Published in final edited form as:

J Neurosci Methods. 2014 May 15; 228: 100–109. doi:10.1016/j.jneumeth.2014.03.006.

Semi-automatic quantification of neurite fasciculation in high-density neurite images by the Neurite Directional Distribution Analysis (NDDA)

Amy M Hopkins^a, Brandon Wheeler^a, Cristian Staii^b, David L. Kaplan^a, and Timothy J. Atherton^b

Amy M Hopkins: Amy.Hopkins@Tufts.edu; Brandon Wheeler: Brandon.Wheeler@Tufts.edu; Cristian Staii: Cristian.Staii@Tufts.edu; David L. Kaplan: David.Kaplan@Tufts.edu; Timothy J. Atherton: Timothy.Atherton@Tufts.edu

^aDepartment of Biomedical Engineering, Tufts University Science & Technology Center, 4 Colby Street, Medford, MA 02155, USA

^bDepartment of Physics and Astronomy, and Center for Nanoscopic Physics, Tufts University Science & Technology Center, 4 Colby Street, Medford, MA 02155, USA

Abstract

Background—Bundling of neurite extensions occur during nerve development and regeneration. Understanding the factors that drive neurite bundling is important for designing biomaterials for nerve regeneration toward the innervation target and preventing nociceptive collateral sprouting. High-density neuron cultures including dorsal root ganglia explants are employed for *in vitro* screening of biomaterials designed to control directional outgrowth. Although some semiautomated image processing methods exist for quantification of neurite outgrowth, methods to quantify axonal fasciculation in terms of direction of neurite outgrowth are lacking.

New Method—This work presents a semi-automated program to analyze micrographs of high-density neurites; the program aims to quantify axonal fasciculation by determining the orientational distribution function of the tangent vectors of the neurites and calculating its Fourier series coefficients ('c' values).

Results—We found that neurite directional distribution analysis (NDDA) of fasciculated neurites yielded 'c' values of ~ 0.25 whereas branched outgrowth led to statistically significant lesser values of $< \sim 0.2$. The 'c' values correlated directly to the width of neurite bundles and indirectly to the number of branching points.

Comparison with Existing Methods—Information about the directional distribution of outgrowth is lost in simple counting methods or achieved laboriously through manual analysis.

© 2014 Elsevier B.V. All rights reserved.

Corresponding author: Amy M Hopkins, amy.hopkins@tufts.edu, +1 (617) 627-2580, Department of Biomedical Engineering, Tufts University Science & Technology Center, 4 Colby Street, Medford, MA 02155, USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The NDDA supplements previous quantitative analyses of axonal bundling using a vector-based approach that captures new information about the directionality of outgrowth.

Conclusion—The NDDA is a valuable addition to open source image processing tools available to biomedical researchers offering a robust, precise approach to quantification of imaged features important in tissue development, disease, and repair.

Keywords

Image processing; axonal bundling; fasciculation; axonal alignment; axon branching; neurite quantification methods; dorsal root ganglia

1.1. Introduction

Research over the last three decades has focused on elucidating the mechanisms regulating neurite bundling, or fasciculation, and defasciculation. Fasciculation events are critical in axon guidance where axons bundle to travel long distances, after initial outgrowth and formation of a growth cone, before branching to reach their target and forming synapses (Barry et al., 2010; Crossin and Krushel, 2000; Wiencken-Barger et al., 2004). Bundling of neurites is mediated by cell adhesion molecules (CAMs), which allow them to bind together, and defasciculating agents such as polysialic acids (PSAs) (Barry et al., 2010; Fu et al., 2006; Voyiadjis et al., 2011). For example, Rutishauser et al. (1988) observed that in regions of neural cell adhesion molecule (NCAM) expression and low PSA, there are increased adhesion and contact-dependent events (Rutishauser et al., 1988). In contrast, with increased amount of PSA, cell-cell interactions are inhibited (Rutishauser et al., 1988).

Fasciculation is critical to axonal pathfinding during both development (Favero et al., 2013; Georgiev et al., 2008; Haupt et al., 2010; Yanicostas et al., 2009) and following neuronal injury (Hoschouer et al., 2009; Kubasak et al., 2005; Lin et al., 2012; Martini and Schachner, 1988). Beasley & Stallcup (1987) reported that NCAM was present on undifferentiated cells in the germinal zones and on differentiating cells in nerve fiber tracts in the rat spinal cord, hindbrain, forebrain, retina, and cerebellum, whereas the nerve growth factor-inducible large external glycoprotein was restricted to the developing fiber tracts in these tissues (Beasley and Stallcup, 1987). Mutations or knock down of NCAM or L1 (a prevalent neural CAM involved in fasciculation (Crossin and Krushel, 2000)) has been shown to disrupt fasciculation of neurites in early postnatal mouse cerebellar cortex microexplant cultures (Fischer et al., 1986), rat hippocampal neurons (Barry et al., 2010), and in humans (Kamiguchi et al., 1998). Post injury, regenerating axons in the peripheral nervous system re-express or upregulate L1 and maintain these levels during regrowth (Kubasak et al., 2005; Martini and Schachner, 1988; Yu and Shoichet, 2005).

Investigation of neurite fasciculation during regeneration is valuable for the development of biomaterial nerve guide conduits assisting re-innervation. A large percentage of spinal cord injury patients suffer from neuropathic pain symptoms that may be caused by aberrant sprouting and reorganization of injured sensory afferent fibers (Hoschouer et al., 2009). Chicken or rat dorsal root ganglion (DRG) explants are frequently employed to model developing or regenerating sensory nerve fibers for basic neuroscience studies (Fang et al.,

2012; Schwartz and Spirman, 1982; Yoshikawa et al., 2008) or development of new therapeutic materials and devices (Conovaloff and Panitch, 2011; Freier et al., 2005; Madduri et al., 2010; Schnell et al., 2007; Wang et al., 2010; Wieringa et al., 2010a; Wieringa et al., 2010b; Yu and Shoichet, 2005).

