A New Method for Automated Identification and Morphometry of Myelinated Fibers Through Light Microscopy Image Analysis

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Abstract Nerve morphometry is known to produce relevant information for the evaluation of several phenomena, such as nerve repair, regeneration, implant, transplant, aging, and different human neuropathies. Manual morphometry is laborious, tedious, time consuming, and subject to many sources of error. Therefore, in this paper, we propose a new method for the automated morphometry of myelinated fibers in cross-section light microscopy images. Images from the recurrent laryngeal nerve of adult rats and the vestibulocochlear nerve of adult guinea pigs were used herein. The proposed pipeline for fiber segmentation is based on the techniques of competitive clustering and concavity analysis. The evaluation of the proposed method for segmentation of images was done by comparing the automatic segmentation with the manual segmentation. To further evaluate the proposed method considering morphometric features extracted from the segmented images, the distributions of these features were tested for statistical significant difference. The method achieved a high overall sensitivity and very low false-positive rates per image. We detect no statistical difference between the distribution of the features extracted from the manual and the pipeline segmentations. The method presented a good overall performance, showing widespread potential in experimental and clinical

settings allowing large-scale image analysis and, thus, leading to more reliable results.

Keywords Medical imaging · Automated object detection · Biomedical image analysis · Image Segmentation · Morphometry

Introduction

The evaluation of peripheral nerves is often complemented by morphometric analysis in both clinical and research settings [1]. Morphometric analysis is described as a series of procedures that allows a quantitative description of structure, particularly revealing minimal morphological differences between form and function [2]. These procedures can be briefly described as the extraction and analysis of a set of relevant features referred as morphometric features.

Morphometric analysis is extensively used in studies concerning nerve repair [3], regeneration [4, 5], and implant and transplant [6, 7]. Moreover, morphometric analysis has also been widely used in the investigations of animal models for diseases known to compromise the peripheral nervous system, such as diabetes [8, 9], hypertension [10, 11], and aging [12, 13], with a promising insight to a better understanding of human neuropathies.

The morphometric analysis process consists of extracting morphometric features to analyze them later. This process can be performed manually or with the aid of computer-based image analysis systems with certain degrees of automation [14]. Therefore, we can classify the methods for morphometric analysis of myelinated fibers as manual, semiautomated, or fully automated. Any method demanding manual attention to individual nerve fibers is considered manual. Semiautomated methods generally request the user assistance in some stages

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of the myelinated fibers identification, while automated methods require no user mediation to identify and measure fibers.

Manual methods are described by many authors as tedious, time consuming, laborious, and difficult to perform correctly [14–17] because of the large number of fibers in each nerve fascicle. The image in Fig. 1, for example, represents a region of the laryngeal recurrent nerve of a rat with 131 myelinated fibers. The whole recurrent laryngeal nerve of an adult rat has around 2000 fibers. Additionally, exhaustion and subjectivity decision can be possible sources of error [18].

To reduce the number of fibers demanding analysis, many researches have adopted different sampling schemes for estimating morphometry features such as fiber diameter, area, or perimeter. Although, according to some authors, the number of fibers required to achieve a good estimation can be reduced, the morphometry of certain nerves still remains unfeasible because of their large population of fibers. For large nerves, where fiber size is highly variable, no sampling method is considered adequate for estimating the fiber size distribution correctly. Therefore, sampling schemes can still represent another possible source of error [15].

Semiautomated methods are still a very exhaustive process for the user in studies of nerves with a large population of fibers, despite of reducing significantly the labor input [16, 17]. Thus, there is a strong motivation for automated morphometry.

Nonetheless, automated morphometry is still prone to error originated from the conversion of misdetected particles and spurious data [17]. Several authors have pointed out the underestimation of small, illegible, or irregular axons by automated morphometry because of their poor contrast and various shapes for automated identification [19, 20]. Image processing methods for morphometry have currently achieved an encouraging advance [15], leading to results accurate enough for diagnosis and research purposes.

