Automatic Quantification of the Axons in the Optical Nerve of a Mouse

Jacinda Clemenzi

Department of Electrical Engineering and Computer Science,

Case Western Reserve University, Cleveland, OH, Email: jlc34@cwru.edu

Abstract

This paper presents a morphological method of automatically identifying and quantifying the nerve bundles in a digital image of the optical nerve of a mouse. Mice are commonly used in biological and genetic experiments. It is often necessary to count the rods and cones in the retina. Because of the curvature of the retina it is often easier to count the axons in the optical nerve. Automating this process of quantification is the goal of this paper.

KEYWORDS

Segmentation, optic nerve, mouse, retina, ganglion, image morphology, automatic quantification

INTRODUCTION

The mouse is a common animal for studying the mammalian nervous system and the effects of genetics and environment on the optical nerve system in particular. Counting the number of rods and cones on the mouse retina is central to studying these effects. The typical mouse retina contains approximately 6.4 million rods and 180,000 cones, though this varies with the mouse strain [5]. Elaborate methods have been devised for counting the number of rods and cones on the curved surface of the retina. These typically involve performing hand counts of stained slides under the microscope at various retinal eccentricities along one retinal axis. The density of rods and cones varies across the retina. By counting the numbers in a sufficient surface area at various eccentricities along the vertical meridian, a reasonable approximation of the total number of rods and cones can be made.

Because of the complexity posed by the curved surface of the retina, it is preferable to count the axon bundles in the optical nerve to estimate the number of rods and cones. Each axon in the optic nerve corresponds to a ganglion cell in the retina. The ratio of ganglion cells to rods and rods to cones has been worked out in previous research [5].

The optic nerve consists of approximately 32,000 to 87,000 axons [9]. The axons are irregular in shape and size. Many of them are mostly round, but some are elongated. The spacing between identifiable axons is also irregular, with many axons in close contact and many that are separated. This irregularity and the low contrast of the prepared microscope slides makes the challenge of quantifying the axons more difficult than other image processing techniques developed to count more regular shaped features. This paper explores combining image morphological methods to segment and quantify the axons in the optical nerve.

BACKGROUND

Image processing techniques have been used to count objects in many digital images. Watershed techniques have generally been most useful in segmenting a background when all the items to be characterized are separated from each other. The watershed method can also be used to find the object boundaries when objects to be counted or characterized are packed closely together. When objects are very similar in size and shape then pattern matching techniques like the Hough transform are useful.

To characterize the properties of red blood cells Ritter and Cooper apply a 4-connected algorithm to segment the uniform background. A graph algorithm is applied to the cell boundaries to characterize the condition of the red blood cell. [7]

Research into using machine learning to classify image components shows much promise. If a set of ground truth or gold standard images are available then they can be automatically segmented and analyzed with a standard set of parameters that characterizes the desired objects. This has been shown to work with images of granulous material and aerial images of forests [6].

Corneal endothelial cell tissue is generally packed tightly together. The watershed algorithm works well here to identify and characterize the cell boundaries [8].

Nerves typically consist of tens of thousands axons that are roughly elliptical in shape. Fok, Chan, and Chin explore a method of identifying axons with an elliptical Hough transform and then identifying the boundaries with an active contour method [3]. The false detection rate with this method is still high.

DESIGN OF THE IMAGE PROCESSING TECHNIQUE

Since the optical nerve of a mouse contains axons of varying sizes and shapes the first processing done to the image was to determine the object size distribution using granulometry. Granulometry is used to find the average red blood cell size by Dempster and Ruberto [2].

The digital image of the optical nerve was converted to grayscale. A disk of increasing size was used to perform a morphological opening on the grayscale image. If f represents the value of each pixel in the original grayscale image than the sum of all those values represents the surface area A.

$$A = sum(f)$$

After applying a morphological opening to the image using a disk structuring element, d_k, of size k = 1, 2, …, the resulting pixel values are represented by f_{dk}. The sum of all the pixel values in f_{dk} represents the surface area after opening the original image with structuring element d_k.

$$A(k) = sum(f_{dk})$$

The reduction in surface area from an opening with structuring element d_k is represented with $R(k)$. Therefore, the size distribution, $D(k)$, can be determined by differentiating $R(k)$.

$$R(k) = 1 - \frac{A(k)}{A}$$

$$D(k) = R(k+1) - R(k)$$

A typical size distribution for the gold standard sample 4 image shows that most of the axons are 7, 9, and 12 pixels large. See Figure 1.

