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Analyzing neurite outgrowth from explants by fitting ellipses

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ABSTRACT

The establishment of appropriate connectivity in the developing nervous system depends on many molecular guidance cues. A key method for studying the response of nerve fibers to such guidance cues is to culture explants of neural tissue in three-dimensional collagen gels. However, most previous analyses of the neurite outgrowth patterns from these explants have been very simple, often measuring only one or two parameters. Here we introduce a more sophisticated method for characterizing neurite outgrowth from explants, based on fitting an ellipse to the pattern of outgrowth. This provides 5 parameters describing the outgrowth: x and y position of the center of the ellipse, the elongation, the area and the tilt. We then apply this method to a large dataset of dorsal root ganglion explants grown in the presence of precisely controlled gradients of nerve growth factor. This analysis reveals a number of new features of these data. For instance, we find that it is the position of the center of the ellipse rather than the shape of the ellipse that is correlated with the strength of the gradient. Together these results show that ellipse-fitting of explant data can give new insights into the biological processes underlying neurite guidance by molecular cues.

1. Introduction

Proper brain function depends on the establishment of appropriate connectivity between neurons during development. In the past few years a large number of molecular cues have been identified which are involved in the guidance of axons and dendrites (hereafter referred to as neurites) to their targets (Tessier-Lavigne and Goodman, 1996; Dickson, 2002; Guan and Rao, 2003; Huber et al., 2003; Plachez and Richards, 2005; Mortimer et al., 2008; Lowery and Van Vactor, 2009). Crucial to both the identification and analysis of the mechanisms of action of these guidance cues have been *in vitro* assays (Guan and Rao, 2003; Pujic et al., 2009). A particularly influential approach, partly due to its relatively close recapitulation of the *in vivo* environment, is the three-dimensional collagen gel system (Lumsden and Davies, 1983, 1986). In this system explants of neural tissue are embedded in a collagen gel and then exposed to either uniform or spatially graded levels of guidance cues (for some representative examples see Tessier-Lavigne et al., 1988; Placzek et al., 1990; Kennedy et al., 1994; Brose et al., 1999; Braisted et al., 2000). The resulting pattern of outgrowth from such explants is then examined, for instance to determine if there was a bias in outgrowth towards or away from a guidance cue gradient.

In some biological situations outgrowth is sparse enough that individual neurite trajectories can be reliably extracted (e.g. Weaver et al., 2003), potentially allowing a detailed analysis of how these trajectories are affected by external guidance cues. However, in many other situations the high density of neurites precludes such approaches. In these cases a common method has been to extract pixels representing neurites (e.g. by thresholding image intensity) and then analyze the spatial distribution of these pixels. Up to now this analysis has usually involved the extraction of only one or two parameters, such as total neurite pixels and the ratio of pixels representing neurite growth from one side or region of the explant compared to the other (e.g. Bilsland et al., 1999; Caton et al., 2000; Rosoff et al., 2004; Mortimer et al., 2009). Although such measures can be robust and informative for addressing certain questions, they are clearly a rather crude characterization of the overall pattern of neurite outgrowth from explants. In particular, they are unable to detect more subtle biases in outgrowth patterns, and how these are influenced by external guidance cues. Such biases may be important for understanding the full range of biological effects that guidance cues exert on neurite growth.

To detect such biases a description of outgrowth is needed which is intermediate between a single parameter and the high-dimensional data of the image itself. Here we argue that fitting ellipses to the pixels representing neurites is a useful way to achieve this. Recent computational advances have made fitting an ellipse to a set of datapoints a robust and efficient procedure (Fitzgibbon et al., 1999). Ellipses provide 5 parameters describing the shape, namely the x and y position of the center, the elongation, the area and the

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tilt. Although still simple, this description provides substantially more information than a single parameter. We illustrate the power of this approach by applying it to a dataset we recently generated of ≈2500 dorsal root ganglion (DRG) explants growing in the presence of precisely controlled gradients of nerve growth factor (NGF) (Mortimer et al., 2009). Using a variety of computational techniques to analyze the resulting five-dimensional space of ellipse parameters, we reveal a number of new and unexpected characteristics of the relationship between gradient parameters and the pattern of outgrowth. Overall, we argue that ellipse-fitting has the potential to provide insight into the effect of guidance cues on explants which goes substantially beyond that obtained based on current methods.