Fig. 1 Transversal cut of the recurrent laryngeal nerve of an adult rat

Concerning fiber segmentation methods, the majority of algorithms proposed or used by authors are generally based on standard methods such as histogram threshold, Hough transform, mathematical morphology, active contours, and edge detection. Many authors choose thresholding as a first step to segment the myelin sheaths of the fibers. Local or global histogram-based threshold [14, 18, 21] only segments images according to each gray level of pixel. However, by neglecting the space relation among pixels and any additional information that could be provided by the color, these algorithms produce many false-positives (FPs).

It is common that the use of mathematical morphology for the analysis of images at their structural level [20–23] to detect the myelin sheaths and axons and to eliminate FPs. However, as a result of the differences in shape from one nerve to another, it is necessary the adjustment of the structuring element and other parameters on these methods. Hough transform, active contours, and edge detection are also usually applied as the next step to analyze the image at a structural level. However, such methods are too complex to implement [17].

Other studies generally use flexible methods which can be adapted to the desired morphology encountered in the fibers. Wang [16] uses the watershed algorithm varying its immersion level to segment sciatic nerve fibers of adult rats. The system uses fuzzy logic to determine the finest level of immersion. Additionally, it uses a rule-based fuzzy method to eliminate false-positives, based on size, thickness, and location. Zhao [17] uses a region-growing procedure for segmenting optic nerve fibers of adult rats using feature information about the eight-neighboring pixels. Fibers are identified by region labeling and separated by using the maximum gradient magnitude of the outer annulus.

In a previous study [22], we have compared two pipelines for fiber segmentation of the laryngeal nerve and vestibulocochlear nerve. The first, based on mathematical morphology techniques, has shown better results on the segmentation of the vestibulocochlear nerve. The second, based on a color-analysis technique, has shown better results for the segmentation of the laryngeal recurrent nerve. The results have shown that although color analysis is a good method for fiber segmentation, it requires a good pixel distribution in the color space to form distinguishable clusters to be considered an effective technique. On the other hand, mathematical morphology has proved to be an effective way of segmenting regular-shaped fibers.

Color analysis has proven to be an efficient way to deal with light microscopy segmentation problems [24, 25] because of the characteristic coloring that structures of interest acquire during the staining process. Additionally, methods so far proposed for the morphometric analysis of myelinated fibers in light microscopy disregard color. Considering that our previous study has demonstrated that color analysis could be feasible depending on the pixel distribution in the color space,

we propose and evaluate herein a new method for the automated morphometry of cross-section light microscopy images of myelinated fibers using color analysis based on a selection of specific color features.

Materials and Methods

Color Analysis Algorithm

Initially proposed in [26], the technique we adopted to identify the myelin sheaths uses vector quantization as a replacement for traditional clustering and aims to divide the feature space according to the least sum of squares, resulting in the most separable clusters. This technique was firstly proposed to act as a multi-thresholding scheme for segmenting color images as a result of the limitations of traditional thresholding schemes. It also proved to be more flexible to the arrangement of pixels in the color space and resulted in better segmentation than traditional multi-thresholding schemes.

Firstly, let a pixel be defined as an input vector $X_j = (x_1, x_2, x_3, \dots, x_n)$, where x_i represents a color feature, and an image I be represented by a set of pixels $I = (X_1, X_2, X_3, \dots, X_m)$. Let a cluster C_i be a partition with every pixel X_j that is closer to its weight vector $W_i = (w_1, w_2, w_3, \dots, w_n)$ than any other weight vector W_k , be defined as

$$X_j \in C_i \Leftrightarrow \forall k (k \neq i) \left\| X_j - W_i \right\|^2 < \left\| X_j - W_k \right\|^2, \quad (1)$$

where W_i is given by every pixel X_j that the cluster C_i has won during the iterations of the algorithm. In each iteration, the clusters compete with each other for one pixel. The winner updates its own weight vector accordingly to the pixel's input vector. Considering this, W_i can be described as:

$$W_i = \frac{\left(\sum_{X_k \in C_i} X_k \right)}{m}, \quad (2)$$

where m is the amount of pixels that C_i has won. In view of these definitions, we describe this algorithm next.