Our group recently investigated chick embryo DRG outgrowth on silk hydrogels as soft substrates for neural tissue engineering (Hopkins et al., 2013). Due to observations of differential fasciculation on various silk hydrogel substrates, we became interested in quantifying the degree of axonal bundling. However, we encountered a limited analysis toolset to quantify fasciculation. The most common method to observe DRG neurite outgrowth is optical microscopy (brightfield or fluorescence). Fasciculation of neurite outgrowth of DRG explants is most identifiable in images by: 1) increased width of bundling neurite extensions, 2) few branching points, 3) low tortuosity, and 4) radial neurite alignment emanating from the explant body. Basic manual tracing tools, such as those offered by the *ImageJ* software (National Institutes of Health, Bethesda, MD) and the Sholl analysis method (Sholl, 1953), are the most common image processing methods employed for analysis of neuron morphology. *ImageJ* has applications for quantification of number of cells (nuclei), axon number and width/length, growth cone area, and alignment, and is the most widely employed method for general analysis of neural cell morphology (Daud et al., 2012; Meng et al., 2012; Thelen et al., 2012; Zhou et al., 2012). The Sholl analysis is another frequently employed image processing method that quantifies neurite branching as a function of distance from the cell body (Sholl, 1953). Although these manual methods provide robust analysis of two of the identifiable features of fasciculation in images (width of bundles and extent of branching), quantification of the directional distribution of neurites would contribute new data to axonal bundling studies that could potentially reveal new information about underlying biological processes. A recent focus has been to develop image-processing tools capable of automatically tracing individual neurites and identifying branches using techniques such as steerable filters (Meijering et al., 2004; Schmitza, 2011). These are very effective if the neurites in the image are sufficiently isolated so that few neurites overlap; however for the DRGs studied in this work this assumption is not valid, and the issue of identifying all individual neurites for such images remains an important open problem. Our proposed technique therefore aims to calculate experimentally relevant geometric properties of the neurites that do not require their connectivity to be fully reconstructed.

A full range of complex quantification techniques exist for relating experimental neurite events to fundamental models of behavior. In the biophysics field for example, many scientists have investigated mathematical models to describe growth cone guidance of axonal growth. Earlier models (e.g. those presented by Goodhill et al.) sought to explain axonal guidance through the observation that both the target and axon (as well as surrounding extracellular matrix) provide attractive and repulsive signaling molecules to the searching growth cone (Goodhill, 1997). Therefore, through investigation of diffusion rates and geometries of growth factor gradients at various distances from the extending axon, one can begin to describe guidance observed under specific conditions (Alenghat and Ingber, 2002; Anton et al., 1999; Jin and Varner, 2004; Wehrle-Haller and Imhof, 2003). This

method evolved into a complex model that simulated local growth factor concentration by applying a continuum model that incorporated many biophysical factors influencing outgrowth (i.e. factor emission from the soma, percent receptors bound, energy supplied to axon, etc.) (Segev and London, 2000). Albeit lacking significant factors (i.e. electrical stimulation), this model successfully simulated a self-wiring network. A more sophisticated analysis yet, Betz et al. investigated the stochastic nature of the growth cone advancement and retraction by solving the Fokker-Planck equation (Beighley et al., 2012; Betz et al., 2006). Although such mathematical modeling is critical for connecting experimental observations with fundamental models of neuronal growth processes, they require significant analysis of single events and are not suitable for high-throughput image processing.

The present work aims to supplement current manual image processing tools with a semiautomated program to quantify axonal fasciculation. The proposed technique samples the orientational distribution of the set of tangent vectors of the neurites directly from images allowing the user to obtain an attractive geometric quantity since its determination does not require a full reconstruction of paths of individual neurites, but is more naturally predicted by fundamental models of neuronal growth (Betz et al., 2006; Pearson, 2011). We apply the presented method to neurite outgrowth of chicken embryo DRGs on our previously characterized platform of silk hydrogels (Hopkins et al., 2013). The image analysis presented was written in *Mathematica* and is based on biophysical observations that increased fasciculation results in neurite outgrowth from circular DRG explants in the radial direction, with less branching and tortuosity. As provided in detail below, quantification of fasciculation by the neurite directional distribution analysis (NDDA) method is described by the orientation distribution function (ODF) of vectors tangent to extending neurites. The method allows the user to provide an image, adjust a single parameter that affects how the image is binarized, select a region of interest and verify that the program has correctly detected the desired neurites; typically this process might take a user 2–3 minutes per image. We show that this quantification method results in a rich dataset that supplements existing methods including the Sholl analysis (Sholl, 1953) (quantification of branching) and manual *ImageJ* computations (neurite width).

1.2. Materials and methods

1.2.1. Silk hydrogel substrate preparation

Silk fibroin hydrogels were formed through ultrasonication of aqueous silk solution extracted from *Bombyx mori* silkworm cocoons as previously described (Hopkins et al., 2013). Briefly, silk solutions were prepared for hydrogel formation by diluting with 10x DMEM (Dulbecco's Modified Eagle Medium, Gibco, 12100-046) and dH₂O to obtain a solution of 4% silk concentration and 1x DMEM. Neurotrophin-3 (NT-3, 40ng/mL, Sigma-Aldrich, SRP3128) and for specific samples fibronectin (FN, Sigma-Aldrich, F2006) or laminin (LN, Invitrogen, 23017-015) were added to the silk solution after sonication before gelation. For further characterization of silk hydrogels in regards to gelation, morphology, and neural cell culture, readers are directed to earlier works of Kim et al. (2004), Hu et al. (2010), and Hopkins et al. (2013) (Hopkins et al., 2013; Hu et al., 2010; Kim et al., 2004).

1.2.2. cDRG isolation and culture

cDRGs were isolated and seeded as described in (Hopkins et al., 2013). Briefly: cDRGs were isolated from E8-9 chick embryos as previously reported by (Dichter & Fishbach, 1977) and in accordance to Tufts ICAUC regulations. Chick embryos were removed from eggs, decapitated, and immobilized on a PDMS microsurgery stage. Once the ventral organs were eviscerated, cDRGs were removed from either side of the spinal column and stored in HBSS (Hank's Buffered Saline Solution, Gibco, 14170120) supplemented with dextrose (6 mg mL⁻¹) and NaHCO₃ (0.35 mg mL⁻¹) for up to 3hrs until seeding. Excess tissue and residual roots were cleaned prior to seeding on hydrogels. Explants attached to the gel within 1h prior to addition of media (2% FBS DMEM, Glutamax, high glucose, (-) sodium pyruvate, 1% PenStrep). cDRGs were cultured on hydrogels for 4 days prior to 10% formalin fixation, immunostaining and fluorescent microscopy imaging.

1.2.3. Immunocytochemistry and fluorescent microscopy

After overnight fixation with 10% formalin, samples were washed with PBS, blocked with 4% normal goat serum (NGS, Sigma-Aldrich, G9023), permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, X100), and incubated with anti- β -tubulin III primary antibody produced in rabbit (Sigma-Aldrich, T2200) at 4°C overnight. The next day, samples were washed 2–3x for 10–15 min while shaking before staining with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, A-11008) shaking at room temperature for 40 min. Samples were washed thoroughly again with PBS prior to imaging with an inverted fluorescent microscope (Leica Microsystems, IL, USA).