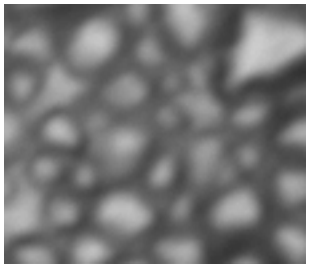
The following morphological procedures were repeated to extract the axons that were the size of each of the local maximums in the size distribution plot.

To identify all the possible locations of axons of an expected size an H-dome extraction method was used. A dome extraction method was presented as a way to locate all the cornea endothelial cells in an image in preparation for a watershed operation to find the boundaries [8].

Before performing a dome extraction the original image was enhanced by performing a tophat transformation and filtered with a smoothing filter ¼ the size of the object size being extracted. The enhanced image is called *D1*.

The essence of the dome extraction is to find all the centers where the middle is light and the pixels gradually darken outwards. A constant intensity value, h, was subtracted from the enhanced image *D1*.

$$D2 = D1 - h$$



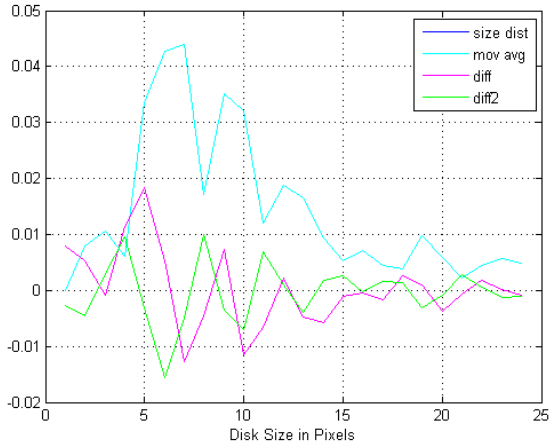


Figure 1: Gold standard sample 4 image and size distribution plot

The value of h should be congruous with the size of the dome expected. In this case a value of 30 was chosen. Perform an image reconstruction using *D2* as the marker image and *D1* as the mask.

$$D3 = imreconstruct(D2, D1)$$

Then subtract reconstructed image, *D3*, from the enhanced image, *D1*, to produce the domes.

$$D4 = D1 - D3$$

A simple Otsu thresholding technique can be applied to *D4* to extract the possible locations of all the axons of an expected size in the image. These can be further improved by applying black and white morphological operations to remove spurs and isolated pixels. See Figure 2 for an example of applying the dome extraction method to one of the gold standard optic nerve images.

A blob analysis was performed on the extracted domes to determine the minor elliptical axis length. If this length was more than twice or less than a quarter of the expected object size then the dome was deleted. See Figure 3 for an example of filter out the domes that are not the target size.

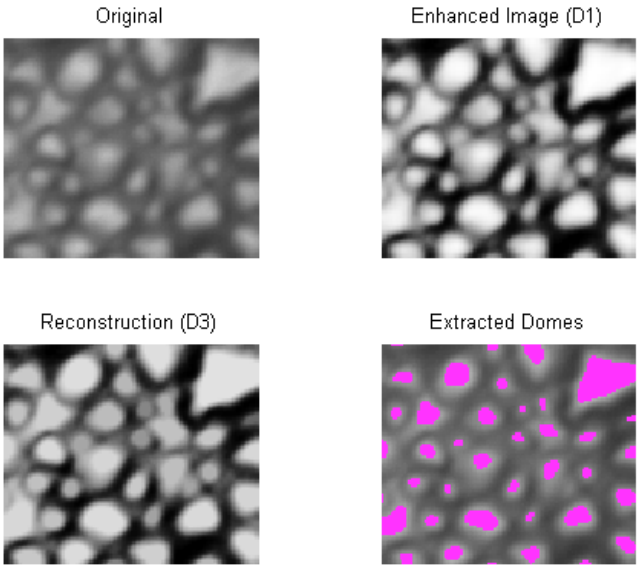


Figure 2: Gold standard sample 4 image and dome extraction

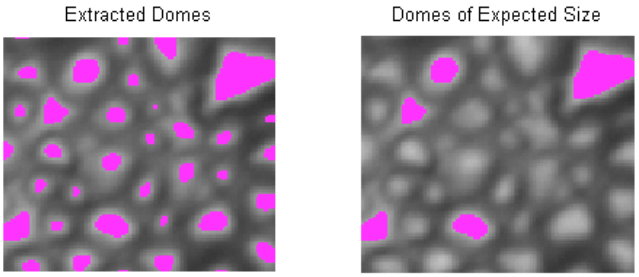


Figure 3: Gold standard sample 4 image with dome extraction and filtered based on 19 pixel expected axon size

