$) and 40 ($n=4$) kDa dextrans measured at 20 μm intervals between 50 and 130 μm from the tip. Slope (at e.g. 100 μm) is defined as the fractional change in dextran concentration at 100 μm from the tip, and is determined from the change in dextran concentration over 10 μm (from 95 to 105 μm), divided by the concentration at 100 μm .

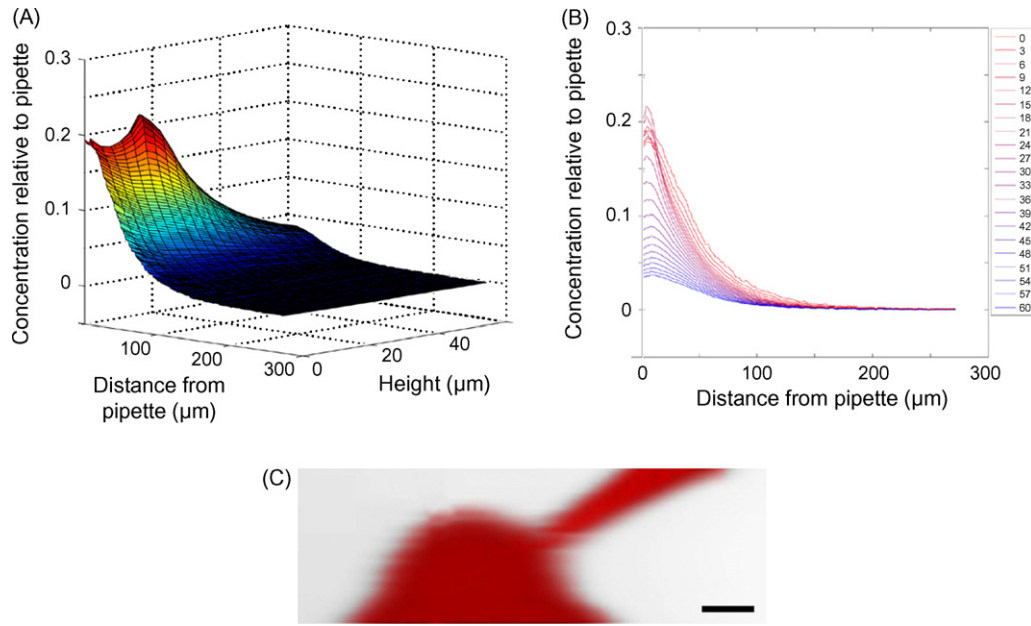


Fig. 3. The effect of charge (of dextran) on gradient shape. Panels A and B show gradient profiles of anionic 10 kDa dextran, using 2 Hz picospritzing frequency and 20 ms pulse duration. Panel C shows a three-dimensional reconstruction of a 10 kDa anionic dextran gradient (scale bar = 20 μm). Compare with Fig. 1D for neutral dextran.

dence of chemotropism, a lack of response may be due to many reasons.

The shape of a stabilized gradient is determined partly by the diffusion constant of the guidance cue and the amount of guidance cue in each ejection (Lohof et al., 1992). This indicates that if equal amounts of guidance cue of unequal molecular weights are ejected to form gradients, the resulting gradients should differ. Since diffusion rate is expected to be roughly inversely proportional to the cube root of the molecular weight, an increase in molecular weight should yield a decrease in the diffusion constant of the guidance cue (Crank, 1975). The net result is an increase in the concentration of the guidance cue at any given point, although theoretically the slope of the gradient at any point should be unchanged. This is important if a particular value of guidance cue concentration is desired, since the parameters will have to be altered in order to compensate for the decreased diffusion of higher molecular weight molecules.

Once the guidance cue reaches the substrate its movement becomes dependent on complex factors, such as the interaction between charges on the guidance cue and substrate. Ejection of a guidance cue with a net positive charge, or partial charges, may result in the adhesion of the guidance cue to the glass via electrostatic attraction. Adhesion through other uncharacterized mechanisms may also occur, leading to a buildup of guidance cue on the glass around the micropipette tip, potentially changing the gradient. Since real guidance cues are likely to contain a complex pattern of surface charges, their concentration at the growth cone is unlikely to be reliably predicted from purely diffusive mechanisms. Furthermore, the net charge on a protein is pH-dependent. Consequently, variations in pH during the assay may cause variations in charge on the guidance cues.

