Automatic Detection, Segmentation and Characterization of Retinal Horizontal Neurons in Large-scale 3D Confocal Imagery


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ABSTRACT

Automatic analysis of neuronal structure from wide-field-of-view 3D image stacks of retinal neurons is essential for statistically characterizing neuronal abnormalities that may be causally related to neural malfunctions or may be early indicators for a variety of neuropathies. In this paper, we study classification of neuron fields in large-scale 3D confocal image stacks, a challenging neurobiological problem because of the low spatial resolution imagery and presence of intertwined dendrites from different neurons. We present a fully automated, four-step processing approach for neuron classification with respect to the morphological structure of their dendrites. In our approach, we first localize each individual soma in the image by using morphological operators and active contours. By using each soma position as a seed point, we automatically determine an appropriate threshold to segment dendrites of each neuron. We then use skeletonization and network analysis to generate the morphological structures of segmented dendrites, and shape-based features are extracted from network representations of each neuron to characterize the neuron. Based on qualitative results and quantitative comparisons, we show that we are able to automatically compute relevant features that clearly distinguish between normal and abnormal cases for postnatal day 6 (P6) horizontal neurons.

Keywords: retinal neurons, segmentation, feature analysis, confocal imagery.

1. INTRODUCTION

Over the past decade, recent advances in cellular imaging technology have made it possible to capture high-resolution 3D images of individual neurons that enable neurobiologists to investigate fundamental questions in neuronal structure, morphological development, and neural disorders. The morphology (i.e., size, shape, and branching structure) of a neuron is the key determinant of its function in the nervous system. With distinct morphological features and distinct functionality, different neurons have remarkably diverse dendrite structures that are generated in particular series of events during development to maintain normal adult life. Developmental anomalies may lead to neural malfunction or be early indicators for variety of neuropathies ranging from Alzheimer’s to schizophrenia.

Although these recent advances in technology provide potentially powerful tools (e.g., confocal laser scanning microscopy) for neurobiologists to collect detailed neuronal data, computational methods to analyze the neuronal structure have not kept pace with imaging technology, and neurobiologists still rely largely on manual analysis procedures. Even with expertise, it is a laborious, biased and time-consuming process to manually analyze the neuron morphology without any systematic approach [1]. Therefore, the automatic characterization of neural structure from 3D confocal imagery is essential for structural analysis of neuron morphology to fully exploit these valuable sources of data.

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Research sponsored by the Laboratory Directed Research and Development Program of Oak Ridge National Laboratory (ORNL), managed by UT-Battelle, LLC for the U. S. Department of Energy. This manuscript has been authored by employees of UT-Battelle, LLC, under contract DE-AC05-00OR22725 with the U.S. Department of Energy. Accordingly, the United States Government retains and the publisher, by accepting the article for publication, acknowledges that the United States Government retains a non-exclusive, paid-up, irrevocable, world-wide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purposes.
In the literature, there exist many works related to automated cell detection, segmentation and analysis of neuronal structure from neuron images [1, 2, 3]. In [3], neuron somata in 3D confocal image stacks are automatically detected and counted by using local thresholding, a marker-based watershed approach and a Gaussian model-based algorithm. Automated labeling of neurites with curvilinear structure in 2D fluorescence images based on the local Hessian matrix and reconstruction of broken branches (dendrites) is proposed in [4]. Cohen et al. [5] proposed an automated neurite tracing technique in 3D confocal imagery for selectively marked neurons based on segmentation, skeletonization, and graph extraction. Kofahi et al. [6] achieved a similar goal by generating seed points to find possible dendrite segments and recursively following the neuronal topology using variable size of directional kernels modeled with a 3-D cylinder. Kerekes et al. [7] introduced a fully automated method for segmenting and characterizing the dendritic tree of a single neuron in pre-cropped confocal image stacks. Although these recent works show good performance for neuronal segmentation on high-resolution imagery of a single neuron, none of them can be directly applied to automatic characterization and classification of multiple retinal horizontal neurons in large-scale 3D confocal imagery. Therefore, further research is needed to adapt existing techniques to automatic characterization and classification of multiple neurons.

