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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.

Keywords: Axon guidance; Turning assay; Pipette assay

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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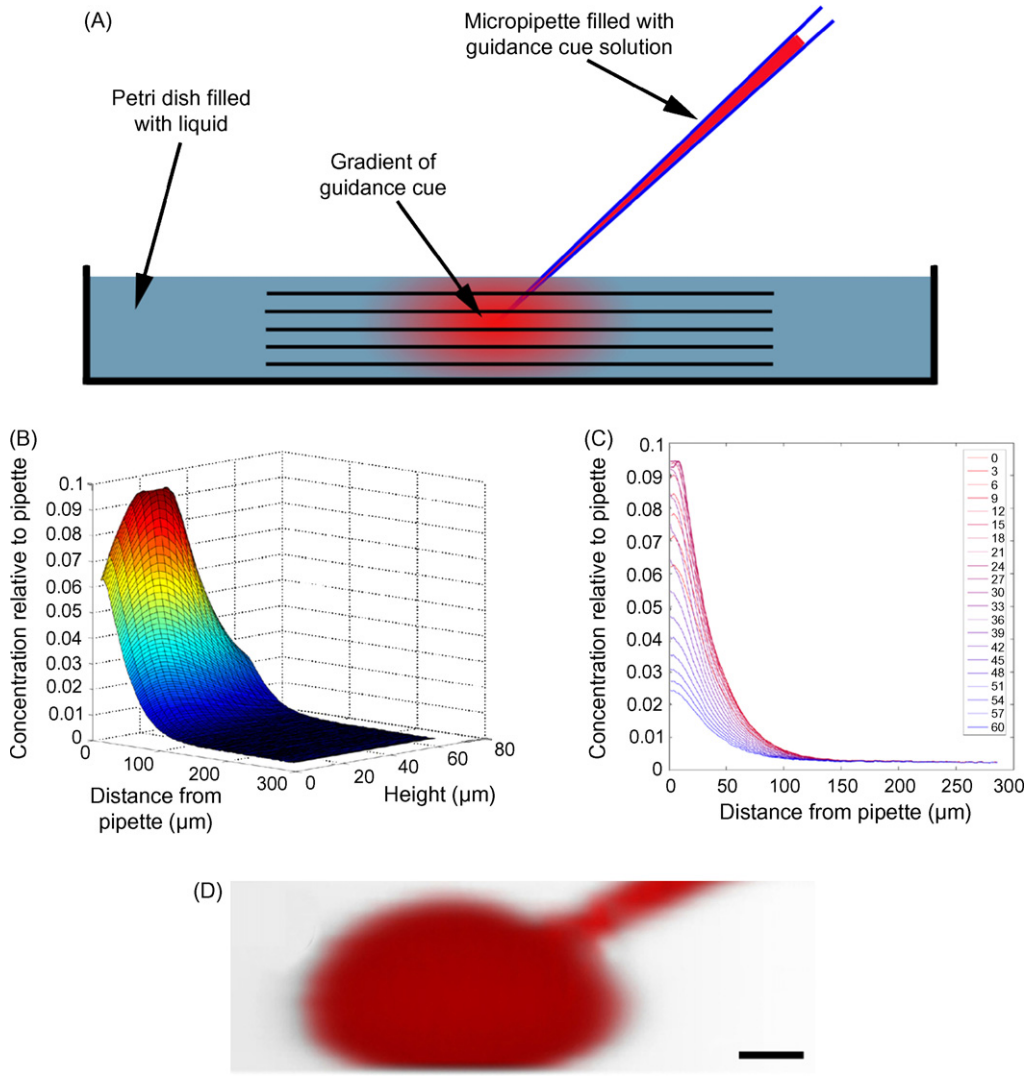

Fig. 1. Method of analysis of the pipette assay. Panel A depicts schematically a gradient produced in a 35 mm diameter Petri dish by a micropipette. Black horizontal lines represent the image planes produced by confocal imaging of a stable gradient in liquid (not to scale). Panel B shows a measured three-dimensional 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