A desirable feature of an axon guidance assay is to be able to vary the amount of guidance cue presented to the growth

cone, for instance, in order to quantify the response over a range of guidance cue concentrations. It should be possible to control the amount ejected, and therefore the concentration at the growth cone, by varying either the pulse duration or pulsing frequency. We found that changes in pulse duration from 10 to 20 ms produced a reliable increase in dextran concentration at the growth cone, but that at longer pulse durations this relationship became unpredictable. The movement of the dextran solution beyond the tip due to jetting is limited to several microns under most picospritzing conditions. However at relatively long ejection duration times, jetting causes the guidance cue liquid to be pushed substantially further away from the tip, displacing the peak of the gradient, and thus reducing its symmetry. This causes variability in the concentration and slope of the gradient (Fig. 4E and F). In one of three experiments using 200 ms pulse duration, we observed a decrease in dextran at the growth cone position (compared to 50 ms).

Similarly, we found that predictably controlling the concentration of guidance cue at the growth cone by changing the pulsing frequency is also difficult. Although changes in pulsing frequency from 0.5 to 2 Hz produced an increase in dextran concentration at the growth cone, higher pulsing frequencies produced only modest (or no) increases which did not scale in proportion to the change in pulsing. It is likely that the non-linear relationships between the concentration of dextran at the growth cone and either pulse duration or pulsing frequency are due, at least partly, to uncharacterized flaws in the equipment. For example, due to the elastic nature of the plastic tubing used to deliver the compressed gas, the slight compressibility of the dextran solution and growth medium, and leakage of gas at joints, the pressure applied to the dextran solution will not increase linearly (over the measured range) with the pulse frequency. Our results suggest using pulsing frequency to control the concen-