In this paper, we propose a method to classify multiple retinal neurons in large-scale 3D confocal image stacks acquired using a two-photon laser scanning microscope (TPLSM) as shown in Fig. 1, in which 35 to 40 neurons are visible from a transgenic mouse at postnatal day 6 (P6). Low spatial resolution imagery and the presence of intertwined dendrites from different neuron cells make the structural analysis of neuron morphology in retinal neuron imagery a challenging neurobiological problem. Therefore, we present a fully automated method for detecting somas (cell body), segmenting the dendrites of each neuron, and characterizing the neurons with respect to the morphological structure of their dendrites. We give the details of the technical approach in the next section. The data acquisition system and experimental results are presented in Section 3. Finally, we provide concluding remarks in Section 4.

Figure 1. 3D confocal image stacks of retinal horizontal neuron layer from a transgenic mouse at postnatal day 6. Between 35 and 40 neurons are visible with a wide variety of dendritic tree structure and complexity due to the presence of clutter and the variability of cell intensity.

2. TECHNICAL APPROACH

In our approach, we characterize the retinal neurons by following four main steps: (1) soma detection, (2) dendrite segmentation, (3) skeletonization and network analysis, and (4) feature extraction. We first localize each individual soma in the large-scale 3D confocal imagery by using morphological operators and active contours. By using each soma position as a seed point, we automatically determine an appropriate threshold to segment dendrites in each neuron. Then, the skeletonization and network analysis step generates the morphological structures of segmented dendrites. Finally, shape-based features are extracted from the network representation of each soma and its dendrites to characterize each neuron in the 3D confocal image stacks. More details about these four steps are given in the following sections.
2.1 Soma detection

Soma detection is essential not only to determine which dendritic segments belong to which neuron but also to extract important morphological characteristics of the dendrites for neuron classification, including order of dendrite branches, length of dendrite and angle of dendritic segments. In our approach, we detect multiple somas in a large 3D whole-field image stack by using a combination of grayscale morphology operators, adaptive thresholding, connected component analysis and active contour.

Morphology operators shrink, enlarge and filter image regions based on a particular geometrical shape. Grayscale morphological erosion, one of the morphology filtering operators, examines the image regions for a particular geometrical structure in a grayscale range [8]. Since the shape of typical somas can be estimated as a sphere, we first filter the image by an erosion operator with a spherical structuring element whose radius is equal to size of the minimum volume enclosing the typical soma. Then, adaptive thresholding converts the resultant image to a binary image to separate the foreground and background regions. The adaptive threshold is set to keep image regions whose mean intensity value is greater than the 50% of the mean of the resultant image. After thresholding, connected component analysis labels all separate image regions as different somas using 26-connectivity, and the centroid of each soma is computed. By using each soma centroid as a seed point, an active contour algorithm automatically determines the boundary of each soma based on the Chan-Vese energy [9]. Each resultant 3D closed surface from active contour segmentation represents the boundary of each soma. Fig. 2(a-b) shows a representative soma segmentation result of a horizontal neuron cropped from larger whole-field image shown in Fig.1. The resulting segmentation is used as the boundary of each soma. Fig. 2c shows the detected intensity volume of the somas in the whole-field image shown in Fig.1.

