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A Graph Watershed Method for Analysis and Quantification of Neurite Branching Structure

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Abstract: A new method for analysing neurite structure in 2D microscopy images is presented. Detected neurite structure is converted into a graph representation and grown from detected cell bodies using a graph-based version of the watershed transformation. In doing so, various annotations of neurite complexity and morphology are characterised to aid studies of neuronal behaviour. We present the results of our algorithm on example images of neurite outgrowth.

Keywords: *neurite outgrowth analysis, linear feature detection*

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1. INTRODUCTION

Analysis of neurite structure is a vital component of the drug discovery process (Kim et al, 2006). Such analyses give researchers the ability to identify compounds that impact on neuronal electrophysiology. Researchers are interested in changes to the length, shape and complexity of neurite structure. Semi-automated approaches for detecting and quantifying neurite structure are commonly used to this end (Meijering et al., 2004). In high content analysis situations, however, semi-automation can be impractical. Xiong et al. (2006) present an automated approach to detection and quantification of neurites which produces image-wide quantification, but not at a cellular level.

In this paper, we present an automated approach to the analysis and quantification of detected neurites. Our procedure uses the watershed algorithm (Soille, 2003, Sect 9.2) on a graph derived from detected neurites and their cell bodies. The watershed algorithm is commonly used in image analysis applications on 2D and 3D images. The watershed can be similarly applied to more arbitrary graph structures. For example, Wegner et al. (1998) present a region-merging technique that applies the watershed transformation to a graph where the vertices are regions in a 2D image. Our approach associates neurite trees with a parent cell body. In the process of growing trees it collects various measures of length, width and complexity such as degree of branching. The methodology described in this paper forms the basis of the software package, HCA-Vision, used by, for example, Vallotton et al. (2007) to analyse neurite branching in cultured cortical neurons.

2. METHOD

2.1. Pre-Processing and Feature Detection

Typical microscopy images of neurite structure contain two classes of objects: the cell bodies (both the cytoplasm and nucleus) and neurites. Both of these object classes are marked by a single fluorescent stain. The images being analysed in this application are 2D grey-scale.

Segmenting the cell bodies is the starting point of our approach. A pre-processing step of a morphological top-hat (Soille, 2003, Sect 4.5) is used to flatten the background. The size of the square structuring element for the top-hat is user supplied and is chosen to be greater than the radius of any cell body. We now remove the neurite structure in the image using a morphological opening (Soille, 2003, Sect 4.1) with a polygonal structuring element. The radius of the structuring element is chosen to be greater than the thickness of any neurite in the image. The image is then thresholded using a method that finds a threshold based on gradient strength information similar to that proposed by Weszka and Rosenfield (1979). A bivariate histogram of Sobel gradient versus grey level is calculated. The average gradient strengths for each grey-level are treated as a histogram. An input parameter then determines the quantile of the distribution to be used as the threshold.

Figure 1: A microscopy image of a single neuron.

To segment the neurites, we use the multiple directional non-maximum suppression (MDNMS) technique proposed by Sun et al. (2006). The result of this technique is skeletonised (Soille, 2003, Sect 5.4) using 4-connectivity. Any neurite structure which lies inside the cell bodies is removed from the image. The sensitivity of the MDNMS approach can lead to over-segmentation of neurite structures. These inaccuracies manifest as small barbs in the skeleton which can be pruned by a debarbing process which removes barbs with length less than a user supplied threshold.

2.2. Neurite Segments

We now divide the skeleton of neurite structure into unique neurite segments. A segment is a linear section of neurite structure between two intersection or branching points. This is done by first finding intersection points in the 4-connected skeleton. An intersection point is characterised by having more than two 4-connected neighbours. Intersection points are then removed from the skeleton and in doing so the neurite structure is divided into segments which remain 4-connected. Each segment is now given a unique label. Figure 2 shows an example image of individual segments detected for the neurites in Figure 1. For this example, the cell body and neurites were first segmented using the approach described in Section 2.1.

