

Research Article

Cluster Analysis of Myelin Nerve Fibers of the Peripheral Nerve

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Abstract

One of the unsolved issues in neuromorphology is the classification of myelin nerve fibers (MNF).

Objective: to use cluster analysis to classify the sciatic nerve MNF.

Material and methods. The work was performed using 5 one-year-old male Wistar rats. Semi-thin sections were stained with methylene blue. MNF morphometry was performed using ImageJ, and statistical processing – using the software environment R.

Results of the study. Ward's and k-means methods were used to cluster the MNF. Three clusters of MNFs are defined and their parameters are determined. The presented algorithm for adapting the literature data to the format of the obtained results includes determining the total average for the combined set of each indicator and the total variance, which is the sum of intragroup and intergroup variances.

Conclusions: 1) for the classification of MNF it is advisable to use cluster analysis; 2) clustering should be performed according to the transection areas of the axial cylinder and myelin sheath; 3) the number of clusters is determined by the agglomerative method of Ward, and their metrics – by the iterative method of k-means; 4) three clusters of MNF of the rat sciatic nerve differ in the transection areas of the fibers, the axial cylinder and the myelin sheath and the percentage of nerve fibers; 5) when comparing identical indicators according to the obtained and literature data, the results were equivalent in the areas of the axial cylinder and myelin sheath and their shape coefficients, despite the fact that the classification of myelin fibers and their morphometry was performed using different methods.

Keywords

sciatic nerve; myelin nerve fibers; axial cylinder; myelin sheath; cluster analysis

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Problem statement and analysis of the latest research

Examining at the microscopic level the myeloarchitectonics of peripheral nerves, usually on their transection sections determine the number of myelin nerve fibers (hereinafter referred to as MNF). The fibers are a priori divided by their diameter or transection area (hereinafter – area) into 3 [5, 6, 9, 13,

18, 24] or 4 groups [1, 10, 11, 12, 15]. Some authors distinguish five groups of myelin fibers [17]. Classification of MNF is also carried out with a certain step according to diameter or area of fibers or their axial cylinders [2]. All approaches to the classification of MNF have a right to exist, because they are chosen according to the purpose and objectives of each study, but such classifications are subjec-

tive. The current lack of a unified approach to the classification of MNF according to morphometric parameters creates great difficulties in the correct understanding of the structural organization of peripheral nerves. This circumstance was the reason for our search for a new method of classification of myelin fibers, based on the use of cluster analysis.

Objective. To use cluster analysis to classify myelin nerve fibers, obtain their clusters, and determine the parameters of each.

1. Materials and Methods

The work was performed using 5 sexually mature (one-year-old) male Wistar rats weighing 210–230 g. Animals were kept and manipulated according to general bioethical norms and principles of animal experiments. Euthanasia of animals was performed under thiopental anesthesia (1 g of dry matter of sodium thiopental was dissolved in 10 ml of isotonic solution and administered intravenously at a rate of 0.1 ml per 100 g of body weight). Collection of sciatic nerves (SN), production of epoxy modules and semi-thin sections and their staining with methylene blue was performed according to conventional methods [16]. Immediately after cessation of respiration of animals, SN was isolated on the thigh and cut into pieces, each 1.5–2 mm long. Fixed for 2 hours in a 2% solution of osmium tetroxide (OsO_4) in 0.1M cacodylate buffer (pH 7.2). After washing and dehydration, the nerve pieces were contrasted in 2% uranyl acetate solution.

Semi-thin sections were examined with the help of a Micros Austria MC300 light microscope and photographed using ToupCam 5.1M UHCCD C-Mount Sony camera with a ToupTek Photonics AMA075 adapter using ToupView v. 3 software.