Initialization

The first cluster is initialized, with its weight vector W_0 equals $W_0 = (0, 0, 0, \dots, 0)$. Additionally, this cluster is assigned a variable *wins* that is initialized to 0. This variable represents the number of victories that this cluster has achieved. Also, we initialize the number of iterations $N_i = 0$. The win threshold θ_i and the maximum number of iterations N_{max} are given, respectively, by Eqs. (3) and (4), which are defined in [24].

$$\theta_i = 400\sqrt{K}, \quad (3)$$

$$N_{max} = \theta_i(2K-3)(K+7) \quad (4)$$

where K represents the maximum number of cluster that can be created.

Competitive Learning

The competitive learning employed in this algorithm can be described as a series of steps: (a) select one random input vector X_j from I . (b) Select a winner cluster C_{winner} that, according to Eq. (1), minimizes the distance between X_j and W_{winner} . (c) Update the winner weight vector according to Eq. 4 and increase by one the *wins* variable of C_{winner} . If *wins* have reached θ_i , create a new cluster C_k , where $W_i = W_{winner}$, set the *wins* variable of both clusters to 0. (d) Increase by one N_i . If $N_i < N_{max}$, repeat from (a).

Animal and Tissue Preparation

This study used images of the recurrent laryngeal nerve, which were obtained from 12 adult rats, and of the vestibulocochlear nerve, which were obtained from four adult guinea pigs. Animals were anesthetized with sodium pentobarbital (Nembutal, 40 mg/kg, i.p.) and perfused through the left ventricle with a 0.05-ml phosphate-buffered saline solution, pH 7.4, and then with 2.5 % glutaraldehyde solution, in 0.1 ml cacodylate buffer, pH 7.2.

Nerves were carefully dissected without stretching, removed in one piece, and placed in the fixative solution for additional 12 h. They were washed in cacodylate buffer, pH 7.2, and processed for epoxy resin embedding. Semi-thin transverse sections of the fascicles were stained with 1 % toluidine blue and observed with the aid of an Axiophot photo microscope (Carl Zeiss, Jena, Germany) under an oil-immersion lens ($\times 100$). Images of 636×474 pixels were captured by a digital camera (TK-1270, JVC, Victor Company of Japan, Ltd., Tokyo, Japan) and then downloaded to the computer.

A total of 54 images were used in the present study. Forty-two were from the 12 recurrent laryngeal nerve, and 12 were from the four vestibulocochlear nerves. Every image was segmented manually by a well-trained neurologist with years of experience in manual segmentation of myelinated fibers for later evaluation of the algorithms of segmentation.

A previous study [27], using the same method for manual segmentation investigated in this study, showed that for manually segmented images, morphometric results were not statistically different among observers, thus validating the method and excluding the need for the manual segmentation by two or more observers.

The experiments with recurrent laryngeal nerve were approved by the Institutional Ethics Committee for Animal Research (CETEA—*Comitê de Ética em Experimentação Animal*, protocol numbers 139/2006 and 169/2006) from the

School of Medicine of Ribeirão Preto, University of São Paulo, Brazil. The experiments with vestibulocochlear nerve were approved by the Institutional Ethics Committee for Animal Research (CEEa—*Comitê de Ética em Experimentação Animal*, protocol number 064/2005) from the Biological Center of the Federal University of Pernambuco, Brazil.

Proposed Method

Color Feature Selection

Because segmentation results of color images are extremely dependent on pixel distribution on their color feature space [26] and the intensity distribution of pixels may vary according to the used color transformation, analyzing and selecting the possible color transformations and their features represent an important step toward good results.

To select the best subset of color features, we analyzed different color models based on the Pearson product–moment correlation coefficient. The different color models taken into account were CieLAB (L^* , a^* , b^*), CieLUV (L^* , u^* , v^*), XYZ (X , Y , Z), and RGB (R , G , B). To perform this analysis, we took a random sample of pixels from every image for each nerve and each color model. Depending on the manual segmentation of each image, these pixels were classified as myelin or non-myelin pixel. Table 1 shows the Pearson correlation coefficient between the color features of the analyzed models and the pixel class for each nerve.

