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Evaluation of neurite outgrowth anisotropy using a novel application of circular analysis

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ABSTRACT

Precise axon growth is required for making proper connections in development and after injury. One method of studying axon guidance and growth is through *in vitro* outgrowth assays that present controlled microenvironments. In this study, we applied circular statistical methods to evaluate directional neurite response. Visualization of data on a circular scale allows more accurate representation of the data, as neurite angles are inherently expressed on a circle. Here, the direction of neurite outgrowth from dorsal root ganglia derived neurons on different substrate types was quantitatively measured. Further, simulations of datasets with known circular parameters reflecting expected neurite angle distributions from different substrate types were also generated. Circular statistical methods were utilized and compared to linear statistical models widely used in the neuroscience literature. For small samples, Rao's spacing test showed the smallest occurrence of Type I errors (false positives) when tested against simulated uniform distributions. *V*-test and Rayleigh's test showed highest statistical power when tested against a unimodal distribution with known and unknown mean direction, respectively. For bimodal samples, Watson's U^2-test showed the highest statistical power. Overall, circular statistical uniformity tests showed higher statistical power than linear non-parametric tests, particularly for small samples ($n = 5$). Circular analysis methods represent a useful tool for evaluation of directionality of neurite outgrowth with applications including: (1) assessment of neurite outgrowth potential; (2) determination of isotropy of cellular responses to single and multiple cues and (3) determination of the relative strengths of cues present in a complex environment.

1. Introduction

Axon guidance during development and after injury has been studied in traditional cell culture and in increasingly complex *in vitro* environments generated with tissue engineering and other biomedical engineering techniques. One approach has been to manipulate the cells' local microenvironment and observe neurite outgrowth in microenvironments containing cues of interest. Studies of axon guidance often use *in vitro* neurite outgrowth assays (Ronn et al., 2000; Smit et al., 2003; Thompson and Buettner, 2006; Weaver et al., 2003) as models to elucidate the growth potential of neurons, the effects of the environment, and the mechanisms underlying the axon growth process.

Quantitative assessment of neurite outgrowth in these assays represents a critical step in gaining specific information on axon growth. Quantitative morphometric analyses depend heavily on microscopy techniques (Meijering et al., 2004; Mitchell et al., 2007) and automated (Karlon et al., 1998; Price et al., 2006; Weaver et al., 2003) or semi-automated (Bilsland et al., 1999; Hynds and Snow, 2002; Thompson and Buettner, 2006) image analysis tools which allow researchers to accurately assess neuronal and neurite growth. Parameters that provide information on neuronal response may include the area of the neuron or neurite (Abosch and Lagenaur, 1993), number of neurites (Abosch and Lagenaur, 1993; Le Roux and Reh, 1994), neurite orientation, neurite length (Abosch and Lagenaur, 1993) and path of migration. One widely used measure for the strength of a guidance cue is the direction of neurite outgrowth following some underlying directional stimulus (Alexander et al., 2006; Bruder et al., 2007; Deumens et al., 2004; Mahoney et al., 2005; Thompson and Buettner, 2006).

The geometry of neurite outgrowth is most meaningfully parameterized in a circular coordinate system centered on the cell and rotationally aligned to the stimulus applied. The distribution of neurite angles in culture can be described by circular statistical parameters, such as mean direction and length of the mean vector, in an analogous manner to linear statistical parameters mean and variance. For both linear and circular parameters, the mean refers to the expected value of a random variable. Length of mean

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vector and variance are both measures of the spread of the data, where the variance represents the average squared deviation from the mean, and length of mean vector is an inverse analogue of the variance. Circular variables have values that fall along a circle and hence have specific properties related to the cyclic nature of the circular scale. The application of these methods to neurite direction is analogous to the application of population biology measures to cellular function.

Statistical analysis of circular variables differs from analysis of linear variables as there are several properties of circular variables that need to be taken into account. Because circular variables are finite and closed when a circular dataset comes back on itself (at 0° and 360°), the zero direction, the designation of magnitude, and the number and size of groups (in the case of grouped data) are arbitrary. In addition, the mean angle of orientation cannot be found by the simple summation of measured values and division by the sample size. The sums of circular variables must be taken either modulo 360° if the sample is circular, or taken modulo 180° if the sample is axial, i.e. where data occur as an undirected line as in the example of geological fractures (Tran, 2007). For axial data in the present study, there is symmetry about the *y*-axis hence there is no distinction between the north–south directions. Analysis for linear variables approximates randomness by using a Poisson distribution; this distribution does not translate to circular variables. In circular statistics, the null hypothesis describing a random distribution is taken to be a uniform distribution, where all directions may occur at equal probability, approximating randomness and reflecting the finite closure of a circle (Fisher, 1993).