2. Methods

2.1. Explant data

DRGs from postnatal days 1–3 rats were cultured in three-dimensional collagen gels for 2 days in the presence of NGF gradients as described elsewhere (Mortimer et al., 2009). The gradients were generated using the technology of Rosoff et al. (2005). Fractional gradient steepnesses of 0.1%, 0.2%, 0.3% and 0.4% per 10 μ m were used, at absolute concentrations varying from 0.001 to 100 nM. 38 different combinations of gradient steepness and absolute concentration were investigated in total, with an average of 66 explants per condition, giving approximately 2500 explants altogether. Explants were then fixed, stained with TUJ1, and photographed under epifluorescence. Examples of the resulting explant images are shown in Fig. 1; for detailed experimental methods see Mortimer et al. (2009). As confirmed by confocal imaging (data not shown) most of the neurite outgrowth was concentrated in roughly a two-dimensional plane, and thus the two-dimensional epifluorescence imaging was sufficient to capture most of the information about neurite outgrowth.

2.2. Ellipse-fitting

As described in Mortimer et al. (2009), images were first thresholded in intensity to produce a binary image. The pixels representing the explant body were then removed, leaving just pixels representing neurites. Ellipses were then fitted to the neurite pixels using a direct least square method (Fitzgibbon et al., 1999) (Fig. 1). This method is robust and computationally efficient as it analytically determines a unique solution. The results were converted to five parameters describing the position and shape of the outgrowth: the *x*- and *y*-axis coordinates of the ellipse center relative to the center of the explant body, the area of the ellipse, the ratio of semi-minor to semi-major axis length or "elongation", and the absolute angle of rotation of the ellipse semi-major axis from the gradient direction or "tilt". Although more complicated than fitting a circle, fitting an ellipse provides 2 more parameters (elongation and tilt) compared to fitting a circle. As illustrated by the results we present these