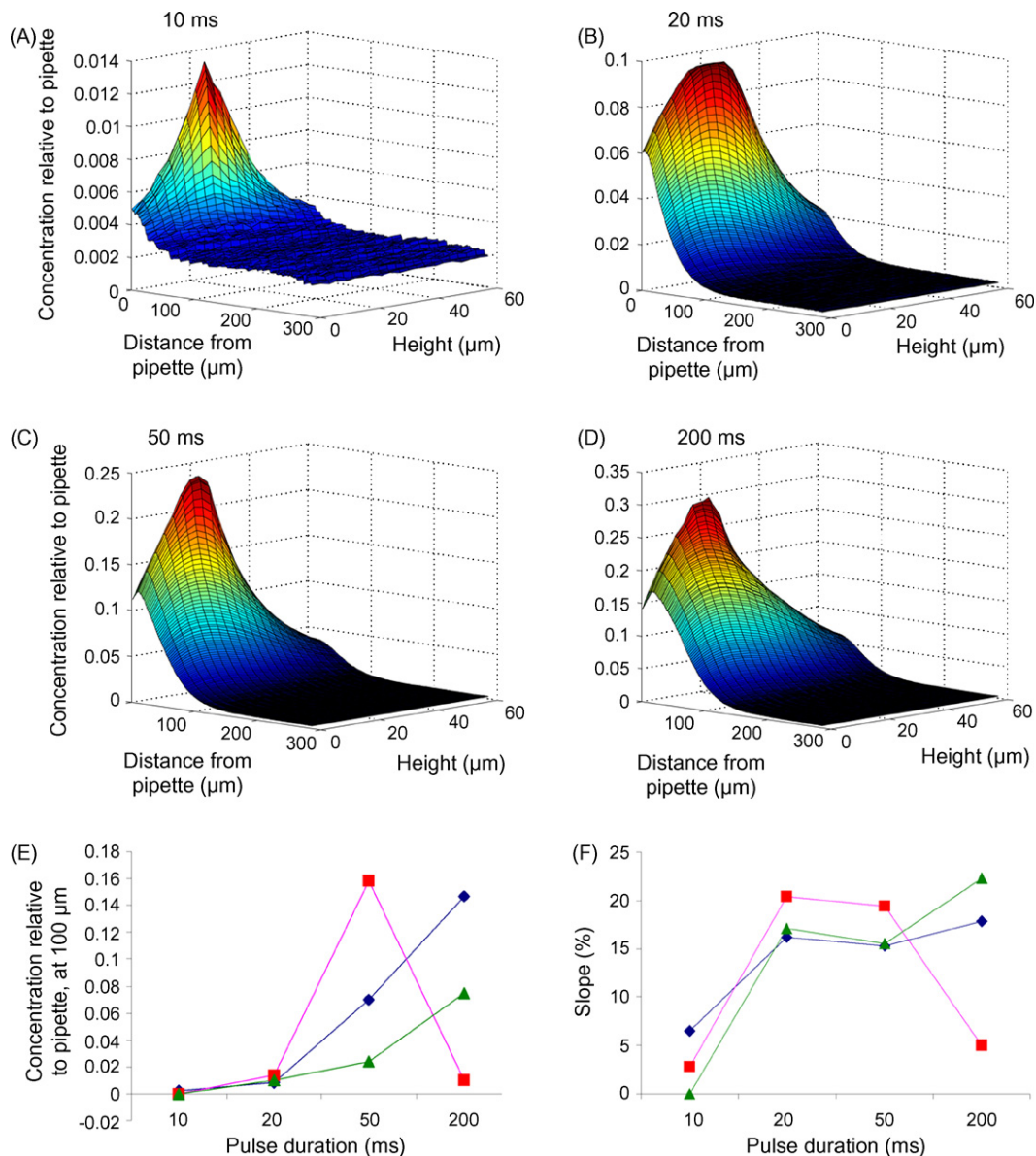


Fig. 4. Effect of pulse duration on gradient shape. Gradients of 10 kDa dextran-TMR produced using pulse durations of 10 ms (A), 20 ms (B), 50 ms (C), and 200 ms (D) and a 2 Hz picospritzing frequency. Bottom panels show the effect of pulse duration (ms) on the concentration of 40 kDa dextran-TMR (nM) (E) and on gradient slope at 100 μm from the pipette tip (F) in three independent experiments in which a single micropipette was used to obtain data for each of the four pulse duration periods (10, 20, 50 and 200 ms).

tration at the growth cone may be limited to a small range of target concentrations.

Another potential source of variation in the concentration of guidance cue at the growth cone may be inconsistencies in the height of the pipette above the floor of the dish. Experiments in this study were mostly performed with a pipette tip height of 25 μm because this was the value which could be achieved consistently without damage to the tip (by collision with the floor). Pipette tip height is not usually reported quantitatively in the literature. We found that variation in the concentration at the growth cone due to other factors (such as currents in the growth medium resulting from heating stages), largely masked any variation when we varied pipette height over a small range (20–40 μm above the floor). This will clearly not hold for heights

approaching the horizontal distance between the axon and the pipette tip. Nevertheless, it remains prudent to maintain consistent pipette heights in order to minimize variability in the concentration of guidance cue at the growth cone.

We also found (data not shown) that currents in the growth medium can often exert strong influences on gradient shape. Such currents may arise from thermal nonequilibrium between the growth medium and the air around the Petri dish, or from uneven heating provided by a thermal stage if used. The resulting distribution of guidance cue can be complex and nonsymmetrical, although it usually assumes the shape of a unipolar outflow from the tip. The direction of the outflow is difficult to predict or control due to the complex pattern of currents within the dish. Axons placed in the vicinity of such a gradient may expe-