L , G , R , X , Y , and Z show a strong correlation with the pixel classification, where L is presented as the most

descriptive feature. L is defined as the luminosity of a pixel; therefore, the strong correlation with the pixels classification is explainable by the distinct darker colors displayed by myelin pixels. B shows a weak correlation because of the low difference in intensity of these color features between myelin and non-myelin pixels. This is due to the used stain, toluidine blue. R , G , X , Y , and Z are more correlated to the myelin coloring due to the same reason.

It is also noticeable that although nerves are different, the most representative color features remain the same because the same material preparation was made for both nerves. However, it can also be seen that the color correlation of the proposed features for the vestibulocochlear nerve is significantly lower.

Fiber Segmentation

The pipeline used in the proposed method to segment myelinated fibers is divided into five steps that are described next. The whole pipeline is based on the technique presented in [26] to roughly identify the myelin sheaths and in [28] to later separate fibers and identify them individually. Figure 2 illustrates the result of each step for an image of the vestibulocochlear nerve.

- Step 1. We apply the competitive clustering algorithm [26] to the image, which results in an image with pixels divided into clusters. L , R , G , X , Y , and Z were the color features used because they demonstrated a strong relationship between the pixel classification with myelin or non-myelin.
- Step 2. Myelin sheaths are particularly darker than any other structure; therefore, the cluster representing the myelin pixels is defined as the one with the lowest centroid value. The image is binarized using the myelin cluster, dividing image pixels into myelin and non-myelin.
- Step 3. To eliminate possible noise caused by wrongly labeled pixels, morphological filtering is applied by using area-closing and area-opening operators. As result of the differences in shape and size between the laryngeal and the vestibulocochlear nerve, different values of area are used.
- Step 4. We apply the clump splitting algorithm [28] using concavity analysis to separate any fibers that remained together in the same binary clump. Because there is no difference in the concavities formed between the two fibers, there is no need to redefine the thresholds for both laryngeal and vestibulocochlear nerves. A topological filter is then applied to eliminate any holeless objects, eliminating most of the FPs. A morphological filter is employed to eliminate any objects touching the

Table 1 Pearson product–moment correlation coefficient between color features and the classification of pixels as myelin or non-myelin for laryngeal and vestibulocochlear nerves. The color models analyzed were CieLAB (L, a^*, b^*), CieLUV (L, u^*, v^*), RGB (R, G, B) and XYZ (X, Y, Z)

Color feature	Pearson coefficient	
	Laryngeal	Vestibulocochlear
L	<i>−0.838</i>	<i>−0.743</i>
a^*	0.626	0.514
b^*	−0.647	−0.553
u^*	−0.225	0.272
v^*	−0.504	−0.504
R	<i>−0.834</i>	<i>−0.700</i>
G	<i>−0.833</i>	<i>−0.740</i>
B	−0.677	−0.669
X	<i>−0.832</i>	<i>−0.736</i>
Y	<i>−0.835</i>	<i>−0.741</i>
Z	<i>−0.722</i>	<i>−0.700</i>

Values displayed in italics are presented as the most descriptive features

Fig. 2 Proposed method pipeline for the segmentation of myelinated fibers

image boundaries. This eliminates fibers only partially appearing in the image, which is a required procedure to avoid wrong measurements, even in the manual morphometric analysis.

Morphometric Feature Extraction

The extracted morphometric features were (1) fiber area, (2) axon area, (3) fiber diameter, (4) axon diameter, and (5) G ratio. The fiber area is calculated as the sum of myelin sheath and axon area. The axon (and fiber) diameter is defined as the smallest distance between a pair of antipodal points belonging to the convex hull of the object, i.e., the minimum diameter. We use the minimum diameter because of the obliquity in the cross-sections of the lamina, which causes a high variability of the maximum diameter and other features, making these inaccurate measures.