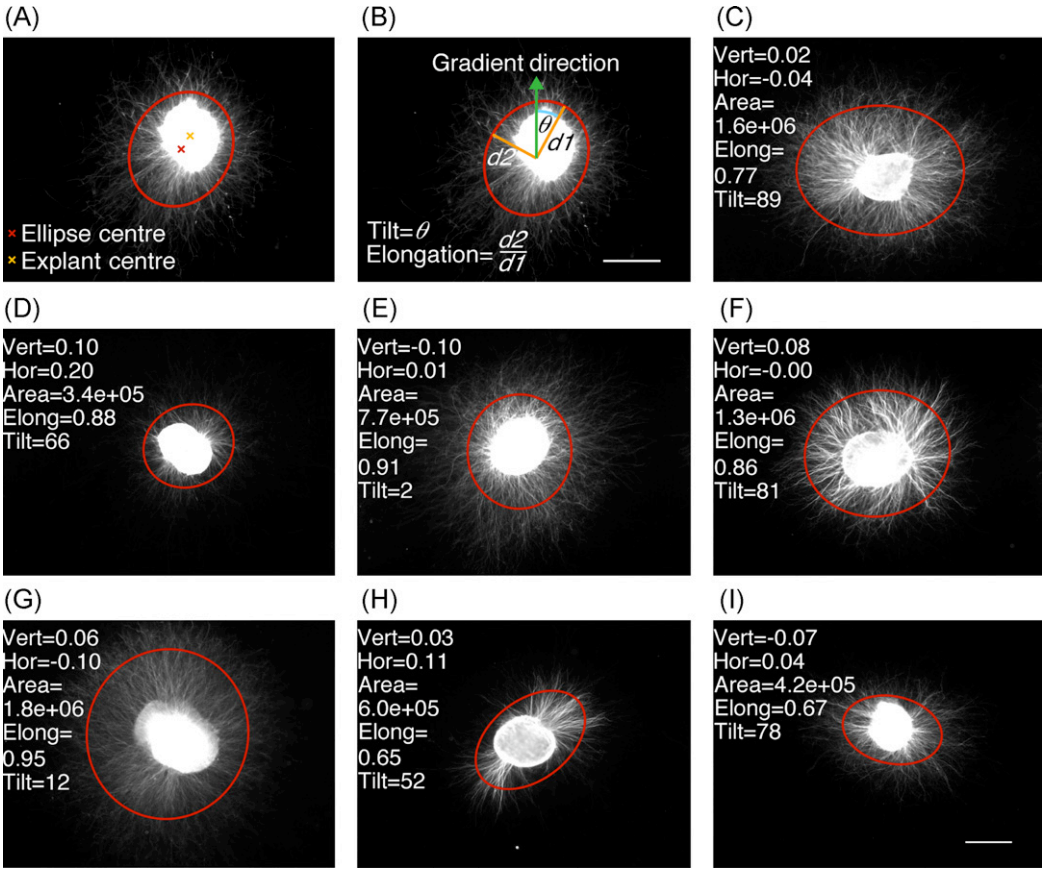

Fig. 1. Ellipse-fitting to explants. Fitted ellipses are shown superimposed on the corresponding raw images of DRGs explant grown for 2 days in a three-dimensional collagen gel. (A) *x* (horizontal) and *y* (vertical) displacement are defined as the position of the center of the ellipse relative to the center of mass of the explant. (B) We refer to the ratio of lengths of the semi-minor to semi-major axis as the elongation, and the angle (in degrees) between the semi-major axis and the gradient as the tilt. Elongation and tilt are defined as shown. (C–I) Examples of ellipses fitted to a variety of DRG explants after 2 days of growth in a three-dimensional collagen gel, together with the ellipse parameters extracted. An NGF gradient was present increasing upwards in the image. Vertical and horizontal displacement are given in units of average explant diameter across all explants, i.e. the number represents the fraction of distance moved relative to the size of a typical explant body. Area is given in units of square microns. Scale bar in B, $I = 500\ \mu$m.

additional parameters can provide useful additional information regarding the pattern of neurite outgrowth. Matlab code to perform ellipse-fitting of a set of general datapoints is freely available from http://homepages.inf.ed.ac.uk/rbf/CVonline/LOCAL_COPIES/FITZGIBBON/ELLIPSE.

2.3. Total outgrowth and guidance ratio

To compare with the results of the ellipse-fitting we used the "total outgrowth" and "guidance ratio" values previously calculated for this dataset in Mortimer et al. (2009). First, each thresholded image was divided into an up-gradient half and a down-gradient half by a line perpendicular to the gradient direction passing through the centroid of the explant body. Denoting pixels representing neurites in the up-gradient half as U and in the down-gradient half as D, total outgrowth was calculated as $U + D$, and the guidance ratio was calculated as $(U - D)/(U + D)$.

2.4. Clustering

To explore the parameter space and discover whether there are distinct types of explants we applied AutoClass (Cheeseman et al., 1988), a Bayes-based cluster method which determines the natural classes within the data. The resulting classification is described probabilistically so that each data point is assigned a probability of class membership. AutoClass requires the user to supply information about how the attributes describe the data, such as their range and whether they are real or discrete. From this information AutoClass determines broad prior distributions for models that will represent the data. These models specify the types of likelihood functions, or probability distributions, of each attribute in each class. Our ellipse-fitting parameters are real independent attributes and were modelled with normal distributions. The joint distribution was calculated from the prior expectations and likelihood functions. The joint distribution was then broken up into smaller regions and AutoClass searched the parameter space within these regions until the marginal joint distribution, a combination of regions, was maximised. For more details see Cheeseman et al. (1988), and for a recent discussion of AutoClass in a biological context see Achcar et al. (2009).

3. Results

Fig. 1 shows how the ellipse parameters were defined, and examples of the resulting ellipse fit for a range of different DRG explants grown for 2 days in three-dimensional collagen in the