In the image of the optical nerve there are sometimes large irregular gaps between the axons. To filter out the larger gaps an opening operation was performed as follows. The original grayscale image was evened by subtracting the background. The background was determined by applying an averaging filter to the original grayscale image with a very large structuring element, nominally 20 times as large as the smallest expected axon size. A histogram equalization was performed on the evened image to produce an image called *E1*. The image *E1* was eroded with a disk structuring element ¼ the expected axon size, *dq*. Allow *s* to represent the expected axon size.

$$q = \frac{s}{4}$$

$$E2 = E1 \ominus d_q$$

Then a morphological opening was performed on the eroded image *E2* with a disk structuring element, d_w, to highlight the regions that are 'open' and unlikely to contain axons.

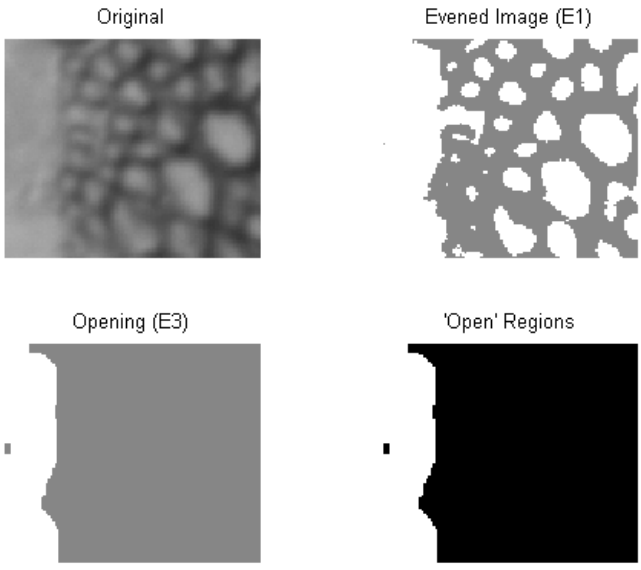


Figure 4: Gold standard sample 6 image with 'open' areas segmented base on 12 pixel expected axon size

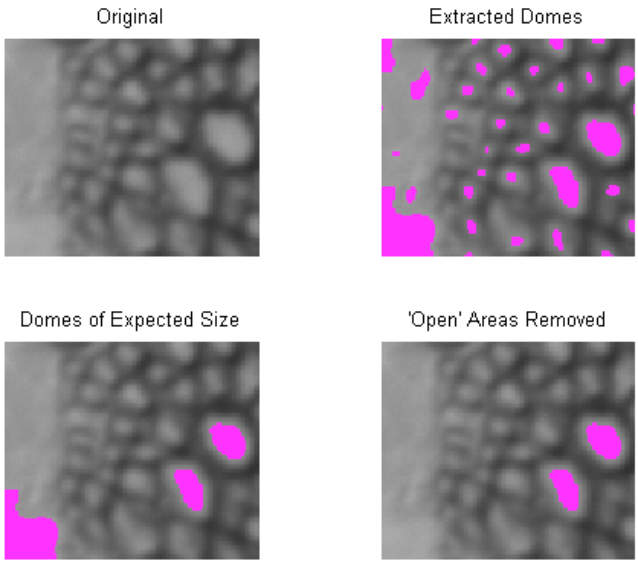


Figure 5: Gold standard sample 6 image with dome removal and filtering based on 19 pixel expected axon size

$$w = \frac{3}{4} \cdot s$$

$$E3 = E2 \circ d_w$$

The Otsu thresholding technique was applied to the opened image *E3* to segment the areas that were 'open' and unlikely to contain axons. Any domes that overlapped these 'open' regions were removed.