1.2.4. NDDA through application of the ODF for quantification of axonal fasciculation

Prior to image processing by the NDDA method, images were modified in *Adobe Photoshop* (Waltham, MA) or *ImageJ* (National Institutes of Health, Bethesda, MD) to eliminate fluorescence background and artifacts. Images were then imported into the NDDA program in *Mathematica 8* (Wolfram, Somerville, MA). The program (see supplementary material) was then initiated and the user was required to input thresholding, identification of center-point, local radial boundaries, and desired concentric annuli dimensions as described in detail below. Analysis results were exported as pdf images (histograms, images) and to *GraphPad Prism 5* ('c' values, La Jolla, CA). Absolute 'c' values were used for analysis. Statistical analysis was applied by *Prism* including two-way ANOVA and student's t-tests. Replicates were n = 10 for fasciculated samples and n = 11 for branched samples. Statistical significance is reported along with results.

1.2.5. Sholl analysis

For application of the Sholl analysis, we employed the ShollAnalysis *ImageJ* plug-in written by the Ghosh laboratory, which counts the number of intersections of neurites as a function of distance from the cell soma or explant (Aizawa et al., 2004; Chen et al., 2005; Ghosh, 2005). Immunofluorescent images were imported into *ImageJ*, the scale was set accordingly, and inner and outer radius of explant body was measured using the line segment tool. The center of outgrowth (i.e. the center of the explant body) was selected and the ShollAnalysis plug-in was run after inputting the following parameters: radius step size = (outer radius)/4;

radius span = radius step size; inner radius = measured radius + (radius step size)/2; outer radius = (measured outer radius) + (radius step size)/2. These parameters were chosen in order to divide analysis into 4 concentric annuli analogous to the NDDA program. The inner and outer radii were offset in order to eliminate data collection of the explant body. Results were input into *GraphPad Prism 5* software (La Jolla, CA) for statistical analysis (two-way ANOVA).

1.2.6. Manual ImageJ analysis of DRG neurites

The width of neurite bundles was measured manually in *ImageJ* using the segmented line tool. Sixteen randomly selected neurites were measured per image. Results were imported into *Prism 5* software for plotting and statistical analysis (t-test, two-way ANOVA).

1.3. Theory and Calculations

This program was written in *Mathematica 8* (Wolfram Research, Champaign, IL) by one of the authors (TJA). The program is intended to operate on images of neurites. Extraneous objects, such as dye aggregates, in the image were removed from the analysis either by setting a region of interest, or alternatively using *Adobe Photoshop* (Waltham, MA) (Figure 1a). The program automatically removes the image background through subtraction of the image convolved with a wide 200 pixel Gaussian function; the user must select a binarization threshold depending on the illumination conditions (Figure 1b). The program calculates the distribution function of the tangent vectors of the neurites as follows: first, the image is divided into blocks of 10 pixels (the size is adjustable). For each block, the program uses *Mathematica*'s *ImageLine* function to calculate the Radon transform and identify its maxima to identify line elements present in the block. The user may specify the threshold that maxima in the Radon transform must exceed for a line to be detected. The Radon transform is a standard, well-established image processing technique for line detection; further details are to be found in standard textbooks (e.g. (Deans, 1983)). The result of these calculations is a set of tangent vectors $\hat{a}_i$ sampled from the image. The program displays all these detected samples overlaid on the original image as a cross-check, permitting the user to make adjustments if required (Figure 1c). Regions of interest can be selected by defining a local radial frame to exclude artifacts such as atypical growth or neurites that have lifted from the substrate (Figure 1d). Once the radial tangent vectors are identified, the ODF is determined as follows:

The ensemble of tangent vectors is initially sampled with respect to the image axes $\hat{x}$ and $\hat{y}$; this can be automatically transformed into an arbitrary user-specified coordinate system and associated pair of reference directions $\hat{x}$ and $\hat{y}$. For example, where neuronal growth is directed by a surface pattern (Beighley et al., 2012), the reference coordinate system $(\hat{x}, \hat{y})$ can be aligned with the pattern and not the image axes. The angle n_i that each sampled tangent vector $\hat{a}_i$ makes with the reference direction $\hat{x}$ is defined

$$\cos\theta_i = \hat{a}_i \bullet \hat{x}.$$

The ensemble of measured angles is sampled from a continuous orientational distribution function:

$$P(\theta)d\theta \quad (1)$$

Here, $P(\theta)$ is the probability that a randomly selected unit vector has an orientation that lies between θ and $\theta + d\theta$. Considering two limiting cases allows for further explanation of the function: if all vectors are precisely aligned with n , $P(\theta)$ becomes the Dirac delta function $\delta(\theta)$, while for completely random isotropic alignment, $P(\theta)$ is constant on the interval $[-\pi, \pi]$. In the present work, we study the sign-independent orientational distribution constructed by identifying $-\hat{a}_i$ with $\hat{a}_i$, so that the range of theta is restricted to $[-\pi/2, \pi/2]$.

Several meaningful scalar measures of alignment can be constructed from the distribution $P(\theta)$, including for example a full-width half-maximum. However, for distributions that may not conform to narrow Gaussian distributions, a particularly useful class of measures is obtained by expanding $P(\theta)$ in orthogonal functions

$$P(\theta) = c_0 + \sum_n (c_n \cos(2n\theta) + d_n \sin(2n\theta)). \quad (2)$$

For isotropic alignment, as discussed above, $c_n = d_n = 0$ for $n \neq 0$ and $c_0 = 1/\pi$. From a set of N measured angles θ_i , one can obtain the appropriate coefficients by requiring

$$P(\theta) = (1/N) \sum_i \delta(\theta - \theta_i), \quad (3)$$

equating

$$c_0 = 1/\pi, \quad c_n = 2/(N\pi) \sum_i \cos(2n\theta_i), \quad d_n = 2/(N\pi) \sum_i \sin(2n\theta_i). \quad (4)$$

The coefficients comprise the successive moments of the distribution function (1) and are nonzero if there is any degree of alignment. Similar measures of alignment are widely used in fields such as polymer chemistry (Ayres et al., 2006; Ayres et al., 2008) and condensed matter physics to quantify alignment (de Gennes 1995). They therefore can be used to quantify fasciculation by the ODF of the space of tangent vectors of axonal trajectories.

The program outputs both the distribution function and the above measures of alignment, as shown in Figure 2. It is straightforward to choose the reference vector n to any unit vector field of interest; here because we are interested in the radial alignment, n is chosen to be the radial unit vector from the center of the explant body. The first output is a histogram displaying the number of tangent vectors from $\pi/2$ to $\pi/2$ relative to the radial tangent vector n (Figure 2a). Greater neurite fasciculation correlates to a greater number of tangent vectors aligned with the radial tangent vector than in other directions (Figure 2a,b). This data can also be examined as a function of distance from the explant body by defining dimensions of concentric annuli for analysis (Figure 2b,c). Spatial resolution of fasciculation is useful for understanding determination factors of branching events. Therefore, 'c' values (i.e. the

nonzero coefficients of the cosine term of the Fourier transform (4) above) can be reported as both an averaged value representing entire outgrowth, or as a function of distance from the explant body (Figure 2c).