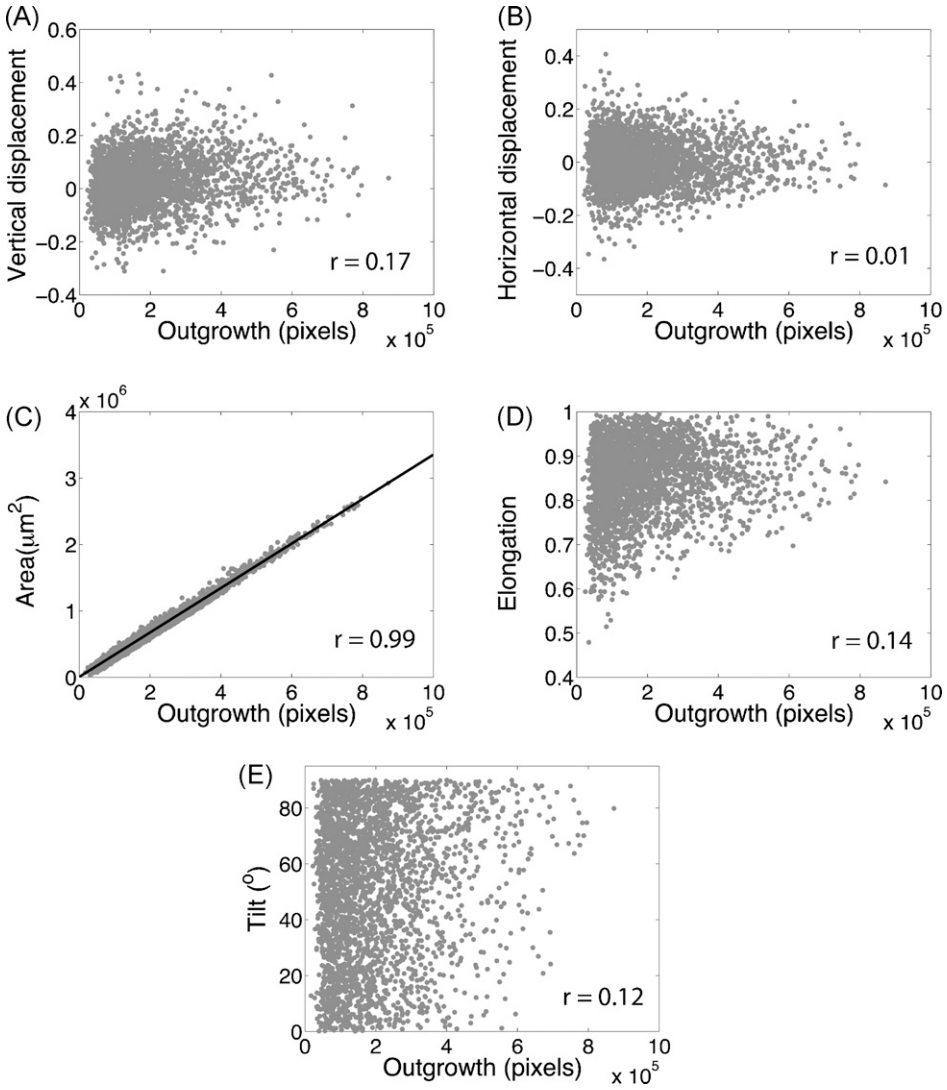

Fig. 2. No correlation between explant size and explant shape. The x-axis in each plot is the total number of neurite pixels as previously determined for each explant by Mortimer et al. (2009). The y-axis in each plot shows each of the 5 ellipse parameters, respectively. There is one point in each graph for each of the 2500 explants. r values quoted are the Pearson correlation coefficients. Only ellipse area has a strong correlation with total outgrowth.

presence of NGF gradients using a variety of gradient parameters (Mortimer et al., 2009). We fitted ellipses for ≈ 2500 DRG explants, obtaining 5 parameters for each; this can be thought of as a five-dimensional space populated by ≈ 2500 points. We then interrogated the structure of this space using a variety of methods, to understand more both about its intrinsic structure, and about which parameters of explant growth are most strongly affected by NGF gradients. For simplicity we will use "explant shape" and "explant size" to mean the shape/size of the overall pattern of outgrowth, rather than the shape/size of the body of the explant.

3.1. Explant shape is independent of explant size

We first addressed the question of whether explant shape is affected by explant size. That is, are larger explants simply expanded versions of smaller explants, or is there a systematic change in shape with size? In previous work, explant size was characterized by summing all pixels representing neurites to give the neurite outgrowth Mortimer et al. (2009). We therefore plotted each ellipse parameter against this outgrowth. Outgrowth strongly predicted ellipse area, but was uncorrelated with all other ellipse parameters (Fig. 2). Thus, there was no change in shape with total outgrowth. This result provides a validation of our ellipse-fitting method: even though the ellipse passes through the "middle" of the distribution of neurite pixels rather than encompassing all pixels, it produces a value with a very strong correlation to the sum of all pixels.

3.2. An external gradient shifts the center of the ellipse but does not change its shape

We next asked how exactly an external NGF gradient changes the distribution of neurite growth. For instance, does the shape become elongated by the gradient? In previous work the overall effect of the gradient on an explant was characterized by the guidance ratio (Mortimer et al., 2009), i.e. the ratio of pixels representing neurites on the up-gradient side of the explant compared to pixels representing neurites on the down-gradient side of the explant. We therefore plotted each ellipse parameter against the guidance ratio. Surprisingly, we found that the only ellipse parameter correlated with the guidance ratio was the vertical displacement of the center of the ellipse (Fig. 3). This suggests that the only effect of the gradient on the explant is to shift the neurite distribution in the direction of the gradient, without changing the overall shape of the neurite distribution.