![Figure 2](http://proceedings.spiedigitallibrary.org/)

**Figure 2.** Representative figures of soma segmentation: (a) 3D confocal image stacks of a horizontal neuron cropped from larger whole-field image shown in Fig. 1; (b) intensity volume of the soma surface in (a); (c) detected intensity volume of the somas in the whole-field image shown in Fig.1.
2.2 Dendrite segmentation

In order to segment the dendrites from background materials, we need to make the following two assumptions. First, intensity values of dendrites and somas are higher than intensity values of background and non-cellular materials. Second, there exists a threshold that distinguishes the all cellular materials from the background. In an ideal segmentation case, an optimal threshold perfectly separates the all cellular materials from the background and extracts the structure of the dendritic tree for every neuron. Since the distribution of the fluorescent material among different cellular regions is not uniform, the intensity values of dendritic segments are diverse in the 3D confocal image stacks. Moreover, the low spatial resolution of large-scale confocal imagery and the existence of intertwined dendrites from different neuron cells both contribute to the difficulty of finding the optimal threshold for dendrite segmentation. Therefore, an exhaustive search method is required to find the threshold value for dendrite segmentation of each individual neuron.

We utilize the dendrite segmentation method proposed in [7] which searches to detect the volumetric explosion of the object of interest as a high initial threshold value is gradually decreased. In this approach, the threshold value is initialized as the maximum intensity value within the soma of interest and gradually steps down until reaching a value where the number of connected voxels to the soma of interest will suddenly “explode” in size as large amounts of background voxels become connected to the soma of interest. The optimal threshold is found by backing off slightly from the value at which the volumetric explosion occurred. More details are presented in [7]. Fig. 3 illustrates the volumetric explosion method for a horizontal neuron (shown in Fig. 2a-b) where in each iteration the total number of connected voxels increases. This procedure is repeated for every soma in the dataset, and segmentation results of each neuron are converted to a binary image for skeletonization step.

![Figure 3. Illustration of volumetric exposure approach for a neuron shown in Fig. 2a-b, where i is the iteration number.](image)

2.3 Skeletonization and network analysis

Skeletonization, a procedure that generates a unit-width line to represent each dendrite, is essential to simplify the analysis of morphological structure of the dendritic tree. We utilize a modified version of the iterative 3-D skeletonization algorithm proposed in [10]. The original thinning algorithm peels iteratively the outermost layer of the binary image of the dendritic tree until only the central voxels of dendrites remain. In this approach, the voxels which have the least number of neighbors are defined as outmost layer and deleted first. Kerekes et al. [7] extended this approach by taking the intensity values of the corresponding voxels into account for the skeletonization of the grayscale dendrite segmentation image. Since dimmest voxels in the outmost layer are deleted first, this approach keeps the brightest voxels of dendrites in the final skeleton. Fig. 4 shows results of the skeletonization algorithm for a single neuron (shown in Fig. 3) and multiple neurons (shown in Fig. 1), respectively.
However, skeletonization does not provide connectivity information of voxels with each other in the same dendrite. Therefore, a further step is required to generate the morphological structure of each dendrite from sets of skeleton points by establishing the connection topology between these skeleton voxels. After skeletonization of each dendritic segment, we establish the network representation of the dendritic tree with a combination of nodes and edges to extract the morphological structure of dendrites. Skeleton voxels with one neighbor (end point) and voxels with at least three neighbors (branch points) are identified as nodes to represent the branching points of the dendrites from either the soma or another dendritic segment. Each skeleton voxel which has exactly two neighbors is identified as a segment point, and connected segment points between two neighboring nodes are grouped to form an edge which represents the corresponding dendritic segment in the skeleton. Thus, the skeleton is converted to a system of edges and nodes.

For network analysis, we first search the connectivity of nodes and edges with each other to determine which edges and nodes compose each dendrite. Then, we trace the connected nodes and edges from the soma node to the end node and group them as an individual dendrite. In order to trace dendrites, tracing agents are generated at each node that is connected to a soma of interest. Each agent starts tracing the connected nodes and edges, saves their information and marks them as visited in order to prevent visiting the same nodes and edges again and avoid looping in the dendrite branches. When a dendrite branches at the next node, we generate additional tracing agents for these new branches to trace them until reaching an ending node or a node that was previously traced by another agent.