1.4. Results

1.4.1. Quantification of fasciculation based on nonzero cosine coefficients of the Fourier transform of the ODF ('c' values)

We found that fasciculated DRG outgrowth yielded an overall 'c' value of 0.254 ± 0.017 whereas branched outgrowth led to statistically significant lesser overall 'c' values of 0.174 ± 0.024 (Figure 3a). Error represents standard deviation of 'c' value measurements from the mean across sampled alignment distributions. Spatially resolved analysis revealed statistically significant differences in 'c' values between branched and fasciculated DRGs (Figure 3b,c). Mean 'c' values of spatially resolved analyses of 11 and 10 branched vs. fasciculated samples, respectively, were 0.265 ± 0.030 vs. 0.325 ± 0.036 for the annulus closest to the cell body (Figure 3c, c1), 0.247 ± 0.034 vs. 0.324 ± 0.041 for the second annulus (Figure 3c, c2), 0.164 ± 0.034 vs. 0.306 ± 0.023 for the third annulus (Figure 3c, c3), and 0.196 ± 0.028 vs. 0.259 ± 0.033 for the fourth annulus (Figure 3c, c4). This data showed that under the current culture conditions, fasciculation does not decrease significantly with distance from the explant body in highly fasciculated samples as compared to branched samples, which do exhibit a significant decrease in 'c' value between c1/c2 and c3/c4 (Figure 3c). Less fasciculation (lower 'c' values) correlates to a more uniformly distributed histogram (Figure 3bi) whereas greater fasciculation (higher 'c' values) exhibits a Gaussian distribution (Figure 3bii).

1.4.2. NDDA complements manual ImageJ width measurements and Sholl analyses of DRG fasciculation

The Sholl analysis is frequently employed for analysis of growth cone guidance and for quantitation of branching as a function of distance from the cell body (Sholl, 1953). Here, we applied the Sholl analysis to DRG explant outgrowth. Additionally, we manually measured the width of neurite bundles, which was expected to increase with increasing fasciculation. Figure 4 presents two cases (one branched, Figure 4ai, and one fasciculated, Figure 4aai) that were analyzed in regards to the Sholl method (Figure 4bi), the NDDA method (Figure 4bii), and axon bundle width (Figure 4c). These comparisons show expected results of greater neurite branching leading to greater number of intersections and decreased neurite thickness (Figures 4b–c). The numbers of intersections quantified by the ShollAnalysis plugin were significantly higher in the highly branched sample than in the fasciculated sample (Figure 4bi). The NDDA revealed the inverse relationship to the Sholl analysis with significantly higher 'c' values for the fasciculated case as compared to the branched sample (Figure 4bii). Finally, we found a positive correlation between the NDDA 'c' values and the width of the neurite bundles as measured in *ImageJ* (Figure 4c). This conclusion was expected since increased bundling of neurites occurs when a greater number of neurites extend closely together resembling a thicker "single" neurite. This behavior is contrasted to extensions with greater branching where bundling does not normally occur simultaneously with tortuous growth.

1.4.3. Comparison of quantitative methods for analysis of axonal fasciculation

Through firsthand experience and reported results, benefits and challenges to several analysis methods emerged. These are presented in Table 1 and discussed further below.

1.5. Discussion

There is a great need in the neurological systems engineering community for fast, automated image processing techniques that yield accurate, quantitative information lending insight on biological processes. Current manual and semi-automated neural analysis methods are either over-simplified or complex and cumbersome for the user. This results in sacrifice of either information or analysis time. The presented program is an intermediate of these two extremes, providing semi-automation with total processing time to 2–3 minutes per image and ease of use while also delivering a vast amount of qualitative (histographical) and quantitative (ODF-based) information regarding axonal bundling (Figure 2). Our long-term goal is to develop a toolkit of fully automated image analysis methods to deliver rich datasets that not only inform users of classical morphological characterization, but are also of functional significance.

Plugins executed within *ImageJ* offer less tedious options while still providing robust analysis one image at a time. NeuronJ is a semi-automatic neurite tracing technique written by Eric Meijering that reduces labor intensiveness by reducing the number of clicks required for neurite tracing (Meijering et al., 2004). NeuronJ computes basic metrics including max neurite length, number of neurites per neuron, and percentage of neurite bearing neurons (Daud et al., 2012), but is limited to two-dimensional images of 8-bit gray-scale or indexed color and may not be accurate when analyzing high cell/neurite density images. The Sholl analysis has also recently been semi-automated by an *ImageJ* plugin created by the Ghosh Laboratory at the University of California, San Diego (Aizawa et al., 2004; Chen et al., 2005; Ghosh, 2005). The Sholl analysis was originally described by Donald Arthur Sholl in 1953 in his publication describing dendritic organization in the neurons of cat cortices (Sholl, 1953). This program computes the number of intersections in concentric annuli as a function of increasing distance from the neuron soma and has been used for decades to study dendrite development and morphology under various conditions (Aizawa et al., 2004; Alcantara-Gonzalez et al., 2012; Chen et al., 2005; Chiang et al., 2011; Milosevic and Ristanovic, 2007; Ristanovic et al., 2006; Schoenen, 1982; Sholl, 1953; Torres-Garcia et al., 2012). Finally, neuroscientists and biomedical engineers have occasionally borrowed from fiber orientation analysis to apply Fast Fourier Transform (FFT) to analyze neurite alignment (Ayres et al., 2006; Ayres et al., 2008; Levitt et al., 2007; Sander and Barocas, 2009; Sivaguru et al., 2010; Voyiadjis et al., 2011). Although the Oval Profile plugin (authored by William O'Connell (Ayres et al., 2006; Ayres et al., 2008)) for *ImageJ* is semi-automatic, customized scripts (i.e. those written in Matlab) are required for production of high-resolution FFT output images and meaningful quantification is difficult due to high contribution of background fluorescence yielding poor signal-to-noise ratios.

To our knowledge, the NDDA method is the first image-processing program to quantify fasciculation based on tangential alignment. Understanding directional distribution of neurite extension is important for growth cone guidance studies as well as gaining

appreciation for the likelihood of reaching the innervation target. This information is lost in simple counting methods and is otherwise achieved laboriously through manual analysis. Furthermore in the NDDA, the reference direction n is not constrained to the radial direction, nor does it necessarily need to be spatially invariant. In the current case, it was desirable to choose the radial vector over all distances since the starting condition included a symmetrically round sphere of cells whose dominant outgrowth pattern is in the radial direction. However, the reference direction can easily be applied toward an increasing gradient of growth factor or other patterned molecule / surface feature, for example, thereby extending the NDDA method to the growth cone guidance community in particular as well as other studies of axonal alignment.