MNF morphometry was performed using ImageJ version 1.47t, developed by the National Institutes of Health (USA) [23] and distributed with the open source without license restrictions, using our original computer program "MorphoTools for ImageJ" [8]. Transverse semi-thin sections of the right sciatic nerve were photographed in random order. At least 10 microphotographs were made for each animal (a total of 58 photofragments with a

total area of about 120 thousand μm^2 were made). To obtain a good optical resolution of the MNF, sections were photographed when magnified $\times 1000$ times. There were determined: the area of the profile fields of the MNF and their axial cylinders; coefficients of shape of nerve fibers and axial cylinders; the area of the myelin sheath, which was calculated as the difference between the areas of each MNF and its axial cylinder. Cluster analysis was performed according to the area of the axial cylinder and the area of the myelin sheath.

Statistical data processing, including cluster analysis, was performed in the software environment R version 3.0. [22]. Clustering was carried out by the transsection area of the MNF and their axial cylinders. Numerical data were presented as mean \pm standard error of mean ($M \pm m$). Taking into account large sample size (1776 MNF), which makes it possible to rely on the assertion of the central limit theorem of probability theory, we can apply Student's t-test to compare the mean values of all metric features (indicators) of different samples. This is also supported by the fact that the standard deviation of the means of all studied indicators was less than half of the arithmetic mean, which indicates the symmetry of the distributions [14]. The difference was considered statistically significant when the significance level $p < 0.05$ was achieved.

2. Results and Discussion

At the first stage, the perimeters of the MNF and their axial cylinders were outlined on microphotographs (Fig. 1). The number of allocated MNFs was 1776.

During the second stage, the MNF was clustered according to two morphometric features: the area of the myelin sheath and the area of the axial cylinder. Ward's agglomerative hierarchical method was used to determine the number of clusters (hereinafter CL), the results of which are presented in the form of a dendrogram (Fig. 2), on the vertical axis of which intercluster distances are marked, and on the horizontal one – MNF numbers, which are combined step by step. To select the number of CL, a "section" of the dendrogram is performed,

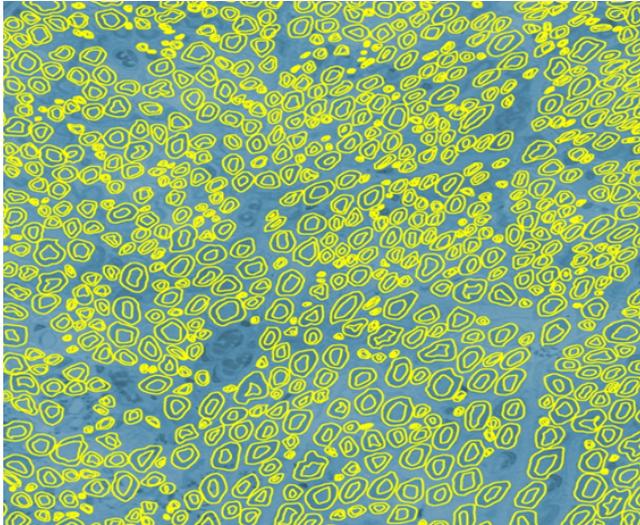


Figure 1. Semi-thin section of the sciatic nerve of one of the rats. The yellow line highlights the contours of the axial cylinder and the myelin sheath of each myelin nerve fiber. Methylene blue staining. Photomicrograph. Magnification: $\times 1000$.

which is recommended to be performed at the step at which the largest change in the intercluster distance took place [20]. The graph shows that such a jump in intercluster distance (about 4.500 units) occurred on the next to last step. In the previous and the last steps, these distances are smaller (within 2300-2500 units). As a result, 3 clusters of MNF were identified, which are outlined by a red line.

During the third stage, in order to determine the morphometric parameters of the MNF of each cluster, the method of clustering of k-means was used. The three CL of MNF in the graph (Fig. 3) are represented in the coordinate field, where the abscissa axis – is the area of the axial cylinders, and the ordinate axis – is the area of their myelin sheath. The MNF of each of the CLs are marked with different colors: CL 1 (red color), CL 2 (green color), CL 3 (brown color). The "star" marker shows the centers of the CLs.