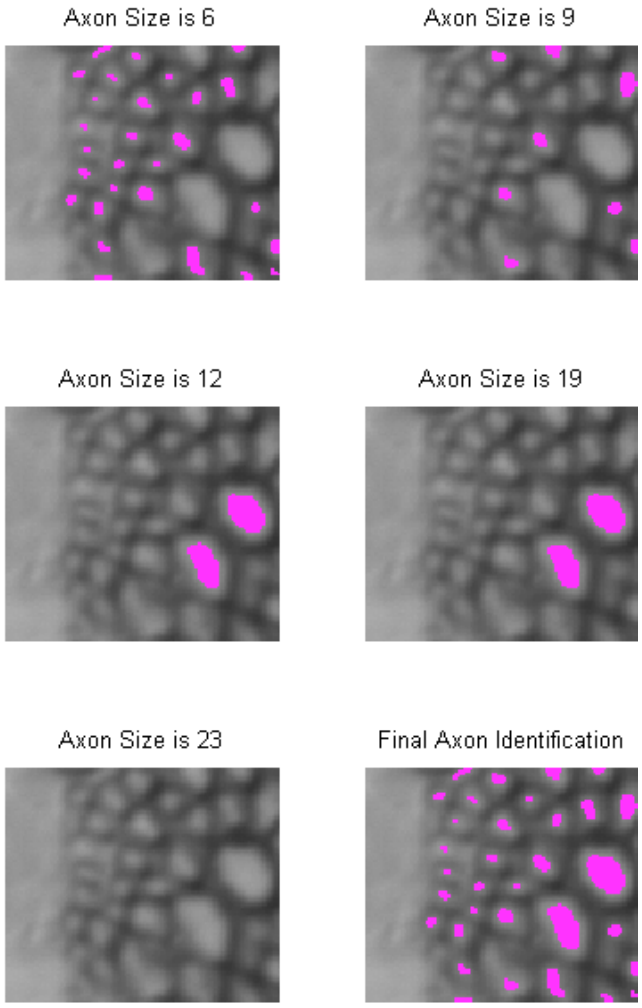


Figure 6: Gold standard sample 6 image axon identification based on expected axon pixel size and the union of all the iterations

The gold standard sample 6 image had a large region on the left side, for instance, that was void of axons. This opening sequence was used to eliminate any false positives that might have appeared in this region. See Figure 4 and Figure 5.

The dome extraction method, expected axon size filtering, and 'open' area removal were performed to the original optical nerve image once for each expected axon size as determined by extracted the local maxima from the size distribution plot. The axons found in each iteration were combined to produce a final estimation of the axon locations. See Figure 6 for the progression of identifying axons in one of the gold standard sample images.

RESULTS AND DISCUSSION

This method for automatically identifying and counting the number of axons in the mouse optical nerve was applied to a series of "gold standard" images. These images were professionally analyzed and the axon locations are known.

The method in this paper correctly identified 80% - 100% of the axons in the gold standard images. The lowest detection rate (80%) was on sample 1, the dark image with very low contrast. The others were in the 94-100% range.

The results of using the morphological method described in this paper to count the axons in the optical nerve of a mouse show that the axons can be easily identified. However, the spaces between the axons tend to be falsely identified as axons, especially if they are approximately the same size as the axons.

The false detection and miss rate is especially high along the borders of the images. This is due to the partial representation of the axon. For better accuracy any axon that has at least one pixel adjacent to the border should be eliminated as potentially misclassified.

The identification of axons in the digital images could be improved by implemented a method of finding closed contours. The analysis of these contours could distinguish between gaps and axons.