2.3. Neighbourhood Graph

A graph of neighbourhood relationships for neurite segments is now built. We first morphologically dilate (Soille, 2003, Sect 3.3) uniquely labeled intersection points, described in Section 2.2, with a 3 x 3 structuring element so that they overlap the ends of neurite segments. Neurite segments which overlap a common intersection point are considered neighbours. This information is initially contained in a bivariate histogram of neurite segments versus intersection points. Non-zero entries in the histogram indicate overlaps between segments and intersection points. A linked list is created by scanning across each row of the histogram, corresponding to an intersection point, and finding non-zero entries indicating neighbourhood relationships among segments.

We now find the segments which are touching cell bodies. We refer to these as `root' segments. We firstly thicken the labeled cell bodies so that they overlap root segments. A thickening is a dilation that preserves an object's label (Soille, 2003, Sect 5.3). Again, we use a bivariate histogram to store the overlap information. Non-zero entries correspond to root segments for a particular cell body.

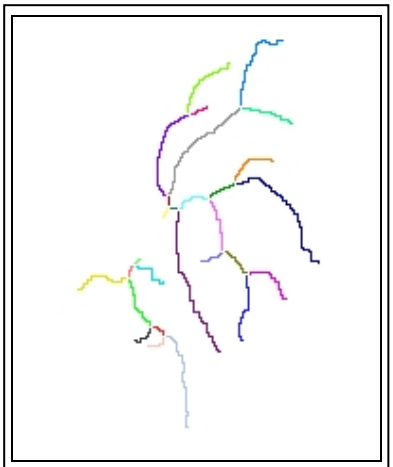

Figure 2: Linear features extracted from the image in Figure 1. Unique segments are colour coded differently.

2.4. Tree Growing Using the Watershed Algorithm

At this stage we associate all neurite segments with a neurite tree. A neurite tree is a connected neurite network extending from a single root segment. Figure 3 shows the two neurite trees, in red and green, for the image in Figure 1. We use the watershed algorithm to derive the association. Typically, in image analysis applications, the watershed is performed on an image, called the segmentation function, which highlights object boundaries. A set of unique seeds are grown on the segmentation function using a priority queue. Seeds are placed in the queue and neighbouring pixels are added with priority given to those with the lowest value in the segmentation function. Pixels are repeatedly taken from the top of the queue and added to the object defined by the pixel's neighbouring seed.

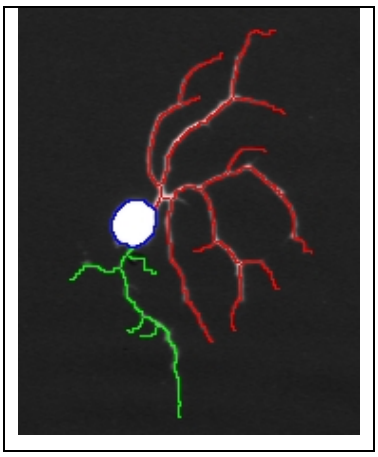

Figure 3. The two neurite trees extracted from Figure 1, colour coded in red and green.

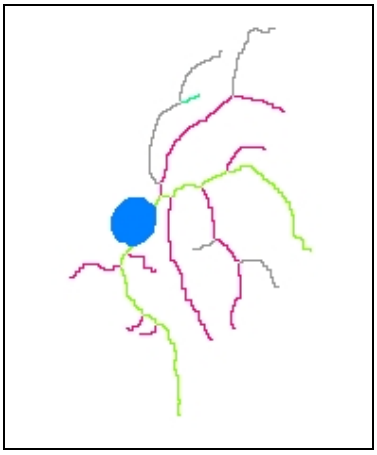

Figure 4. The branching layers derived from Figure 1, primary branches are green, secondary branches are pink and tertiary branches are grey.