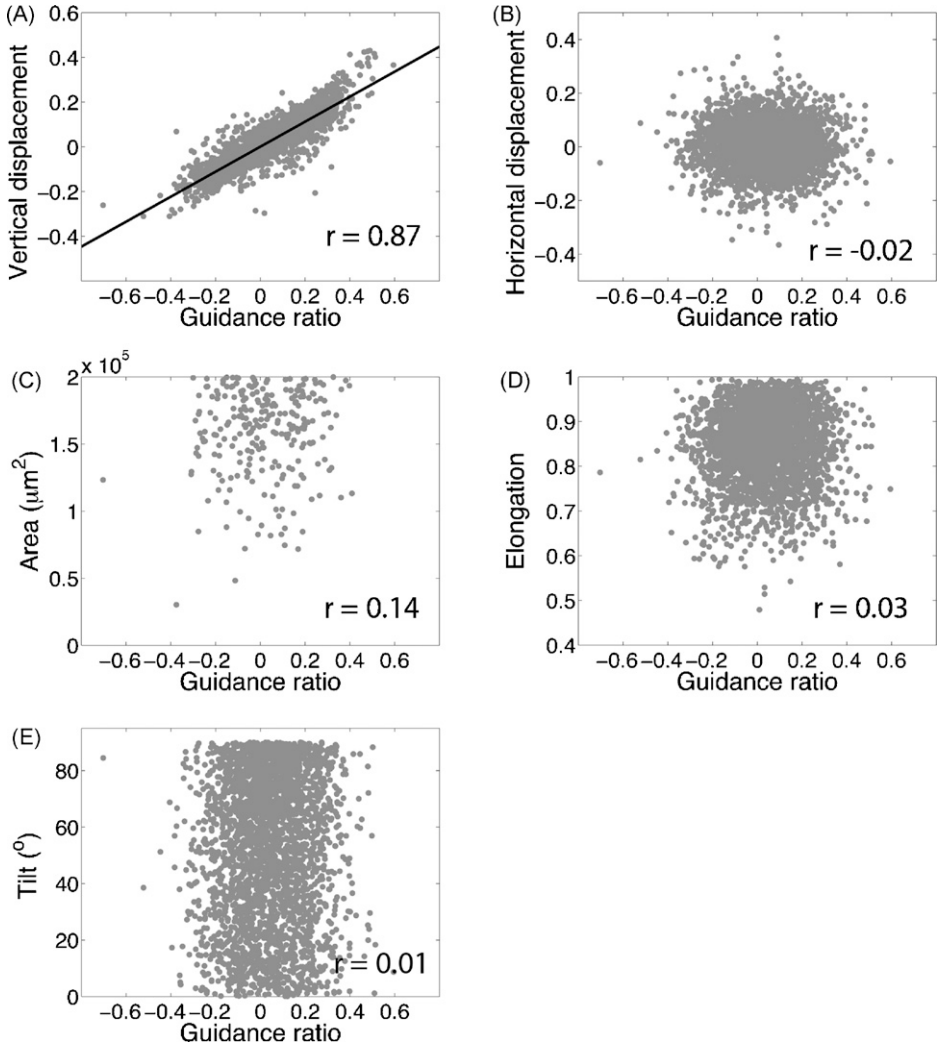

Fig. 3. An external gradient shifts the center of the ellipse but does not change its shape. The *x*-axis in each plot is the guidance ratio as previously determined for each explant by Mortimer et al. (2009). The *y*-axis in each plot shows each of the 5 ellipse parameters, respectively. There is one point in each graph for each of the 2500 explants. *r* values quoted are the Pearson correlation coefficients. Only the vertical center displacement has a strong correlation with the guidance ratio.

3.3. Correlations between explant shape and gradient parameters

So far we have only investigated correlations with the guidance ratio, rather than correlations with the gradient parameters of steepness and concentration. We therefore plotted each ellipse parameter as a function of these gradient parameters (Fig. 4). This confirms that ellipse area and ellipse vertical displacement vary systematically with steepness and concentration (Fig. 4A and C), as was previously observed for outgrowth and guidance ratio, respectively (Mortimer et al., 2009). There was no systematic pattern of variation between the gradient parameters and either the horizontal center displacement (Fig. 4B) or the absolute angle between the tilt and the gradient direction (Fig. 4E). However, there was a very slight (≈ 5%) increase in the elongation of the explants at very high (100 nM) NGF concentrations compared to very low (0.01 nM) concentrations. We suspect that this is caused by an increased tendency towards axon fasciculation at high concentrations, which emphasizes the influence of "hotspots" of growth (see example in Fig. 1H).