Figure 3 shows that in the cluster structure of the sciatic nerve of the rat CLs do not overlap each other, while located next to each other and are not separated by areas of empty space. This indicates that the MNFs of all CLs together form a single structural system [7, 19, 21] – myeloarchitectonics

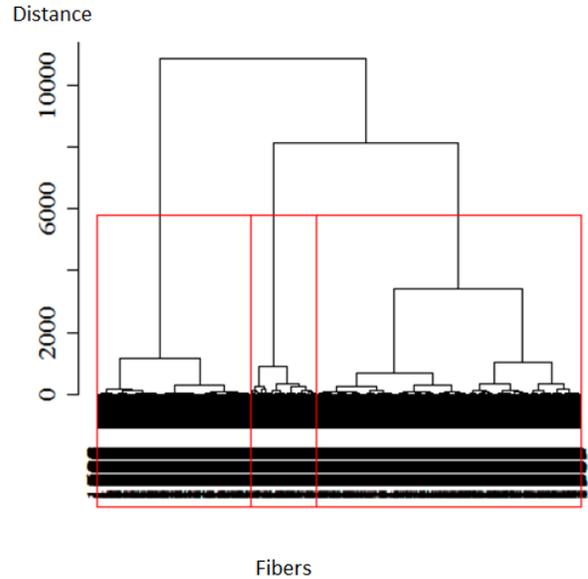


Figure 2. Dendrogram of clustering of myelin nerve fibers (metric – Euclidean, method – Ward's). The red line outlines 3 clusters.

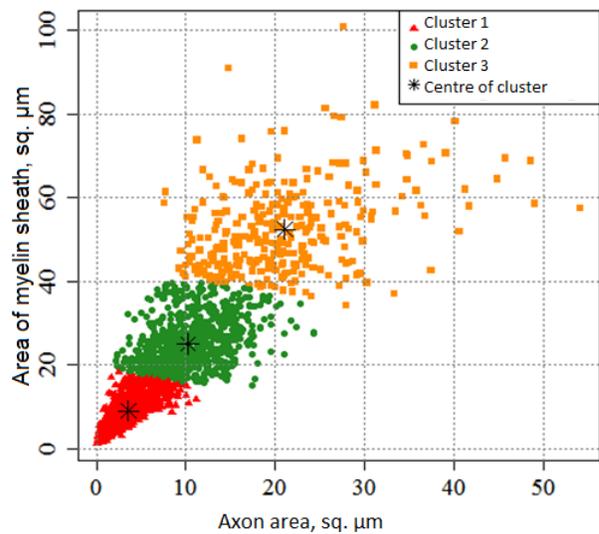


Figure 3. Cluster structure of the set of studied myelin nerve fibers of the sciatic nerve of the rat in the coordinate field

of rat's SN. Clusters of MNFs differ in two metric signs, according to which their classification is carried out, i.e., in the areas of axial cylinders and their myelin shells, as well as in the grouping of

elements. The centers of the clusters are located along the ascending line, which is characteristic of the normal structural systems [21]. The elements of CL 3 are most diffusely placed, many of them deviate significantly from its center.

The values of morphometric parameters of MNF of each of the clusters were determined by the method of clustering of k-means, namely: the area of MNF, the area of their axial cylinders and myelin sheath, the coefficients of MNF shape and their axial cylinders (Table 1).

Multiple pairwise comparison of the obtained clusters with each other is important, according to the different metric features, the results of which are represented in Table 2.

Table 2 shows that all CL according to the indicators, which are highlighted in bold, differ statistically significantly. Achieved by pairwise multiple comparison, a high level of statistically significant difference between the areas of the axial cylinder and the myelin sheath indicates that the classification of MNF is performed convincingly correct. According to the shape coefficients of myelin fibers and their axial cylinders, the CLs do not differ statistically significantly. Thus, using cluster analysis, we've classified the rat sciatic nerve MNF according to two morphometric features (the area of the profile field of the axial cylinder and its myelin sheath), identifying among their study three clusters (groups), which are justified from the standpoint of multidimensional statistics. In addition, a number of morphometric parameters of the MNF of each cluster were obtained. The latter makes it possible at the fourth stage of the study to compare our results with others presented in the literature. We were most interested in the results of experimental studies, in which there was studied the sciatic nerve of a laboratory rat and they determined the same indicators as we did. Based on this, we compared our results with the data of the famous neuromorphologist SB Herashchenko [2] (Table 3).