We use the watershed methodology to grow neurite trees. The framework for the watershed is, however, different to that which is used for 2D images. Instead we have nodes of a graph instead of pixels. The nodes are the individual neurite segments and our seeds are the root segments as found in the above section. Root segments are initially put in a priority queue and neighbouring segments are added with priority given to segments with the highest average brightness. The average brightness is calculated over the pixels that form the skeleton of the neurite segment. Brightness was chosen as the priority as it is a key visual indicator for determining the primary and non-primary branches of the neurite trees. Other criteria for the priority could be used such as the relative orientation of the segments. See Section 2.5.2 for further details on primary branches and branching layer. Neurite segments are repeatedly taken from the top of queue and associated with their neighbouring neurite tree until all segments have been removed from the queue.

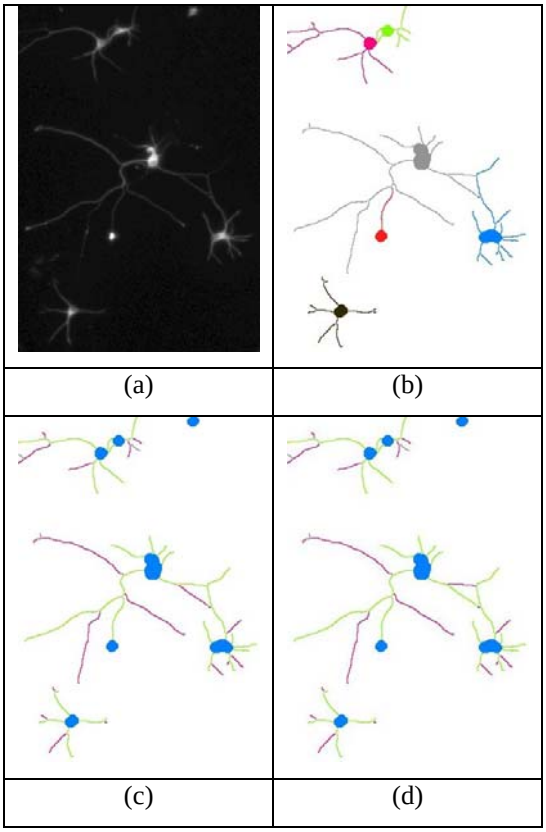

Figure 5: (a) A microscopy image of multiple neurons. (b) An image depicting the relationship between neurons and their neurite structure. Neurons and their neurite structure are coded with the same colour. (c) An image depicting the branching layer assignments. Primary branches are green, secondary branches are pink and tertiary branches are grey. (d) Branching layer assignments produced by NeuronJ

2.5. Neurite Segment and Neurite Tree Quantification

Various measurements can be accumulated for each neurite during the tree growing process. These measurements can in turn be accumulated on a per neurite tree or per cell basis. It is also common to report measurements on a per image basis. There are two groups of measurements collected during the watershed process: those relating to neurite length, width or brightness and those relating to neurite complexity.

2.5.1 Length, Width and Brightness

Length measurements are heavily used by researchers using microscopy images of neurite outgrowth. Before the tree growing process is initiated, the length of each neurite segment is estimated (Dorst, 1987). The average width of each segment is also computed using the method proposed by Lagerstrom et al. (2008). The average brightness of the segment is computed not only to guide the watershed process, but as a reportable measure in itself. As each neurite segment is removed from the queue we can accumulate the length back to the cell body for the segment, the longest path back to the neuron body for the neurite tree and the total length of the neurite tree. In a similar fashion, the average width of the neurite tree and the total area of the neurite tree are accumulated. The average brightness and integrated intensity of the neurite tree are accumulated as the tree growing progresses. Once the trees have been grown, neurite coverage area can be calculated. Neurite coverage area is defined by the area of the convex hull of a neurite structure.

2.5.2 Complexity

A variety of neurite complexity measures for describing neurite morphology are also collected via the tree growing process. Neurites display behaviour where a dominant or primary branch extends from a neuron body with secondary branches extending from the primary branches and so on. On a per neurite scale, we refer to this as branching layer. Root segments are given a primary branching layer, which is coded as 1. As segments are removed from the queue, a neurite segment inherits its parent's branching layer if it is the child segment with the highest average brightness. The remaining child segments are given a branching layer equal to their parent's branching layer plus one. Figure 4 shows the branching layer assignments for each neurite segment in Figure 1. The average branching layer per neurite tree is accumulated as the tree is grown. The situation where a neurite segment has more than one offspring is a branching point. The number of branching

points per neurite tree is collected. The number of extreme neurite segments i.e. those with no children is also collected.