If the tilt (which varies between 0 and 90 degrees) were randomly distributed one would expect its average value to be 45 degrees. However, an unexpected finding was that, for almost all gradient conditions, the tilt was slightly greater than 45 degrees (Fig. 4E). A more detailed analysis of the distribution of these angles is shown in Fig. 4F. From this it is clear that there is a tendency for the ellipses to lie slightly "on their side" relative to the gradient direction. As discussed further below we suspect this may have been caused by a slight nonuniformity in the illumination of explants when the images were captured.

3.4. Principal Component and cluster analyses

Up to now we have examined correlations between ellipse parameters and experimental parameters believed to be important in controlling the pattern of neurite outgrowth. To complement this we also performed some exploratory data analyses on the five-dimensional space of explants to investigate the intrinsic structure of this space.

We first performed a Principal Component analysis (Table 1). However this was not particularly informative since the eigenvalues of the 5 components were all quite similar, indicating that the

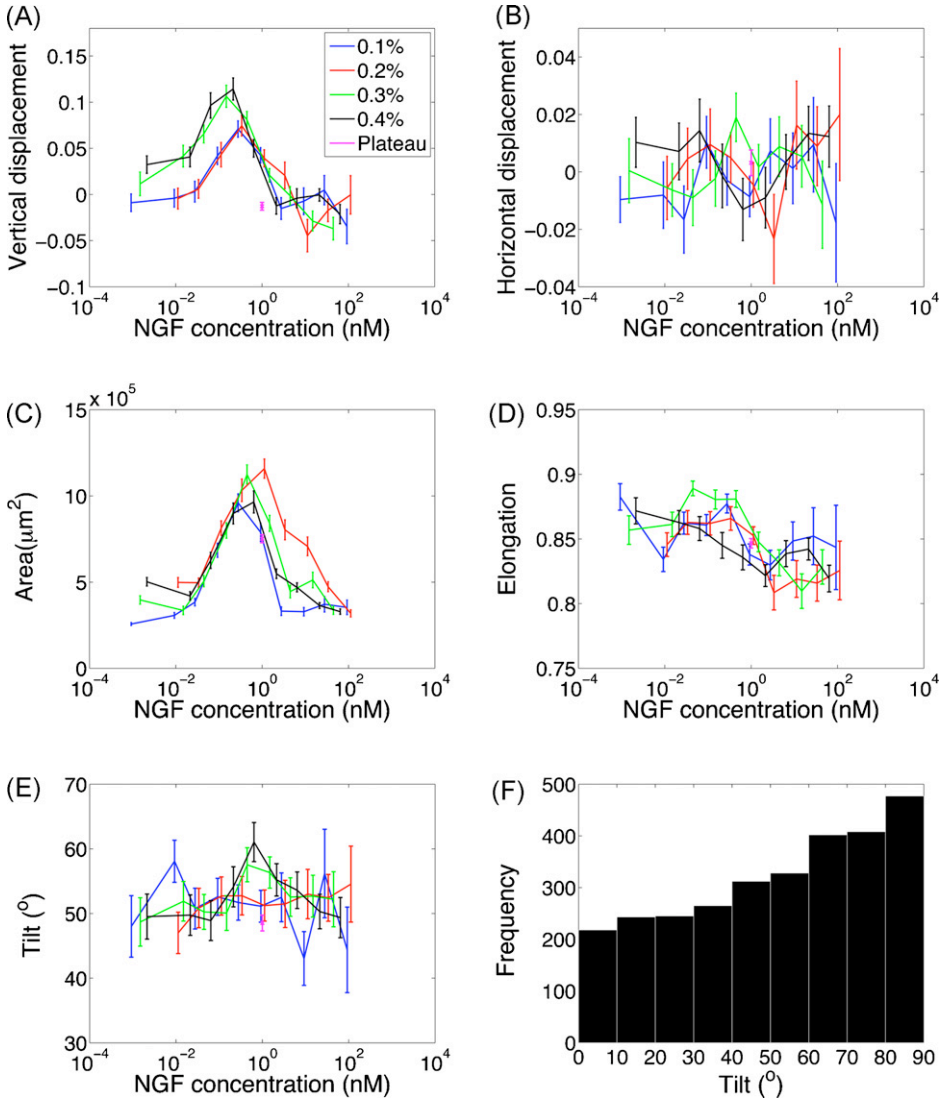

Fig. 4. Correlations between explant shape and gradient parameters. The *x*-axis in plots A–E is the NGF concentration at the explants, and the *y*-axis in each plot shows each of the 5 ellipse parameters, respectively. In each of these plots there is one point for each of 38 different combinations of gradient steepness and concentration (see Mortimer et al. (2009) for more details of gradient conditions), with the different colors representing different gradient steepnesses as shown in the key. Steepness refers to the fractional change in NGF concentration across 10 μm. *F*, number of explants with tilt falling into the bins indicated on the *x*-axis. There is a trend towards large tilts, indicating that the explants tend to be slightly elongated in the direction orthogonal to the gradient direction.

Table 1
Principal components of fitted ellipse parameters. Vertical displacement and overall size dominate the first principal component. However all principal components have similar eigenvalues, indicating that the variance is fairly evenly spread over all dimensions.

	PC1	PC2	PC3	PC4	PC5
Eigenvalue	1.24	1.09	1.00	0.94	0.73
Vertical displacement	0.56	0.02	0.02	−0.73	0.40
Horizontal displacement	0.08	−0.07	−0.99	0.01	−0.03
Area	0.71	0.04	0.07	0.17	−0.67
Elongation	0.33	−0.68	0.07	0.48	0.44
Tilt	0.26	0.73	−0.04	0.46	0.43