As Figure 3 demonstrates, the NDDA successfully distinguishes between changes in overall fasciculation (Figure 3a) as well as growth as a function of distance from the explant body (Figure 3b–c). We observed that although many samples with high levels of branching experienced $c1-2$ values ~ 0.25 similar to those of more fasciculated phenotypes (~ 0.32), ‘ c ’ values of the branched samples decreased significantly with increasing distance from the explant body whereas ‘ c ’ values of fasciculated samples remained high (Figure 3c). These results are expected in the context of DRG outgrowth since neurites in close proximity near the explant body may have greater opportunity to interact and bundle versus points further away that may experience a greater variety of directional cues from many sources. ‘Fasciculated’ versus ‘branched’ growth was determined visually in these studies since fasciculation occurred under several different cues including fibronectin and laminin. Since it is still in question whether neurites come together due to random movement of individual axons, repulsive and attractive signals from neighboring cells/neurites, or traction-like forces exerted by filopodia on each other, an analysis method able to spatially resolve branching events allows for the opportunity to elucidate these mechanisms (Hentschel and van Ooyen, 2000).

We also found that the NDDA provides supportive and complementary information to other metrics for fasciculation assessment including the number of intersections (i.e. the Sholl analysis) and widths of neurite bundles. In this way, the NDDA contributes directional distribution information to a previous dataset based solely on growth dimensions. Biological variability among samples (e.g. number of cells per explant, shape of explant body, number of adherent cells per sample, etc.) as well as procedural variation (e.g. immunostaining technique, image settings, etc.) can skew conclusions based on these measurements since they are purely scalar in nature.

In addition to contributing complementary information to manual *ImageJ* and Sholl analyses, the NDDA method has several benefiting factors as summarized in Table 1. Firstly, the NDDA method does not require a binary or high-resolution image such as the ShollAnalysis plug-in. We found this to be a significant advantage since even low levels of background fluorescence as a result of the hydrogel substrate can negatively affect binary conversion and skeletonization. Whereas the majority of our images could be analyzed by NDDA, only a select few (most at higher magnification) could yield meaningful results from the Sholl method (data not shown).

The NDDA method also successfully analyzes images of neurites at varying magnifications, fluorescence intensities, and cell densities without loss of precision or accuracy. This can be appreciated through comparison of NDDA results of 4x images in Figure 3 (average overall 'c' values of 0.174 and 0.254 for branched and fasciculated samples, respectively) to 10x images in Figure 4 (overall 'c' values of 0.121 and 0.217 for branched and fasciculated samples, respectively). The ability to process high densities of neurites allows for the user to obtain valuable information of growth trends otherwise obtained through laborious processing or lost through methods requiring individual neurite resolution. NDDA also does not require ideal signal:noise ratio due to its forgiving line detection techniques which find maxima in the Radon transform of small subsets of the image. This allows for images taken at separate time points and different experiments to be compared collectively. The increased sensitivity of the edge-detection method also allows for accurate analysis across images of varying neurite density.

The NDDA method also allows for studying arbitrary subsets of an image, such as annuli. This feature avoids contamination of results by artifacts caused by excessive background, dye aggregates, or perturbed samples (Figure 1), and allows for maximum extraction of meaningful data from images with poorer resolution (such as those whose definition is lost through conversion to binary). Although images can be modified to eliminate some of these artifacts before analysis, it is faster and more convenient to define a region of interest during the analysis procedure if need be. Furthermore, some artifacts such as tissue that has lifted from the substrate during washing steps are difficult to remove through image processing.

The ability to analyze an image without significant modification of the image beforehand can also significantly decrease processing time. Methods that require skeletonization must sometimes be traced by hand with programs such as *NeuronJ* or *NeuriteTracer* before analysis. Often, large areas of high fluorescence (such as seen in the DRG explant bodies) must be removed prior to image processing. These preparation steps can significantly increase processing time per image, decreasing one of the main attractions of semi-automated analysis methods. In the NDDA, the ability to slightly modify the images in regards to fluorescence thresholding and radial boundary limitations within the program sequence greatly increases accuracy without sacrificing processing time.

The main objective of image analysis is to gain understanding of the underlying biological mechanisms governing the observed growth changes. Therefore, the relationships among the analysis method, resulting data, and conclusions about the biological condition are critical to understand. Although the ODF of the tangent vectors fully characterizes the "bias" of random walk models and provides a natural connection between stochastic and continuum approaches as discussed in the introduction, we have yet to explore questions with biological basis through employment of the NDDA. We have shown in the present work that moments ('c values') of the orientational distribution function of tangent vectors as a quantitative measure of neurite morphology are able to distinguish between visually discernable fasciculated and non-fasciculated explants. These quantities should be able to be calculated by existing programs that trace individual, isolated neurites (Meijering et al., 2004; Schmitza, 2011). There are also natural quantities that might be predicted from fundamental models of neuronal growth and we are presently working on such a model incorporating

fasciculation to enable correlation of growth processes with these observed parameters. We also aim to generalize to neuronal guidance in three dimensions, where the presented NDDA can be modified to assess the set of spherical harmonics. Finally, we expect the NDDA to translate directly to quantifiable biological phenomena in other areas of regenerative medicine such as blood vessel tortuosity and molecular diffusion through the extracellular space. These studies will further illustrate the strength and robustness of the NDDA method for quantification of imaged features important in tissue development, disease, and repair.

1.6. Conclusions

The present work reports a novel semi-automated image processing method for the quantification of neurite bundling. The NDDA method is based on the distribution of vectors tangent to neurites in fluorescent micrographs. The method does not require pre-processing and provides normalized quantification across micrographs of various fluorescence intensities, magnifications, and resolutions since it does not rely on scalar counting methods. The NDDA successfully discerned between DRG outgrowth that was highly branched or fasciculated both in terms of averaged and spatially resolved directional distributions. The NDDA is a valuable addition to open source image processing tools available to biomedical researchers with potential to expand further to 3D image representations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the NIH P41 Resource Center (EB002520) and AFIRM for support of this work.