In a data set with multiple neurons, it might be possible that two separate dendrites from the same soma or two different somas become to close each other. Due to the low resolution of image acquisition system, dendrite segmentation and skeletonization algorithms render these two separate dendrites connected. Therefore, it is required to break these connected dendrites into two separate dendrites. Usually, an individual dendrite extends to the environment smoothly and does not change its direction sharply. However, when two separate dendrites are combined with each other because of the low resolution image, there is a sharp angular transition from dendrite to dendrite. In order to find where these dendrites are connected, we compute the instantaneous angles between small pieces of dendrite starting from one soma ending to another soma. The transition point is determined to be the point of highest change in instantaneous angle that is greater than a pre-defined threshold. Then, we break this combined dendrite into two pieces at the closest node point to the transition point, add the new dendrites to our register and remove the previous connected dendrites. In our algorithm, we initially set the size of the dendrite piece to five voxels and the pre-defined threshold to 60 degrees.

Finally, we group the path of each tracing agent as an individual dendrite to extract topological features of each neuron, such as number of dendrites, length and angles of dendrites, and types of dendrites.

2.4 Feature Extraction

A retinal neuron can have three types of dendrites: apical (extending upward), basal (extending downward), and horizontal. Moreover, each dendrite exhibits distinct morphological features (e.g., emanating point, angle, height). At postnatal day 6 (P6), the dendrites in a normal horizontal mouse neuron typically become horizontal and dendrites emanate from the top of the soma and extend into the environment horizontally as shown in Fig. 5(a). However, in the case of Rb-deficient mice, abnormal cell development more frequently occurs in the retina, resulting in the formation of
abnormal dendrite morphology (i.e., lingering apical or basal dendrites), which becomes most evident around P6. Instead of only horizontal dendrites, apical and basal dendrites also appear in the dendrite morphology. Apical dendrites emanate from the top pole of the soma and extend to upper retinal layers as shown in Fig. 5(b) whereas basal dendrites emanate from the bottom pole of the soma and extend to lower retinal layers as shown in Fig. 5(c).

In order to characterize the morphology of dendrites and classify them as apical, basal and horizontal, we extract the following features from morphological structure of dendrites: (1) emanating point, (2) angles of dendritic segments with respect to emanating point, (3) angles of dendritic segments with respect to previous dendritic segment, (4) length of dendrite, (5) height of dendritic segments in z-axis, (6) angle of best fitting plane, and (7) distance to best fitting plane. Fig. 6 illustrates the angular feature extraction from the morphological structure of a dendrite.

Based on these extracted features, we introduce a probabilistic model that computes the probability of a dendrite being apical, basal or horizontal. In this model, we first generate a linear probability density function (pdf) for the emanating point feature where the height of the emanating point from the bottom of the soma is normalized by the height of the soma. Second, each dendritic tree is partitioned into apical, basal or horizontal segments based on the angles of dendritic segments with respect to emanating point and previous dendritic segment. Based on this partitioning, another linear distribution function is computed which indicates the percentage of dendrite segments being apical, basal or horizontal. In addition, we fit the z-height of dendritic segments into a Gaussian distribution, and the angle of best fitting plane and distance to best fitting plane into linear distribution functions. Finally, we multiply each probability function and classify each dendrite into different classes by comparing the probability of being apical, basal and horizontal.
3. DATA AND EXPERIMENTAL RESULTS

3.1 Data acquisition
Retinal data was acquired from Chx10-Cre; RbLox/–; GAD67-GFP transgenic mice at postnatal day 6 by using a two-photon laser scanning microscope (TPLSM). In order to capture the 3D image stacks with maximum resolution, the entire the retina was scanned in 0.5 μm step-size increments in x, y, (spatial resolution) and z (depth resolution) axis which is recorded in a 3D image stack (volume). In each image with the 40x objective, 15-20 developing horizontal neurons were captured (e.g., Fig. 3a-b). Each volume is approximately 512x512x256 voxels in size.