G ratio is defined in Eq. (5) as the ratio between the fiber diameter and axon diameter [29]. This feature represents the neural impulse conduction speed, correlating the myelin sheath thickness and axon size. This ratio has been useful for theoretical extrapolations related to saltatory conduction. G ratio values between 0.6 and 0.7 are those in which the driving speed of myelinated fibers has been determined maximum.

$$G = \frac{\text{axon_diameter}}{\text{fiber_diameter}} \quad (5)$$

Proposed Method Evaluation

In order to properly assess the proposed method, we carried out two evaluations. The first evaluation is a quantitative analysis comparing the fiber segmentation result of each image produced by the proposed method with the respective manual

segmentation of that image. The second evaluation is a comparative analysis between the morphometric features extracted from the manual and the proposed method segmentation. Both evaluations are described in detail next.

Fiber Segmentation Evaluation

Let A be the binary image resulted from the segmentation of the pipeline, and B be the binary image resulted from the manual segmentation. The following metrics were used for the evaluation of the proposed method:

Area similarity (AS)

This metric represents the similarity between the areas of A and B . This metric is calculated as the ratio between two times the intersection of the areas of A and B , by the sum of the areas of A and B [16]. Therefore, the AS can be defined as

$$AS = \frac{2(A \cap B)}{A + B} \quad (6)$$

A larger value of AS indicates a better agreement between A and B .

Area overlap error (AOE)

The AOE is a measure of the difference between A and B . This metric is calculated as the difference between the union and intersection of the areas of A and B , divided by the area of B .

$$AOE = \frac{(A \cup B) - (A \cap B)}{B} \quad (7)$$

Sensitivity (SS)

This metric allows us to quantify our method capacity to correctly identify fibers. Assuming that the false-negatives (FNs) represent the number of fibers that were not detected and the number of true-positives (TPs) is

equal to the number of fibers that were correctly detected, we can define SS as

$$SS = \frac{TP}{TP + FN}. \quad (8)$$

False-positive rate (FPR)

The FPR measures the percentage of FPs detected by the proposed pipeline. FPs cause bias in the extracted feature distributions [21]. This is a greater issue for automated morphometric analysis methods since we are inserting spurious data into the real feature distributions. The FPR is defined as

$$FPR = \frac{FP}{TP + FP}. \quad (9)$$

Extracted morphometric feature evaluation

To evaluate significant differences between the morphometric features extracted from fibers segmented manually and segmented by the proposed method, we firstly created histograms for each extracted feature distribution. Then, we tested the manual and automatic distributions for significant statistical differences. Firstly, we applied the Kolmogorov–Smirnov normality test, with significance for $p < 0.05$. If the distributions succeed in the normality test, we compare them using t test, with significance for $p < 0.05$; otherwise, we compare them using the Wilcoxon test, with significance for $p < 0.05$.

In these last comparisons, a value greater than 0.05 shows that there is no significant difference between the distributions.

Results and Discussion

For the assessment of our results, 54 images were tested and the results for each nerve were averaged for each defined metric. The same algorithm was applied to both the recurrent laryngeal and vestibulocochlear nerves, except for an adjustment made in the values of parameters for area closing and area opening operators in step 3 of the pipeline. Figures 3 and 4 compare the segmentation results of the proposed and manual methods for the vestibulocochlear and recurrent laryngeal nerves, respectively. It is noticeable that too irregular fibers usually pass undetected by our method. This happens because of the concavities constituting the boundaries of these fibers, which causes them to split and be eliminated by the pipeline.

Table 2 shows a summary of the results achieved for all the images evaluated. In order to evaluate the nerves individually, the mean and the standard deviation for the images related to the recurrent laryngeal nerve and to the vestibulocochlear nerve were calculated separately. The differences between the results presented for the laryngeal and vestibulocochlear nerves were expected due to the better correlation of the color features and class of the pixels in the laryngeal nerve, which were shown in Table 1. This shows us that the pixels in the laryngeal nerve images have a distribution in the color space more favorable to the used clustering algorithm than the pixels in the vestibulocochlear nerve.