References

- Aizawa H, Hu SC, Bobb K, Balakrishnan K, Ince G, Gurevich I, Cowan M, Ghosh A. Dendrite development regulated by CREST, a calcium-regulated transcriptional activator. *Science*. 2004; 303:197–202. [PubMed: 14716005]
- Alcantara-Gonzalez F, Rebeca Mendoza-Perez C, Zaragoza N, Juarez I, Enrique Arroyo-Garcia L, Gamboa C, De la Cruz F, Zamudio S, Garcia-Dolores F, Flores G. Combined administration of cerebrolysin and donepezil induces plastic changes in prefrontal cortex in aged mice. *Synapse*. 2012; 66:938–49. [PubMed: 22826038]
- Alenghat FJ, Ingber DE. Mechanotransduction: all signals point to cytoskeleton, matrix, and integrins. *Science's STKE : signal transduction knowledge environment*. 2002; 2002:pe6.
- Anton ES, Kreidberg JA, Rakic P. Distinct functions of alpha(3) and alpha(v) integrin receptors in neuronal migration and laminar organization of the cerebral cortex. *Neuron*. 1999; 22:277–89. [PubMed: 10069334]
- Ayres C, Bowlin GL, Henderson SC, Taylor L, Shultz J, Alexander J, Telemeco TA, Simpson DG. Modulation of anisotropy in electrospun tissue-engineering scaffolds: Analysis of fiber alignment by the fast Fourier transform. *Biomaterials*. 2006; 27:5524–34. [PubMed: 16859744]
- Ayres CE, Jha BS, Meredith H, Bowman JR, Bowlin GL, Henderson SC, Simpson DG. Measuring fiber alignment in electrospun scaffolds: a user's guide to the 2D fast Fourier transform approach. *J Biomater Sci-Polym Ed*. 2008; 19:603–21. [PubMed: 18419940]
- Barry J, Gu Y, Gu C. Polarized targeting of L1-CAM regulates axonal and dendritic bundling in vitro. *European Journal of Neuroscience*. 2010; 32:1618–31. [PubMed: 20964729]

- Beasley L, Stallcup WB. The nerve growth factor-inducible large external (NILE) glycoprotein and neural cell-adhesion molecule (N-CAM) have distinct patterns of expression in the developing rat central nervous system. *J Neurosci*. 1987; 7:708–15. [PubMed: 3559708]
- Beighley R, Spedden E, Sekeroglu K, Atherton T, Demirel MC, Staii C. Neuronal alignment on asymmetric textured surfaces. *Applied Physics Letters*. 2012:101.
- Betz T, Lim D, Kas JA. Neuronal growth: A bistable stochastic process. *Physical Review Letters*. 2006:96.
- Chen YC, Wang PY, Ghosh A. Regulation of cortical dendrite development by Rap1 signaling. *Mol Cell Neurosci*. 2005; 28:215–28. [PubMed: 15691704]
- Chiang L-Y, Poole K, Oliveira BE, Duarte N, Sierra YAB, Bruckner-Tuderman L, Koch M, Hu J, Lewin GR. Laminin-332 coordinates mechanotransduction and growth cone bifurcation in sensory neurons. *Nature Neuroscience*. 2011; 14:993–U79.
- Conovaloff A, Panitch A. Characterization of a chondroitin sulfate hydrogel for nerve root regeneration. *Journal of neural engineering*. 2011:8.
- Crossin KL, Krushel LA. Cellular signaling by neural cell adhesion molecules of the immunoglobulin superfamily. *Developmental Dynamics*. 2000; 218:260–79. [PubMed: 10842356]
- Daud MFB, Pawar KC, Claeysens F, Ryan AJ, Haycock JW. An aligned 3D neuronal-glial co-culture model for peripheral nerve studies. *Biomaterials*. 2012; 33:5901–13. [PubMed: 22656449]
- Deans, SR. *The Radon Transform and some of its Applications*. John Wiley & Sons; 1983.
- Dichter MA, Fischbach GD. Action potential of chick dorsal root ganglion neurons maintained in cell-culture. *J Physiol-London*. 1977; 267:281. [PubMed: 559758]
- Fang L, Wang Y-N, Cui X-L, Fang S-Y, Ge J-Y, Sun Y, Liu Z-H. The role and mechanism of action of activin A in neurite outgrowth of chicken embryonic dorsal root ganglia. *J Cell Sci*. 2012; 125:1500–7. [PubMed: 22275431]
- Favero CB, Henshaw RN, Grimsley-Myers CM, Shrestha A, Beier DR, Dwyer ND. Mutation of the BiP/GRP78 gene causes axon outgrowth and fasciculation defects in the thalamocortical connections of the mammalian forebrain. *J Comp Neurol*. 2013; 521:677–96. [PubMed: 22821687]
- Fischer G, Kunemund V, Schachner M. Neurite outgrowth patterns in cerebellar microexplant cultures are affected by antibodies to the cell-surface glycoprotein-L1. *J Neurosci*. 1986; 6:605–12. [PubMed: 3512794]
- Freier T, Montenegro R, Koh HS, Shoichet MS. Chitin-based tubes for tissue engineering in the nervous system. *Biomaterials*. 2005; 26:4624–32. [PubMed: 15722132]
- Fu M, Vohra BPS, Wind D, Heuckeroth RO. BMP signaling regulates murine enteric nervous system precursor migration, neurite fasciculation, and patterning via altered Ncam1 polysialic acid addition. *Developmental Biology*. 2006; 299:137–50. [PubMed: 16952347]
- de Gennes, PG. *The Physics of Liquid Crystals*. Oxford University Press; 1995.
- Georgiev D, Taniura H, Kambe Y, Takarada T, Yoneda Y. A critical importance of polyamine site in NMDA receptors for neurite outgrowth and fasciculation at early stages of P19 neuronal differentiation. *Experimental Cell Research*. 2008; 314:2603–17. [PubMed: 18586028]
- Ghosh, A. Ghosh Lab Software. UCSD The Ghosh Lab; 2005.
- Goodhill GJ. Diffusion in axon guidance. *Eur J Neurosci*. 1997; 9:1414–21. [PubMed: 9240399]
- Haupt C, Kloos K, Faus-Kessler T, Huber AB. Semaphorin 3A-Neuropilin-1 signaling regulates peripheral axon fasciculation and pathfinding but not developmental cell death patterns. *European Journal of Neuroscience*. 2010; 31:1164–72. [PubMed: 20345923]
- Hentschel HGE, van Ooyen A. Dynamic mechanisms for bundling and guidance during neural network formation. *Physica A*. 2000; 288:369–79.
- Hopkins AM, De Laporte L, Tortelli F, Spedden E, Staii C, Atherton TJ, Hubbell JA, Kaplan DL. Silk Hydrogels as Soft Substrates for Neural Tissue Engineering. *Advanced Functional Materials*. 2013:1–10.
- Hoschouer EL, Yin FQ, Jakeman LB. L1 cell adhesion molecule is essential for the maintenance of hyperalgesia after spinal cord injury. *Exp Neurol*. 2009; 216:22–34. [PubMed: 19059398]