3. EXPERIMENTAL RESULTS

The performance of the algorithm was assessed on images of embryonic cortical neurons from Sez-6 null mice. Analysis and quantification of these images underpins a study of the effect of deleting the Sez-6 gene. Images of the cultured neurons were captured from an inverted microscope. The images typically contained one or a small number of neurons. The results of our algorithm were compared to results achieved using the semi-automated solution, NeuronJ (Meijering, 2004).

Figure 1 shows a subsection of one of the assessment images. The cell body was detected using the gradient strength based method described in Section 2.1. Linear features were detected using the MDNMS approach and are shown in Figure 2. Figure 4 shows the branching layer results described in Section 2.5.2. The branch layer assignments correspond exactly with those achieved with NeuronJ. Measurements derived from our method are shown in the first result column in Table 1.

Figure 5(a) shows another image from the assessment images. In this example there are multiple neurons in the scene. Cell bodies were again detected using the gradient strength based method described in Section 2.1. Linear features were detected using the MDNMS approach. Figure 5(b) shows an image displaying cell assignment of neurite structure. Neurite structure is colour coded the same as its parent cell. In images where the neurite structure is non-overlapping, this is not an issue, but it is a feature of our assessment images. The results of our algorithm are identical to those produced by NeuronJ. Figure 5(c) shows the branching layer results. Figure 5(d) shows the branching layer results achieved using NeuronJ. There is an almost exact correspondence between the branching layer results. The only difference is the swapping of the primary and secondary branches stemming from the bottom right of the central large cell. In this case, the correct result is subjective. This outcome is typical of comparisons between our approach and NeuronJ on this set of images. Measurements derived from our method for this image are shown in Table 1.

Figure 1		**Figure 5**					
		green	**pink**	**grey**	**red**	**blue**	**black**
L.max	123	89	150	194	59	108	40
L.total	785	170	340	755	59	324	196
N.roots	2	3	3	6	1	7	4
N.seg	28	9	13	16	1	17	12
N.ext	15	6	8	11	1	12	8
N.branch	13	3	5	5	0	5	4
BL.max	4	3	3	3	1	2	2
BL.mean	2.0	1.4	1.5	1.5	1.0	1.2	1.3
I.int	115475	10800	12979	58681	13290	30477	14476
A.cover	14676	2611	8368	30771	640	5853	4305
A.neur	961	294	456	1028	76	443	278

Table 1: Per cell neurite outgrowth quantification results for the images in Figure~\ref{fig.eg1raw} and Figure~\ref{fig.eg2raw}. L.max: Length of longest neurite; L.total: Total length of neurite outgrowth; N.roots: Number of root segments; N.seg: Number of segments; N.ext: Number of extreme segments; N.branch: Number of branch points; BL.max: Maximum branch layer; BL.mean: Mean branch layer; I.int: Integrated intensity; A.cover: Neurite coverage area (area of the convex hull of neurite outgrowth); A.neur: Area of neurite outgrowth.

For an image of dimensions 512 by 512 pixels, on an Intel Pentium 4 with 2.66GHz CPU, the approach takes around 0.2 seconds. The time is heavily dependent on the density of features. In large images with dense neurite outgrowth and cell bodies, the approach can take up to 10 seconds.

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4. CONCLUDING REMARKS

We have presented a new method for analysing neurite structure in 2D microscopy images. The approach subjects a graph representation of detected neurite outgrowth to the watershed algorithm. We treat the cell bodies of the neurites as seed points in the watershed process. The process allows us to compile a collection of measurements annotating neurite complexity and morphology. Such measurements underpin biological research into neuronal behaviour. The results of applying this method to example images of neurite outgrowth demonstrate the effectiveness of the approach.

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