As one can see from Table 3, the area of MNF CL 1 corresponds to the area of MNF of the smallest caliber ($S < 20 \mu m^2$), and CL 2 – to the area of MNF of the next caliber ($40 > S > 20 \mu m^2$), which are represented in this work [2]. Instead, MNF CL

3 combines (by fiber areas) nerve fibers of the three largest calibers ($60 > S_1 > 40$; $80 > S_2 > 60$ and $100 > S_3 > 80 \mu m^2$), which the author identified according to the aim and objectives of the study. In this format it is impossible to make a statistical comparison between the indicators obtained in two different laboratories. However, in descriptive statistics there is a mathematical apparatus, which in the presence of two or more sampling rate of the same indicator allows us to determine its total mean according to the total population and calculate the total variance [3, 4]. These are the following formulas:

$$M_{total} = \frac{\sum N_j M_j}{\sum N_j}, \text{ where :} \quad (1)$$

M_{total} – the total mean for the total population (in our case, the total mean of the three groups of any morphological indicator), M_j – group mean of group j ; N_j – the volume of group j .

$$D_{total} = D_{intrarg} + D_{intrerg}, \text{ where :} \quad (2)$$

D_{total} – total variance, i.e., the variance of the values of the morphological feature according to the total population relative to its total mean.

$$D_{intrarg} = \frac{\sum N_j D_j}{n}, \text{ where :} \quad (3)$$

$D_{intrarg}$ – intragroup variance, i.e., the arithmetic mean of group variances weighted according to the group volumes; N_j – group volume j ; D_j – dispersion of group j ; n – the volume of the total (combined) population.

$$D_{intrerg} = \frac{\sum N_j (M_j - M)^2}{n}, \text{ where :} \quad (4)$$

$D_{intrerg}$ – intergroup variance, i.e., the variance of group means relative to the total mean for the entire sample; N_j – group volume j ; M_j – group mean of the group j ; M – general mean; n – the volume of the total population.

Table 1. The value of metric indicators of myelin fibers of different clusters of rat's the sciatic nerve (M ± m).

Indicator	Clusters		
	CL1	CL 2	CL 3
area of MNF	12.49 ± 2.88	35.25 ± 3.62	73.49 ± 6.71
area of axial cylinder	3.54 ± 1.05	10.23 ± 1.65	21.03 ± 3.51
area of myelin sheath	8.95 ± 1.98	25.02 ± 2.65	52.45 ± 4.66
Coefficient of the shape of MNF	0.85 ± 0.06	0.86 ± 0.07	0.86 ± 0.07
Coefficient of the shape of axial cylinder	0.73 ± 0.006	0.75 ± 0.05	0.73 ± 0.05

Notes: area – transsection area; CL – clusters; MNF – myelin nerve fibers; (M ± m) – mean value ± standard error of the mean.

Table 2. The results of a pairwise multiple comparison of different clusters of myelin nerve fibers of the sciatic nerve.

Comparison of CL according to	Statistical significance	
	CL 1	CL 2
area of MNF		
CL 2	p < 0.001	–
CL 3	p < 0.001	p < 0.001
area of axial cylinder		
CL 2	p < 0.001	–
CL 3	p < 0.001	p < 0.001
area of myelin sheath		
CL 2	p < 0.001	–
CL 3	p < 0.001	p < 0.001
coefficient of the shape of MNF		
CL 2	p > 0.05	–
CL 3	p > 0.05	p > 0.05
coefficient of the shape of axial cylinder		
CL 2	p > 0.05	–
CL 3	p > 0.05	p > 0.05
percentage (%) MNF		
CL 2	p < 0.02	–
CL 3	p < 0.001	p < 0.001

Notes: CL – clusters; MNF – myelin nerve fibers.