- Hu X, Lu Q, Sun L, Cebe P, Wang X, Zhang X, Kaplan DL. Biomaterials from Ultrasonication-Induced Silk Fibroin-Hyaluronic Acid Hydrogels. *Biomacromolecules*. 2010; 11:3178–88. [PubMed: 20942397]
- Jin H, Varner J. Integrins: roles in cancer development and as treatment targets. *Br J Cancer*. 2004; 90:561–5. [PubMed: 14760364]
- Kamiguchi H, Hlavin ML, Yamasaki M, Lemmon V. Adhesion molecules and inherited diseases of the human nervous system. *Annu Rev Neurosci*. 1998; 21:97–125. [PubMed: 9530493]
- Kim UJ, Park JY, Li CM, Jin HJ, Valluzzi R, Kaplan DL. Structure and properties of silk hydrogels. *Biomacromolecules*. 2004; 5:786–92. [PubMed: 15132662]
- Kubasak MD, Hedlund E, Roy RR, Carpenter EM, Edgerton VR, Phelps PE. L1 CAM expression is increased surrounding the lesion site in rats with complete spinal cord transection as neonates. *Exp Neurol*. 2005; 194:363–75. [PubMed: 16022864]
- Levitt JM, Hunter M, Mujat C, McLaughlin-Drubin M, Muenger K, Georgakoudi I. Diagnostic cellular organization features extracted from autofluorescence images. *Optics Letters*. 2007; 32:3305–7. [PubMed: 18026288]
- Lin JF, Pan HC, Ma LP, Shen YQ, Schachner M. The Cell Neural Adhesion Molecule Contactin-2 (TAG-1) Is Beneficial for Functional Recovery after Spinal Cord Injury in Adult Zebrafish. *Plos One*. 2012;7.
- Madduri S, Papaloizos M, Gander B. Trophically and topographically functionalized silk fibroin nerve conduits for guided peripheral nerve regeneration. *Biomaterials*. 2010; 31:2323–34. [PubMed: 20004018]
- Martini R, Schachner M. Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and myelin-associated glycoprotein) in regenerating adult mouse sciatic nerve. *J Cell Biol*. 1988; 106:1735–46. [PubMed: 2453520]
- Meijering E, Jacob M, Sarria JCF, Steiner P, Hirling H, Unser M. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytom Part A*. 2004; 58A:167–76.
- Meng F, Hlady V, Tresco PA. Inducing alignment in astrocyte tissue constructs by surface ligands patterned on biomaterials. *Biomaterials*. 2012; 33:1323–35. [PubMed: 22100982]
- Milosevic NT, Ristanovic D. The Sholl analysis of neuronal cell images: Semi-log or log-log method? *Journal of Theoretical Biology*. 2007; 245:130–40. [PubMed: 17084415]
- Pearson YE, Castronovo E, Lindsley TA, Drew DA. Mathematical Modeling of Axonal Formation Part I: Geometry. *Bulletin of Mathematical Biology*. 2011; 73:2837–64. [PubMed: 21390561]
- Ristanovic D, Milosevic NT, Stulic V. Application of modified Sholl analysis to neuronal dendritic arborization of the cat spinal cord. *Journal of Neuroscience Methods*. 2006; 158:212–8. [PubMed: 16814868]
- Rutishauser U, Acheson A, Hall AK, Mann DM, Sunshine J. The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science*. 1988; 240:53–7. [PubMed: 3281256]
- Sander EA, Barocas VH. Comparison of 2D fiber network orientation measurement methods. *J Biomed Mater Res Part A*. 2009; 88A:322–31.
- Schmitza SK, Hjorth JJJ, Joemai RMS, Wijntjes R, Eijgenraam S, de Bruijn P, Georgiou C, de Jong APH, van Ooyen A, Verhage M, Cornelisse LN, Toonen RF, Veldkamp W. Automated analysis of neuronal morphology, synapse number and synaptic recruitment. *Journal of Neuroscience Methods*. 2011; 195:1850193.
- Schnell E, Klinkhammer K, Balzer S, Brook G, Klee D, Dalton P, Mey J. Guidance of glial cell migration and axonal growth on electrospun nanofibers of poly-epsilon-caprolactone and a collagen/poly-epsilon-caprolactone blend. *Biomaterials*. 2007; 28:3012–25. [PubMed: 17408736]
- Schoenen J. The dendritic organization of the human spinal cord - the dorsal horn. *Neuroscience*. 1982; 7:2057–87. [PubMed: 7145088]
- Schwartz M, Spiran N. SPROUTING FROM CHICKEN-EMBRYO DORSAL ROOT-GANGLIA INDUCED BY NERVE GROWTH-FACTOR IS SPECIFICALLY INHIBITED BY AFFINITY-PURIFIED ANTIGANGLIOSIDE ANTIBODIES. *P Natl Acad Sci USA -Biol*. 1982; 79:6080–3.
- Segev I, London M. Untangling dendrites with quantitative models. *Science*. 2000; 290:744–50. [PubMed: 11052930]

- Sholl DA. Dendritic organization in the neurons of the visual and motor cortices of the cat. *Journal of Anatomy*. 1953; 87:387. [PubMed: 13117757]
- Sivaguru M, Durgam S, Ambekar R, Luedtke D, Fried G, Stewart A, Toussaint KC. Quantitative analysis of collagen fiber organization in injured tendons using Fourier transform-second harmonic generation imaging. *Opt Express*. 2010; 18:24983–93. [PubMed: 21164843]
- Thelen K, Jaehrling S, Spatz JP, Pollerberg GE. Depending on Its Nano-Spacing, ALCAM Promotes Cell Attachment and Axon Growth. *Plos One*. 2012:7.
- Torres-Garcia ME, Solis O, Patricio A, Rodriguez-Moreno A, Camacho-Abrego I, Limon ID, Flores G. Dendritic morphology changes in neurons from the prefrontal cortex, hippocampus and nucleus accumbens in rats after lesion of the thalamic reticular nucleus. *Neuroscience*. 2012; 223:429–38. [PubMed: 22858596]
- Voyadjis AG, Doumi M, Curcio E, Shinbrot T. Fasciculation and Defasciculation of Neurite Bundles on Micropatterned Substrates. *Ann Biomed Eng*. 2011; 39:559–69. [PubMed: 20872249]
- Wang HB, Mullins ME, Cregg JM, McCarthy CW, Gilbert RJ. Varying the diameter of aligned electrospun fibers alters neurite outgrowth and Schwann cell migration. *Acta Biomaterialia*. 2010; 6:2970–8. [PubMed: 20167292]
- Wehrle-Haller B, Imhof BA. Integrin-dependent pathologies. *J Pathol*. 2003; 200:481–7. [PubMed: 12845615]
- Wiencken-Barger AE, Mavity-Hudson J, Bartsch U, Schachner M, Casagrande VA. The role of L1 in axon pathfinding and fasciculation. *Cereb Cortex*. 2004; 14:121–31. [PubMed: 14704209]
- Wieringa PA, Wiertz RWF, de Weerd E, Rutten WLC. Bifurcating microchannels as a scaffold to induce separation of regenerating neurites. *Journal of Neural Engineering*. 2010a:7.
- Wieringa PA, Wiertz RWF, de Weerd EL, Rutten WLC. In vitro Verification of a 3-D Regenerative Neural Interface Design: Examination of Neurite Growth and Electrical Properties Within a Bifurcating Microchannel Structure. *Proc IEEE*. 2010b; 98:389–97.
- Yanicostas C, Herbomel E, Dipietromaria A, Soussi-Yanicostas N. Anosmin-1a is required for fasciculation and terminal targeting of olfactory sensory neuron axons in the zebrafish olfactory system. *Molecular and Cellular Endocrinology*. 2009; 312:53–60. [PubMed: 19464344]
- Yoshikawa M, Senzaki K, Yokomizo T, Takahashi S, Ozaki S, Shiga T. Roles of Runx1 in the expression of calcitonin gene-related peptide and neurite fasciculation of dorsal root ganglion neurons. *Neuroscience Research*. 2008; 61:S86-S.
- Yu TT, Shoichet MS. Guided cell adhesion and outgrowth in peptide-modified channels for neural tissue engineering. *Biomaterials*. 2005; 26:1507–14. [PubMed: 15522752]
- Zhou S, Shen D, Wang Y, Gong L, Tang X, Yu B, Gu X, Ding F. microRNA-222 Targeting PTEN Promotes Neurite Outgrowth from Adult Dorsal Root Ganglion Neurons following Sciatic Nerve Transection. *Plos One*. 2012:7.