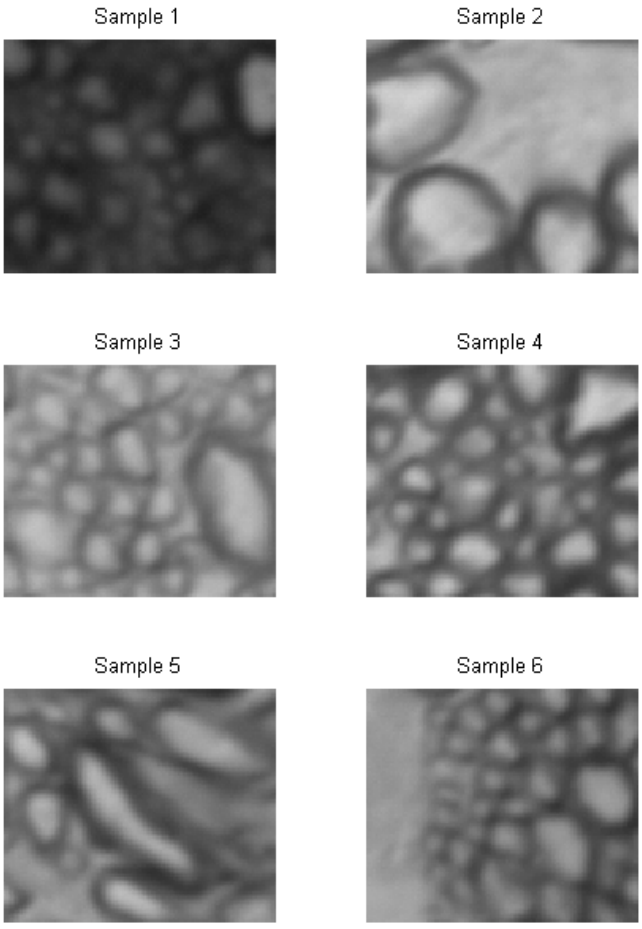


Figure 7: Gold standard optical nerve images

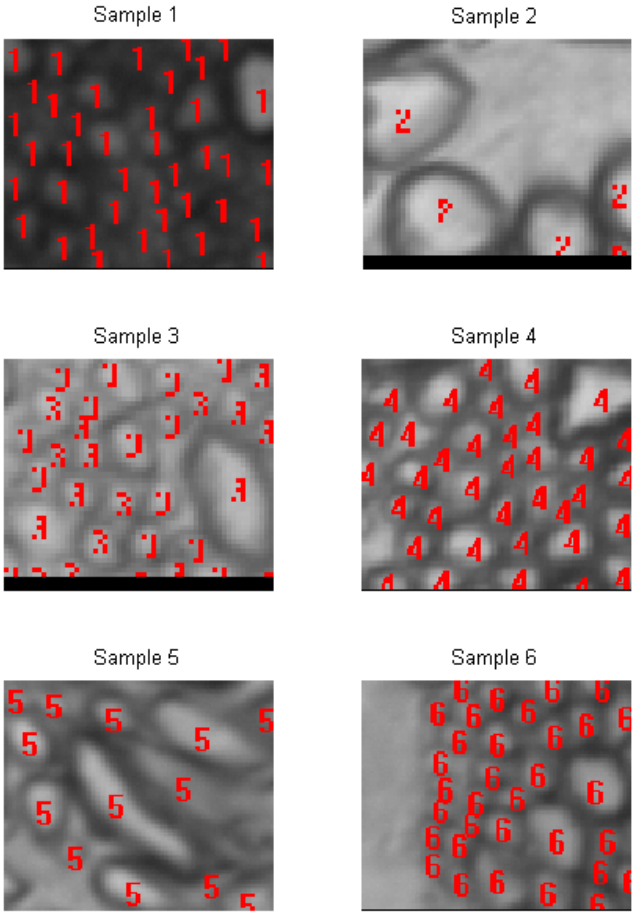


Figure 8: Gold standard optical nerve images professionally counted

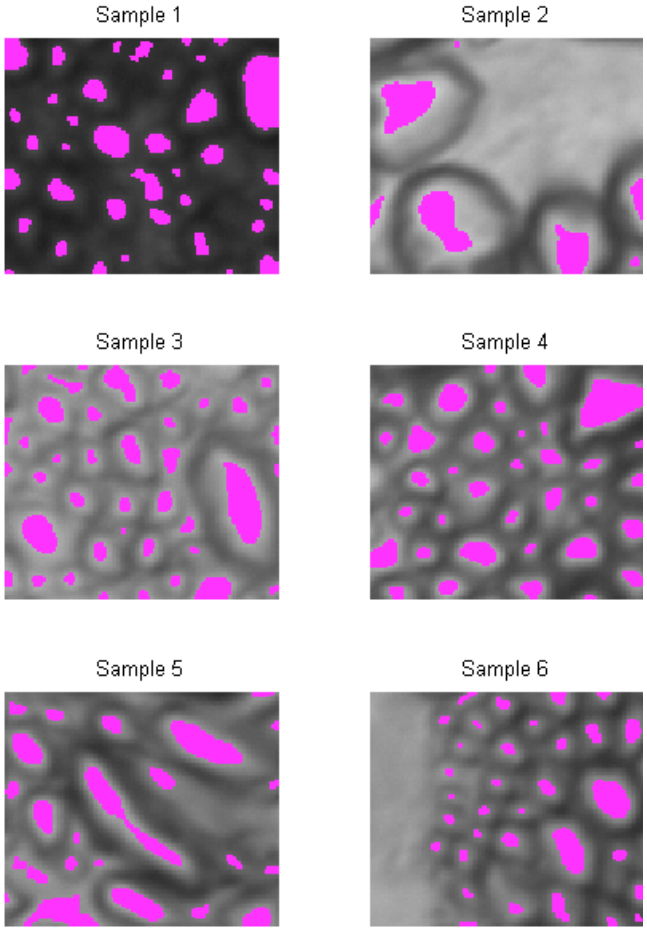


Figure 9: Gold standard optical nerve images counted using the method in this paper