Neurite outgrowth angles are generally simple distributions, requiring display of data and summary of a single random sample usually with single or bimodal groups. As such, a null hypothesis of uniformity and randomness is generally appropriate, with the objective to assess the uniformity of a given distribution of neurite angles cultured in different environmental conditions. When the comparison of two or more samples of neurites cultured in different conditions is of interest, regression analysis and statistical models may be useful for description and prediction of cell response. Circular statistical methods complement traditional linear statistical methods to describe and draw inferences about the population of neurons and neurites being studied (Batschelet, 1981; Fisher, 1993). We propose in this study that in many cases, circular statistical methods allow us to more robustly describe the complexity of neurite outgrowth phenomena.

In this work, we employed circular statistical models to evaluate directional growth in a variety of representative *in vitro* neurite outgrowth assays. Multiple statistical methods were used to evaluate *in vitro* neurite outgrowth ranging from Gaussian based models and non-parametric methods to hypothesis testing for circular samples. Here we report a comparison of circular and linear data presentation and statistical methods for evaluation of several types of neurite outgrowth patterns.

2. Methods

2.1. Substrate preparation

Three types of substrates were used to evaluate the use of circular statistical methods on directionality of neurite outgrowth: adsorbed uniform protein coating on glass, adsorbed protein stripes and adsorbed protein gradients. Uniform protein coating was performed by incubating protein solution for 1 h on acid washed glass coverslips, washing twice with sterile water and air drying.

Micropatterned laminin (LN, 50 μg/mL) and chondroitin sulfate proteoglycans (CSPG, 10 μg/mL) stripes of 10-mm length, 50-μm width and 50-μm pitch were stamped onto glass coverslips via micro-contact printing techniques as described in Bruder et al. (2006). Briefly, grooved polydimethyl siloxane (PDMS) stamps fabricated using the method described in Goldner et al. (2006), were submerged in 10% sodium dodecyl sulfate in deionized water, rinsed in water, and incubated with 50 μg/mL mouse LN in Hank's balanced salt solution without calcium or magnesium (HBSS-CMF) for 1 h. Glass coverslips were plasma activated with a plasma cleaner/sterilizer (PDC-32 G, Medium RF level, 1 min, in air), and incubated in contact with stamps overnight to achieve adsorbed alternating stripes of either LN or CSPG.

Protein gradients were generated with the use of a microfluidic gradient mixer, fabricated using soft lithography techniques in a modification of the method of Dertinger et al. (2002), described in Li et al. (2008). Briefly, the gradient mixer pattern was designed in AutoCAD and transferred to a silicon wafer using photolithography. Using the silicon wafer as a template and PDMS as an elastomeric replica, soft lithography was used to fabricate the gradient mixer. The polymeric gradient mixer and a glass slide were irreversibly bonded by plasma activation of both surfaces for 1 min. Single cue gradients of LN or CSPG opposite bovine serum albumin (BSA, 3%, a neutral molecule for neurite guidance), were generated as substrates to evaluate neurite directionality. Protein solutions of LN or CSPG and BSA were pumped through the gradient mixer, at 0.2 μL/min and allowed to interdiffuse and adsorb overnight. The glass substrates containing the adsorbed protein gradients were used as the substrates on which to culture dorsal root ganglia (DRG) neurons.

2.2. Cell culture

DRG were dissected from the spinal columns of postnatal (P0–P4) rat pups and cleaned of axons, blood, and connective tissue. DRG were incubated in 0.05% trypsin–EDTA in HBSS-CMF at 37 °C for 60 min and dissociated by trituration. Cells were plated onto substrates in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin with 50 ng/mL nerve growth factor (NGF). Cells were seeded at a density of 100,000 cells/mL, on uniformly coated glass or micropatterned substrates and 12,500 cells/mL on gradient substrates. Phase contrast microscopy using a 10× objective was performed using a Nikon Eclipse TE2000-S, and images were captured with Hamamatsu-ORCA outputting to Openlab v.4.05 after 24 h in culture.

2.3. Image analysis

To evaluate direction of neurite outgrowth on uniform substrates and micropatterned protein stripes, the angles of all neurites in at least six fields of view were measured as the angle between the vector from the cell body to the tip of the neurite and the vertical axis (0°, Fig. 1D), using the measure tool in OpenLab software on phase contrast images. To evaluate directional bias of neurites on gradient substrates, the angles (θ) of the longest neurites of all neurons adhered to the gradient channel were measured as described above.

2.4. Linear statistical analysis

Linear descriptive statistics such as mean and standard deviation were calculated by equations described in Table 1. Conventional statistical tests were performed using SPSS 14. Linear probability density functions were tested against circular data as comparison (SPSS 14; listed in Table 2). For the χ^2-test, the angle data was grouped into three groups: neurites growing towards the left (210–330°), right (30–150°) and vertical (0–30°, 150–210°,