Highlights

- We developed a semi-automated image processing method to quantify axonal bundling.
- The method is based on the distribution of the orientation of extending neurites.
- Coefficients from the orientation distribution function indicate degree of bundling.
- Advantages include analysis of low-resolution images with high neurite density.

Figure 1.

Image processing for NDDA for quantification of fasciculation. **(a)** Fluorescent microscopy images (4x) of DRG explant outgrowth on silk hydrogels before **(i)** and after **(ii)** artifact removal with Adobe Photoshop. Scale bars are omitted during processing to prevent interference with analysis program. **(b–d)** Image processing steps within Mathematica program. **(b)** Background removal and binary conversion. **(c)** Tangent vector assignment. **(d)** Local radial frame definition to eliminate floating neurites. Neurites immunostained with α - β -III tubulin. Scale bars = 150 μ m.

Figure 2. NDDA output and 'c' values for quantification of fasciculation. **(a)** Histogram of overall DRG outgrowth summarizing counts of tangent vectors from ϕ_1 to ϕ_2 . **(b)** Spatial resolution of fasciculation analysis. Histograms (left) of corresponding highlighted annulus (right). Greater numbers of tangent vectors in alignment with the radial direction (zero angular separation) correspond to greater fasciculation. Scale bars = 150µm. **(c)** Quantification of fasciculation of overall outgrowth as well as selected outgrowth shown in **(b)**. Higher 'c' values correspond to greater fasciculation. Scale bars are omitted during processing due to interference with analysis program.

Figure 3.

Quantification of fasciculation based on NDDA 'c' values. **(a)** Total 'c' values of branched as compared to fasciculated samples. $n = 10$, error = SEM, $*p = 0.0171$ (t-test). **(b)** Representative output histograms and images of NDDA of branched **(i)** and fasciculated **(ii)** samples. Scale bars omitted due to interference with analysis program. Scale bars = $150\mu\text{m}$. **(c)** Spatially resolved NDDA fasciculation quantification. c1-4 represent annuli with increasing distance from explant body as displayed in **(b)**. Error = SEM, $*p = 0.0385$ (t-tests), $***p = 0.0004$ (2-way ANOVA).

Figure 4.

NDDA method supplements the Sholl analysis and manual ImageJ neurite width calculations. **(a)** Immunofluorescent images (neuronal specific β -III Tubulin) of highly branched **(i)** and fasciculated **(ii)** DRG outgrowth. Scale bars = 100 μ m. **(b)** Quantification of # of intersections and ODF 'c' values of images in **(a)** by the Sholl analysis **(i)** and NDDA **(ii)**, respectively. **(c)** Comparison of NDDA 'c' values with manual measurements of neurite width. ** $p=0.0036$, *** $p<0.0001$ (Student's t-test).

Table 1

Comparison of analysis methods for evaluating neurite fasciculation.

Analysis method	Pros	Cons
Neurite directional distribution analysis (NDDA)	<ul style="list-style-type: none"> • Semi-automated • Quantitative • Processes any image format • Does not require conversion to binary/skeletonization • Extends to analysis of any anisotropic image features • User-determined reference direction • Raw images accepted • Thresholding available within NDDA user interface • Visually displays tangent vector distribution overlayed on image and in histogramical form • User-determined spatial resolution available • Radial boundary limits available • Easily extended to 3D image stacks (i.e. confocal) • Can be applied to images of both high density neurites and single cells • Vector-based 	<ul style="list-style-type: none"> • Longer processing time (~2–5 min/image) • Requires closed source Mathematica software
Sholl analysis	<ul style="list-style-type: none"> • Semi-automated • Fast processing time (<1 min/image) • Quantitative • Open source software 	<ul style="list-style-type: none"> • Requires 8-bit images • Requires high image resolution • Potential information loss through conversion to binary and skeletonization • Requires manual measurements (i.e. inner and outer radius) prior to analysis, thereby increasing processing time • Most accurate on images of single cells • Image processing must be performed prior to analysis • Scalar based = easily affected by biological and technical variability
Manual ImageJ quantifications	<ul style="list-style-type: none"> • High accuracy • Myriad of available metrics (i.e. length, width, tortuosity, directionality of neurites relative to desired reference values) • Image processing available within ImageJ • Open source software • Quantitative • Versatile dataset 	<ul style="list-style-type: none"> • No automation • Long processing time (10 min+/image) • Scalar based = easily affected by biological and technical variability

Analysis method	Pros	Cons
NeuronJ	<ul style="list-style-type: none"> Assists in manual tracing Versatile dataset Quantitative Open source software 	<ul style="list-style-type: none"> Manual process Long processing time (10 min+/image) Requires 8-bit images Requires high resolution for accuracy Scalar based = easily affected by biological and technical variability
Oval Profile plug-in (i.e. FFT, Radial Sums)	<ul style="list-style-type: none"> Semi-automated Fast processing time (<1 min/image) Vector-based 	<ul style="list-style-type: none"> Requires square image More accurate for analysis of anisotropy Requires high resolution images Requires image processing (i.e. removal of explant body fluorescence and artifacts)