3.2 Experimental Results
In this section, we show test results from applying our proposed algorithm to multiple retinal neurons in large-scale 3D image stacks. In two sets of experiments, we tested our automatic detection, segmentation and characterization algorithm with wild-type mice and knockout (Rb-deficient) mice at postnatal day 6. Each set of experiments consists of four different 3D image stacks collected as described in the data acquisition section above. There was no available ground truth for these datasets.

Figure 7. (a) Side view of confocal image volumes of retinal horizontal neurons layer at postnatal day 6 from wild type mouse (sdd37_3_a2); (b) side view of network analysis results of confocal image volumes with soma volume shown; (c) top view of the same confocal image volumes of retinal horizontal neurons in (a); and (d) top view of the network analysis results in (b).
A visual comparison of segmentation results from wild-type mice and knockout (Rb-deficient) mice at postnatal day 6 is shown in Fig. 7 and Fig. 8. In Fig. 7(a, c), side and top views of confocal image volume of retinal horizontal neurons layer at postnatal day 6 from a wild type mouse are shown, respectively. Side and top views of soma detection and dendrite segmentation results of this confocal image volume are shown in Fig. 7(b, d), respectively. We observe that every soma of the wild type mouse retina is aligned at the same level in the retina and every dendrite of these neurons emanates from the top of the soma and extends into the environment horizontally. Thus, we can classify every dendrite in the wild type mouse retina as horizontal.

In Fig. 8(a, c), side and top views of confocal image volume of retinal horizontal neurons layer at postnatal day 6 from a knockout (Rb-deficient) mouse are shown, respectively. Side view and top views of soma detection and dendrite segmentation results of this confocal image volume are shown in Fig. 8(b, d), respectively. We observe that every soma of the knockout mouse is aligned at the same level in the retina as in wild type mice. However, dendrites of some neurons show different characteristics than typical horizontal neurons. Some dendrites emanate from the bottom of the soma and extend vertically. Therefore, there exist several apical and basal dendrites in addition to the horizontal dendrites in the retina of the mutant type mice.

In order to extend our study on the characterization of retinal neurons in 3D confocal image stacks, we generate quantitative results in addition the qualitative comparison by applying the automatic detection, segmentation and characterization algorithm for eight different datasets. Quantitative results for eight mouse retinae are shown in Table 1.
Table 1. Classification results of the retinal images from different wild type and knockout mice.

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<th>Dataset Name</th>
<th>Mouse Type</th>
<th>Total Number of Cell</th>
<th>Total Number of Dendrites</th>
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<td>32</td>
<td>0</td>
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<tr>
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<tr>
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<td>Mutant</td>
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<td>Mutant</td>
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<td>153</td>
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<td>11</td>
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<tr>
<td>Sdd37_4_a3</td>
<td>Mutant</td>
<td>18</td>
<td>43</td>
</tr>
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</table>

We observe that every dendrite in the retina of the wild type mice is horizontal dendrite, whereas in the retina of the knockout mice there exist several apical and basal dendrites in addition to the horizontal dendrites. Thus, we observe a clear separation between the two mouse genotypes in terms of the number of apical and basal dendrites.

4. CONCLUSIONS

In this paper, we presented a new, fully automated segmentation and characterization approach for multiple retinal horizontal neurons in large-scale 3D confocal image stacks. In our approach, we first localize each individual soma by using morphological operators and active contours. By using each soma position as a seed point, we automatically determine an appropriate threshold to segment dendrites of each neuron. Then, skeletonization and network analysis extracts the morphological structure. Finally, network-based features of each neuron are used to discriminate between various classes of neurons as normal and abnormal. Based on qualitative results and quantitative comparisons of the numbers of apical and basal dendrites, we show that our algorithm is able to provide a clear distinction between the two mouse genotypes considered in the experiments. We thus believe that our approach represents a useful tool for future statistical analysis of large datasets of retinal neuron imagery.

REFERENCES