However, initially for the data provided by SB Herashchenko [2], it is necessary according to the standard errors of the values of the indicators listed

in table 3, to calculate in each of the three groups of MNFs, the largest in size, their variances according to formulas 5 and 6:

$$\sigma_j = m_j \sqrt{N_j}, \text{ where :} \quad (5)$$

σ_j – group standard deviation of group j ; m_j – group standard error of group j ; N_j – volume of group j .

$$D_j = \sigma_j^2, \text{ where :} \quad (6)$$

D_j – group variance of group j ; σ_j – group standard deviation of group j .

Knowing the group variances, according to formulas 3, 4 and 2, they calculate the total variance, and according to formula 1 they determine the total mean for each indicator. This recalculation is represented in Table 4.

Having determined the total variance for each indicator, according to formulas 7 and 8, their standard deviations and standard errors are determined.

$$\sigma_{total} = \sqrt{D_{total}} \quad (7)$$

$$m_{total} = \frac{\sigma}{\sqrt{n}} \quad (8)$$

Having performed such statistical transformations of literature data, we represent them in the following format (Table 5).

Table 3. Comparison of the obtained results with the literature data according to the areas of myelin fibers, their axial cylinders and myelin sheath, according to the coefficients of shape of myelin fibers and their axial cylinders, ($M \pm m$).

According to the literature data [2]					
S MNF of different calibers (μm^2)	< 20	20–40	40–60	60–80	80–100
S of AC (μm^2)	3.28 ± 0.11	9.97 ± 0.14	17.22 ± 0.28	25.94 ± 0.45	35.00 ± 1.42
S of myelin (μm^2)	9.26 ± 0.21	19.85 ± 0.18	31.60 ± 0.29	42.22 ± 0.43	50.55 ± 1.29
C _f of AC	0.74 ± 0.009	0.67 ± 0.006	0.63 ± 0.008	0.64 ± 0.013	0.64 ± 0.030
C _f of MNF	0.80 ± 0.005	0.77 ± 0.004	0.75 ± 0.005	0.75 ± 0.008	0.73 ± 0.020
According to the results of our study					
	CL 1	CL 2	CL 3		
S of MNF of different clusters (μm^2)	12.49 ± 2.88	35.25 ± 3.62	73.49 ± 6.71		
S of AC (μm^2)	3.54 ± 1.05	10.23 ± 1.65	21.03 ± 3.51		
S of myelin (μm^2)	8.95 ± 1.98	25.02 ± 2.65	52.45 ± 4.66		
C _f of AC	0.73 ± 0.06	0.75 ± 0.05	0.73 ± 0.05		
C _f of MNF	0.85 ± 0.06	0.86 ± 0.07	0.85 ± 0.07		

Notes: S – transsection area; AC – axial cylinders; Cf – shape coefficient; CL – clusters; MNF – myelin nerve fiber; myelin – myelin sheath; Mean \pm SE – mean value \pm standard error of the mean.

Table 4. Step-by-step calculation of the total mean and total variance of the values of morphometric parameters according to the data of the work [2]

Indicator	Groups MNF according to S (μm^2)	Group M_j	Group m_j	Group σ_j	Group D_j	M_{total}	D_{total}
S of AC (μm^2)	40 – 60	17.22	0.28	0.84	0.74	26.05	59.54
	60 – 80	25.94	0.45	1.29	1.66		
	80 – 100	35.00	1.42	4.26	18.15		
S of myelin (μm^2)	40 – 60	31.60	0.29	0.87	0.76	41.46	65.94
	60 – 80	42.22	0.43	1.29	1.66		
	80 – 100	50.55	1.29	3.87	14.98		
Cf of AC	40 – 60	0.63	0.008	0.02	0.0004	0.64	0.00347
	60 – 80	0.64	0.013	0.05	0.0025		
	80 – 100	0.64	0.030	0.09	0.0081		
Cf of MNF	40 – 60	0.75	0.005	0.012	0.000144	0.74	0.00442
	60 – 80	0.75	0.008	0.024	0.000576		
	80 – 100	0.73	0.020	0.06	0.0036		