Fig. 3 Fiber segmentation result of the proposed method compared to the manual segmentation for the vestibulocochlear nerve. **a** Proposed method segmentation. **b** Manual segmentation. **c** Overlap of the original image and the contour of the automated segmentation (red lines). **d** Overlap of the manual segmentation and the contour of the automated segmentation (red lines)

Fig. 4 Fiber segmentation result of the proposed method compared to the manual segmentation for the laryngeal recurrent nerve. **a** Proposed method segmentation. **b** Manual segmentation. **c** Overlap of the original image and the contour of the automated segmentation (*red lines*). **d** Overlap of the manual segmentation and the contour of the automated segmentation (*red lines*)

When compared to our previous study [22], the proposed method has shown an improvement for the segmentation of the laryngeal nerve and the vestibulocochlear nerve, as we can see by comparing Tables 2 and 3. We performed a statistical analysis and reached the following results: for the laryngeal nerve, all metrics presented statistical significant improvement, except the FPR metric; for the vestibulocochlear nerve, only the AOE presented statistical significant improvement, whereas SS presented better results in the earlier study. It is important to notice that, for morphometry, AOE is a more relevant metric than SS because AOE leads to more accurate morphometric measures. We accredit such improvement to the combination of operations defined in the segmentation pipeline; plus, the feature selection done in the early stages was used to choose the most representative color features. This selection allowed the embedding of a priori knowledge to the competitive clustering algorithm. Moreover, the clustering of the pixels in the color space provides a more flexible and adjustable threshold to most images when compared to more traditional threshold schemes such as those presented in

[14, 18, 21] and a simpler approach when compared to more complex methods such as those presented in [16, 17].

Nevertheless, the usage of different stains or the difference in adherence of the stain to different fibers may affect the pixel distribution of the myelin sheaths, affecting the overall system segmentation performance and requiring proper adjustments. Our method was adjusted for the toluidine blue stain.

The use of morphological operations as filters results in a loss of fibers because of the variation of fibers in form and size of the same nerve. A visual evaluation of the segmented images revealed that the majority of the fibers not detected or eliminated from the process by morphological filters had small diameters. This has been an issue present in other studies. Nevertheless, this was not an important source of error in our method, as shown by FPR in Table 2.

Considering the morphometric feature distribution, we show in Fig. 5 the histograms for each of the extracted features, comparing the manual and the proposed method segmentations for the laryngeal nerve. The histogram for the minimal fiber diameter shows that most of the fibers

Table 2 Evaluation of the fiber segmentation pipeline in our current study

Nerve	Metric							
	AS_{μ}	AS_{σ}	AOE_{μ}	AOE_{σ}	SS_{μ}	SS_{σ}	FPR_{μ}	FPR_{σ}
Laryngeal	0.946	0.037	0.110	0.078	0.949	0.044	0.023	0.034
Vestibulocochlear	0.760	0.076	0.491	0.159	0.757	0.114	0.004	0.006

AS area similarity, AOE area overlap error, SS sensitivity, FPR false-positive rate

Table 3 Evaluation of the fiber segmentation pipeline in our earlier study

Nerve	Metric							
	AS_{μ}	AS_{σ}	AOE_{μ}	AOE_{σ}	SS_{μ}	SS_{σ}	FPR_{μ}	FPR_{σ}
Laryngeal	0.908	0.054	0.179	0.131	0.907	0.097	0.019	0.031
Vestibulocochlear	0.688	0.113	0.791	0.244	0.810	0.107	0.001	0.003

AS area similarity, AOE area overlap error, SS sensitivity, FPR false-positive rate

that are lost in the segmentation process are those with a small diameter ($<4\mu\text{m}$). The histogram for the axon diameter shows a small overestimation of the number of axons with a diameter smaller than $3\mu\text{m}$. This behavior is also present in the histograms for the axon area. Such behavior is expected because of the differences between the manual and the automatic segmentations. This can also be seen in the histograms for the fiber area, where the comparison between the histograms of the manual and proposed methods have their major discrepancies most exclusively for small fibers. These same characteristics are present in the histograms for the vestibulocochlear nerve.

As for the statistical tests, Table 4 shows the results for the applied statistical tests. As we said in the “[Extracted](#)

[Morphometric Features Evaluation](#)” section, if the distribution is normal, the p value is obtained by the t test; otherwise, it is obtained by the Wilcoxon test. Considering significance for $p < 0.05$, we found no statistical differences between the extracted features of the manual segmentation and our pipeline segmentation.