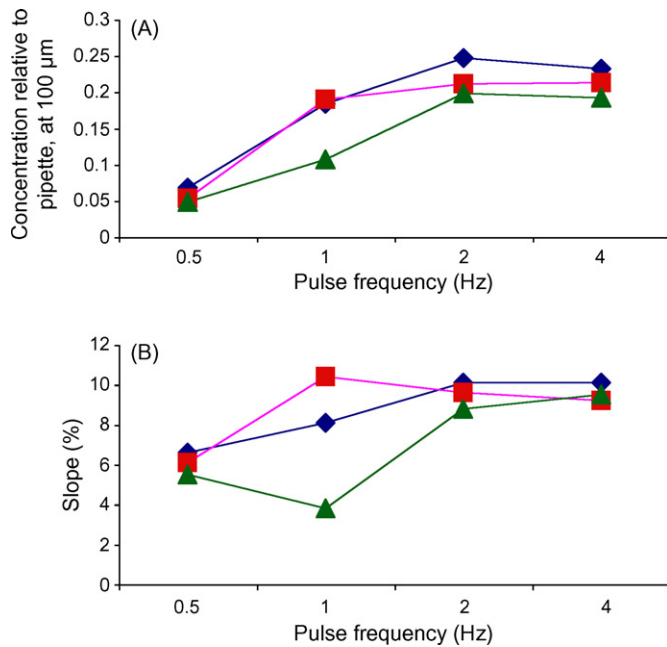


Fig. 5. Effect of pulsing frequency on gradient shape. The concentration (A) of the 70 kDa dextran gradient, and the dextran gradient slope (B) at 100 μm from the pipette, as a function of pulse frequency (using a 20 ms pulse frequency). Both graphs show the results of three independent experiments in which a single micropipette was used to obtain data for each of the four pulse frequencies.

rience guidance cue concentrations ranging from almost zero, to values approaching that found within the pipette. Without directly observing the gradient in each experiment, it is difficult to be certain that currents are not present. Inclusion of a fluorescent dye (such as a high molecular weight dextran-TMR) in the guidance solution would allow monitoring of the gradient during ejection. Although eliminating currents in the medium is difficult, attempts can be made to minimize them. This can be achieved, for example, by ensuring that all components of the assay (including growth media, plasticware and microscope components) have thermally equilibrated prior to the start of picospritzing. The use of an incubation chamber in conjunction with a heating stage can ensure the reduction of thermal boundaries between the air and the liquid in the Petri dish, and a heating collar around the objective lens can reduce its ability to function as a heat sink. By improving the uniformity of heating in a Petri dish, the presence of currents can be reduced, leading to an improvement in the symmetry of the gradient, and consequently, the reproducibility of the assay.

The generation of a guidance cue gradient in the pipette assay requires its ejection from a micropipette with a narrow bore size at the tip. The narrowing of the pipette, and the use of positive pressure for ejection, can lead to the obstruction of the bore at the tip by insoluble particulates present in the guidance cue liquid, or glass particles present in the capillary. Unless a visible marker is used, it is difficult to determine if the tip is completely or partially blocked during pipetting. We used compressed gas to remove particulates from capillaries prior to pulling them. Even so, we found that approximately 10% of our pipettes blocked if used for more than 1 h. Since the quantity of guidance cue delivered to the growth medium varies as the square of the micropipette

internal diameter at the tip, variation in this diameter can lead to large variation in the concentration of guidance cue at the axon.

Ejection pressure is also a critical determinant of how much guidance cue is delivered into the growth medium. Standard gas regulators have ranges measured in hundreds of psi. Since ~ 3 psi is sufficient for the ejection of nanolitre quantities of fluid, it is unlikely that such regulators will be adequate to enable gas pressure to be measured or set reproducibly. Lastly, if the guidance cue is dissolved in materials with a density different to that of the medium the cells are growing in, it may either move upwards (due to negative buoyancy) or sink. Both may lead to a change in the gradient structure compared to that predicted by diffusion alone.

Overall we found considerable variability in the values of the concentration and gradient slope, particularly for the 40 and 70 kDa dextrans when long pulse durations are used, or when measurement is made at large distances from the pipette tip. The concentration of 70 kDa dextran varied between 0.07 and 0.13 (approximately 2-fold) relative to the intrapipette concentration, while the slope of the 70 kDa dextran gradients varied between approximately 6 and 25% (approximately 4-fold). Least variability occurred when short pulse durations (< 50 ms) were used and with measurement made < 110 μm from the pipette tip.

The importance of determining accurately the concentration at the growth cone of material ejected from the pipette is highlighted by recent studies which demonstrate a relationship between concentration and function, as well as a relationship between concentration and degree of function. For instance, Kolpak et al. (2005) found that at low concentrations, sonic hedgehog functions as a positive factor for retinal ganglion cell axonal growth, while at high concentrations, it functions as a negative factor. Similarly, netrin-1 has been shown to function as a facilitative or inhibitory regulator of angiogenesis depending on its concentration (Yang et al., 2007). Furthermore, Rosoff et al. (2004) found that guidance of dorsal root ganglion neurites by NGF only occurred over a limited range of concentrations. Thus, a knowledge of, and careful control over, the concentration in the pipette assay can be important when trying to gain reliable insights into the underlying biological mechanisms of axon guidance.

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