variance is roughly equally distributed over all dimensions. We therefore turned to a cluster analysis using AutoClass (Cheeseman et al., 1988; Achcar et al., 2009), a Bayesian clustering algorithm which automatically chooses an appropriate number of clusters. This found 8 classes, exemplars of which are shown in Fig. 5. AutoClass also returned a set of 5 numbers indicating the relative importance of each of the 5 dimensions in determining cluster membership. Interestingly these numbers were 1 for tilt, 0.21 for elongation, 0.15 for area, 0.05 for vertical displacement and 0.02 for horizontal displacement. This indicates that for this dataset the tilt is by far the most important variable in determining into which class each explant falls.

4. Discussion

The response of explants to molecular gradients in three-dimensional gels is widely used to probe mechanisms of neurite growth and guidance in the developing nervous system. However, the characterization of patterns of growth from such explants has so far been relatively simplistic. Here, we have proposed a method for describing these patterns using a computationally efficient ellipse-fitting procedure that returns 5 informative parameters describing the neurite growth. The amount of work involved in this procedure is no greater that more standard methods based on counting pixels, since the ellipse-fitting is fully automated. The ellipse parameters obtained to describe neurite growth allow a more nuanced analysis of the outgrowth pattern than more standard methods. We illustrated this by applying the method to a large dataset of DRG explants grown in the presence of precisely controlled NGF gradients. This revealed a number of hitherto unrealised effects.

Firstly, we found that the outgrowth pattern was independent of the amount of outgrowth (Fig. 2). This was not obvious a priori: there could have been subtle changes in the form of growth as axons increased in length or density. That this was not the case confirms that ignoring the total amount of outgrowth is reasonable when investigating asymmetries in outgrowth. Secondly, we found that an external ligand gradient had surprisingly little effect on the shape of the outgrowth (Fig. 3). Instead, the main effect of the gradient was to shift the center of the ellipse away from the center of the explant. Again this was not obvious a priori: for instance one could imagine that the gradient might have stretched the outgrowth along the gradient axis. This finding provides a new constraint for computational models of axonal trajectories (e.g. Goodhill et al., 2004; Xu et al., 2005).

Thirdly, our analysis revealed a previously unrecognized small asymmetry in our gradient assay (Mortimer et al., 2009), whereby the tilt of the fitted ellipses showed a slight bias towards values larger than 45 degrees (Fig. 4E and F). This was not accompanied by any horizontal shift of the center of the ellipse, suggesting that it was not caused by a nonuniformity of the NGF gradient. Rather, we suspect that the illumination brightness of the microscope stage on which the explants were photographed had a slight nonuniformity,