The histograms of the G ratio of a normal nerve are expected to present most of the values between 0.6 and 0.7, as shown by both methods in the current study with no differences between the two methods. Fibers with larger or smaller G ratios can occur in normal nerves due to the remodeling process that occur in the axon during life span. The most important fact for us is that the proposed method was able to reproduce the shape of the normal histograms, with peaks at 0.6–0.7 values, as expected.

Fig. 5 Histogram for each of the extracted morphometric features

Table 4 Statistical differences between measures obtained from the manual and the automatic segmentation. A *p* value greater than 0.05 means no significant difference

Nerve	Measure	<i>p</i> value
Laryngeal	Fiber area	1.000
	Fiber diameter	1.000
	Axon area	0.882
	Axon diameter	1.000
	G ratio	1.000
Vestibulocochlear	Fiber area	0.462
	Fiber diameter	0.350
	Axon area	0.853
	Axon diameter	0.811
	G ratio	0.663

The comparison between our method and other methods in the current literature is difficult due to many differences between (1) the image sets used by each research, (2) the type of nerves that are studied, and (3) the different metrics that each author uses. Nevertheless, in Table 5, we show the results achieved by related efforts and the nerves they used. By analyzing and comparing them to our proposed method, we can state that our method presents an encouraging performance.

Conclusion

This paper presented a method for the automatic morphometry of myelinated fibers from different nerves. We have implemented and tested a method based on the competitive clustering for segmenting myelin sheaths and concavity analysis for later separation of connected fibers. Our method was based on the analysis of color features

to produce a better segmentation depending on the pixel distribution on the feature space. Furthermore, it became clear that the usage of feature selection was an essential step toward the improvement of the overall method performance when compared to our earlier study.

According to the domain specialists, the final results can be used to replace the manual segmentation in most cases. Nevertheless, the user's visual evaluation over the segmented image is still recommended to reduce possible errors.

Since our method is based in clustering pixels in a color feature space, its performance can be reduced if applied to grayscale images. Another remark is that one of the main challenges in using a clustering approach is to determine the optimum values of clusters to be used for achieving the best segmentation in each image. In this study, it has been determined empirically as four. However, it was noticeable that this number is variable depending on the image. Additionally, classifying pixels that belong to the cluster with the lowest centroid as myelin sheath pixels is not an absolute truth since in some cases, artifacts can form such clusters.

We currently aim to develop a methodology for determining the optimum number of clusters to be used in an image and to classify a cluster as belonging to the myelin sheath. This will further improve the achieved results. Also, in the future, we intend to carry out further tests and evaluate our method in other nerves, such as the vagus and sural nerves. This will provide additional information for the general improvement of the method. Additionally, we intend to test the reproducibility of our method applied to nerves processed histologically in different periods of time and also coming from multiple specimens.

Our method has shown widespread potential in experimental and clinical applications, eliminating many of the tedious and time-consuming tasks associated with nerve morphometry and allowing large-scale processing and analysis of images, leading to more accurate and reliable results.

Table 5 Other automatic methods with their respective results and used nerves

Ref.	Authors	Nerve	Results
[16]	Wang Y-Y et al.	Sciatic nerve of adult rats	$AS\mu$ value of 0.91, $AS\sigma$ value of 0.01, mean of modified Hausdorff distance (μm) equal to 0.68 and standard deviation equal to 0.05, mean of true-positive rate equal to 96 % and standard deviation equal to 1.41 %.
[17]	Zhao X et al.	Optical nerve of the right eye of adult rats	The authors did not use any quantitative method for comparing their results against the manual extracted features.
[18]	Auer RN	Sural nerve of an adult human	The author reports that a comparison with manually generated histograms revealed a consistently greater size measurement of fibers using the automatic method.
[20]	Fok YL et al.	Not specified	Overall detection rate (SS) of 0.93 and false alarm rate (FPR) of 0.03.
[21]	Romero E et al.	Sciatic nerve of a female cat	0.92 % of FPR and 6.36 % of missed detections. No significant statistical difference between histograms generated from manual and automatic extracted features.

AS area similarity, *SS* sensitivity, *FPR* false-positive rate

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