Notes: S – transsection area; AC – axial cylinders; Cf – shape coefficient; MNF – myelin nerve fibers; myelin – myelin sheath; M_j – group mean of group j ; m_j – group standard error of group j ; σ_j – group standard deviation of group j ; D_j – group variance of group j ; M_{total} – the total mean of the total population of each indicator; D_{total} – the total variance of each indicator.

Table 5. Comparison of the obtained results with the adapted literature data.

According to the literature data [2]			
S MNF of different calibers (μm^2)	< 20	20 – 40	40 – 100
S of AC (μm^2)	3.28 ± 0.11	9.97 ± 0.14	26.05 ± 2.57
S of myelin (μm^2)	9.26 ± 0.21	19.85 ± 0.18	41.46 ± 2.71
C_f of AC	0.74 ± 0.009	0.67 ± 0.006	0.64 ± 0.023
C_f of MNF	0.80 ± 0.005	0.77 ± 0.004	0.74 ± 0.023
According to the results of our study			
	CL 1	CL 2	CL 3
S of MNF of different clusters (μm^2)	12.49 ± 2.88	35.25 ± 3.62	73.49 ± 6.71
S of AC (μm^2)	3.54 ± 1.05 P > 0.50	10.23 ± 1.65 P > 0.50	21.03 ± 3.51 P > 0.20
S of myelin (μm^2)	8.95 ± 1.98 P > 0.50	25.02 ± 2.65 P > 0.10	52.45 ± 4.66 P > 0.05
C_f of AC	0.73 ± 0.06 P > 0.50	0.75 ± 0.05 P > 0.10	0.73 ± 0.05 P > 0.10
C_f of MNF	0.85 ± 0.06 P > 0.50	0.86 ± 0.07 P > 0.20	0.85 ± 0.07 P > 0.10

Notes: S – transsection area; AC – axial cylinders; C_f – shape coefficient; MNF – myelin nerve fibers; myelin – myelin sheath; **P** – the achieved level of statistical significance according to the values of the bilateral Student's t-test [4].

As it can be seen from Table 5, when comparing identical indicators according to the results of our research and the data of SB Herashchenko [2], the difference between the mean values of all indicators is statistically insignificant (**P > 0.05 - 0.50**), i.e., the results are identical.

3. Conclusions

1. The obtained results testify to the expediency of the use of cluster analysis for the classification of myelin nerve fibers of the peripheral nerves and are justified from the standpoint of multidimensional statistics.
2. Clustering of myelin nerve fibers is rationally to be performed according to the transsection areas of the axial cylinder and myelin sheath.
3. To determine the number of clusters one should use agglomerative (hierarchical) Ward's method, and to establish their metrics and represent the cluster structure in the coordinate field – an iterative method of clustering of k-means.
4. In the sciatic nerve of an intact one-year-old

male rat of the Wistar line, three clusters of myelin nerve fibers were identified, which are statistically significantly different in four signs: fiber areas, axial cylinder and myelin sheath, and fiber percentage.

5. When comparing identical indicators according to the obtained and literature data, the results were equivalent in the areas of the axial cylinder and myelin sheath and their shape coefficients, despite the fact that the classification of myelin fibers, as well as their morphometry, was performed using different methods.

4. Prospects for further research

It is advisable to cluster the sciatic nerve of MNF using a larger sample size (at least 10 animals). It will be interesting to compare the obtained data with the results of the cluster analysis of the MNF, performed according to their three metric features. It is promising to compare the defined classification of MNFs with their physiological classifications.

Conflict of Interest

The authors stated no conflict of interest.

Financial Disclosure

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