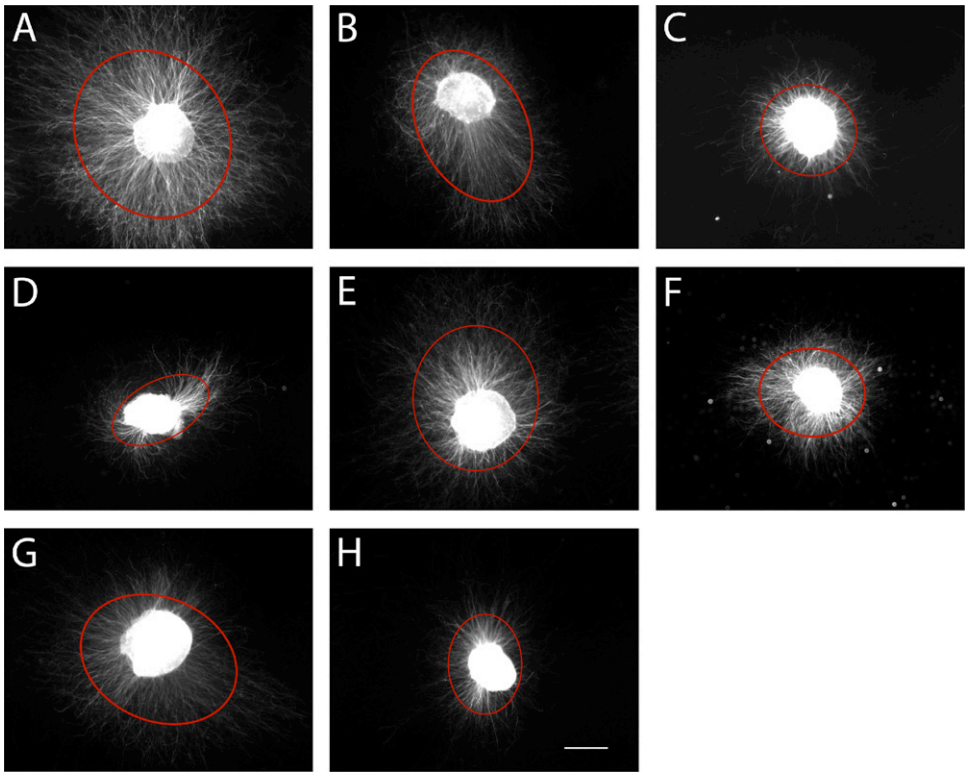

Fig. 5. Cluster analysis. AutoClass was applied to the five-dimensional space of ellipse parameters and produced 8 classes. Exemplars of each class are shown; it can be seen that these represent different types of patterns of neurite outgrowth. For instance (A) represents explants which are large and relatively symmetric, (B) represents large and highly elongated explants, (C) represents small and symmetric explants, and (D) represents small and highly elongated explants. Scale bar in $H = 500\,\mu m$.

such that average pixel brightness increased slightly moving along a tilted axis. This suggests that flat-fielding of the explant images (which was not performed for this dataset) may be important for eliminating such biases.

Lastly, the multidimensional space describing each explant allows exploratory data analyses to potentially identify distinctly different categories of explants which might not have been otherwise apparent. We illustrated this by performing a cluster analysis on our set of explants (Fig. 5). This revealed the importance of tilt in determining class membership. DRG explants can show "hotspots" of growth in regions where bundles of nerves exited the DRG in the intact system before explanting into the collagen gel (see Fig. 1H for example), strongly influencing the tilt. Since normally DRG explants will be placed in an *in vitro* environment in a random orientation one would expect this tilt to be randomly distributed with a mean close to 45 degrees, which is what we observed (see Fig. 2E and 3E). However, the importance of this variable in determining cluster membership suggests that it may be advantageous to attempt to minimise its effect, since it has the potential to obscure more subtle effects due to external gradient conditions. This could be done by, for instance, labelling the orientation of DRGs with colored beads whilst still *in situ*, so that they can then be placed in a consistent orientation in the collagen. A related application of the clustering approach is that it can provide objective criteria by which potentially "problematic" explants, such as those with very strong hotspots of growth, can be excluded from further analysis if desired.

Here we have illustrated the ellipse-fitting method on a dataset of DRGs responding to NGF. However is also applicable to many other combinations of tissue and chemotropic factors, and is particularly suitable for situations where outgrowth is too dense for individual neurite trajectories to be reconstructed. Overall, we suggest that ellipse-fitting provides a promising alternative to more standard methods for analyzing patterns of neurite outgrowth from explants. In particular, it provides the potential for deeper insight into the rules which govern neurite growth as both internal and external parameters are varied.

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