Table 1: Comparison of axon counts

Image	Gold Standard	Morphological Method
1	40	37
2	5	7
3	32	39
4	36	35
5	13	23
6	33	33

Table 2: Success and failure rates

Image	% Match	Miss Rate	False Detection Rate
1	80%	20%	13%
2	100%	0%	40%
3	94%	6%	28%
4	94%	6%	3%
5	100%	0%	77%
6	94%	6%	6%

Table 3: Breakdown of axon counts

Image	Match	Not Detected	False Positives
1	32	8	5
2	5	0	2
3	30	2	9
4	34	2	1
5	13	0	10
6	31	2	2

Table 4: Location of false positives

Image	Interior False Positives	Edge False Positives
1	3	2
2	1	1
3	3	6
4	0	1
5	4	6
6	0	2

Another method than might improve the detection rates would be to implement a method of machine learning based on a neural network approach such as k-means. This requires properly segmenting the gold standard images and specifying a series of identifying properties sufficient to uniquely characterize the axons.

The algorithm was applied to a larger sample image of a mouse optical nerve. The counted image is shown in Figure 10.

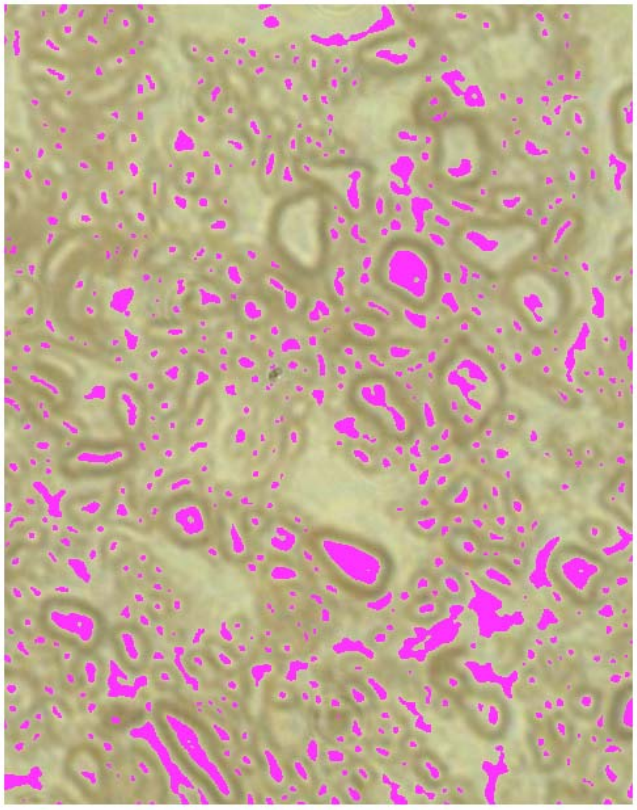


Figure 10: Example mouse optical nerve image with counted axons highlighted

The results of applying the algorithm to the example image were not perfect. The total number of counted axons was 640. However, at least one large axon was missed and several gaps between axons were falsely identified as axons. Several large axons also appeared to have been counted twice. In several spots there were apparently water marks that also caused false identification of axons.

Further refinement of the algorithms presented in this paper are necessary to improve the accuracy of automatically quantifying axons in digital images of mouse optical nerves.

SUMMARY

A morphological image processing method was presented to automatically quantify the number of axons in the mouse optical nerve. The algorithm detected 80% - 100% of the axons in the prepared gold standard digital images, however the false detection rate was as high as 31% for highly elliptical axons if all axons located on the border of the image were ignored.

It was shown that this algorithm worked best on smaller images where axons are generally similar and only slightly elliptical. Further work to improve the false detection rate would be to find and characterize the axon boundaries since the gaps between axons tend to be non-elliptical with sharp corners.

Automated quantification of optical nerve axons will aid in the study of the mouse neural system and transgenic experiments.

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