Path. Res. Pract. 185, 567–571 (1989)

Automated Morphometric Study of Human Peripheral Nerves by Image Analysis

S. Torch[1], Y. Usson[2] and R. Saxod[1]

[1]*Laboratoire de Biologie Animale, Equipe de Neurobiologie du Développement, Université Joseph Fourier, Grenoble, France;* [2]*TIM 3 (UA CNRS 397) Equipe de Reconnaissance des Formes et de Microscopie Quantitative, CERMO, Université Joseph Fourier, Grenoble, France*

SUMMARY

In this paper we describe a program using the image analyzer SAMBA, which allows an automatic analysis of silver stained semithin nerve sections. The operator can interactively delimit the contour of the fascicle to be analysed by means of a digitizing tablet connected to the system which generates a mask of the region. Segmentation of the fibre images is conducted as a function of brightness threshold defined by the operator. Fibre clusters are automatically separated using morphological procedures like dilatation. Morphometric parameters such as the external and axonal diameters, myelin sheath thickness and circularity are measured. We are now testing this method on normal and pathological human superficial peroneal nerves. Preliminary results are promising and the development of adequate statistical analysis of morphometric data will provide us with a new tool for the diagnosis of peripheral neuropathies.

Introduction

Morphometric assessment of human peripheral nerves is a widely and routinely applied method for the quantitative analysis of biopsies for routine diagnosis. Generally, such quantitative analysis is carried out at the light or ultrastructural level by means of semi-automatic techniques and usually in conjunction with sampling techniques, especially when the caliber of fibres is under study. We have previously shown that, due to the extreme heterogeneity of local densities of myelinated fibres and size distributions encountered in fascicles, sampling methods are unsatisfactory[5,8].

Thus, the constraint of analyzing a whole nerve fascicle obviates the use of time-consuming semi-automatic techniques. Therefore, a specially designed program was developed for the histologic image analyzer SAMBA that permits the automatic analysis of semi-thin nerve cross sections. In this report, we present the main features of this program.

Material and Methods

Tissue Preparation

This study was performed on a series of normal ("controls") human superficial peroneal nerves. Specimens were taken from subjects in post-traumatic coma requiring circulatory and respiratory assistance. Segments of 3 cm length of the sensory portion of the nerves were exposed and excised from the distal third of the leg. Sensory nerve conduction velocity and action potential amplitude were measured in each case and appeared normal. Nerve segments were fixed for 6 h, in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, postfixed for 90 min in osmium tetroxide, dehydrated in a series of alcohols, embedded in Epon and polymerised at 60°C for 12 h. Semithin transverse sections were cut at 1 μm, and silver stained according to a method previously described[7].

Morphometric Measurements

Image acquistion: Using the commercially available SAMBA system[2], nerve preparations were first analysed at low magnifi-

0344-0338/89/0185-0567$3.50/0

cation, in order to delimit interactively the contour of the nerve fascicle to be measured by means of a digitizing tablet connected to the system.

This delimited region was subdivided into elementary fields of 256×256 pixels, and the scanning system replaced the preparation in each field to be measured. Each field was divided into vignettes of 64×64 pixels, each subdivided into four equal parts (Fig. 1). At this stage of the image analysis, the spatial resolution is 0.5 μm for 1 pixel (× 63 objective in oil immersion). Myelinated fibres were measured in each vignette, provided they were located in the lower right quadrant of the graph, and did not touch the inferior or right side of this zone (Fig. 1). The overlapping of the fields was conducted in such a way that no fibre was lost or measured twice.

Image segmentation: Fibre segmentation was conducted as a function of pixel brightness. Each point of the analysed image was coded into 256 gray-scale levels. Myelin sheaths appeared as dark objects on a bright background and consequently pixels whose gray-level values were smaller than the threshold value chosen by the operator (Fig. 2) were considered to belong to myelin sheaths. Conversely, the remaining pixels, whose gray-level values were greater than this threshold value, were considered as non-myelin structures (Fig. 2). The system generated a binary image (or logic mask) of the myelin sheaths (Fig. 3). Inversion of the image generates a logic mask of the axons. From the difference of the previous inverted image and the logic mask of axons, an inverted image of the fibres was constructed. Its inversion led to the construction of a logic mask for the fibres. The fibre masks and corresponding axon masks were extracted one by one from the fibre binary image and the measurements of the inner and outer diameters were made on the axon binary masks and on the fibre binary masks, respectively.

Separation and segmentation of fibre clusters: A special subroutine was used when the image analysis led to detection of a single external contour enclosing two or more internal contours.

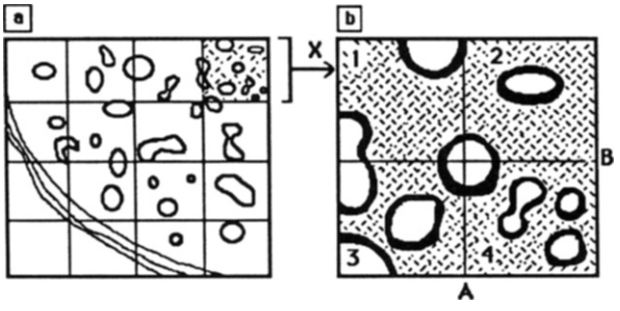

Fig. 1. Each field of 256×256 pixels (picture points) is subdivided into vignettes of 64×64 pixels (a). In (b), a magnified vignette is represented. Each vignette is subdivided into 4 zones (1, 2, 3, 4) sized 32×32 pixels (16 μm square). Fibres located within zone 4 are measured provided they do not touch A or B side.

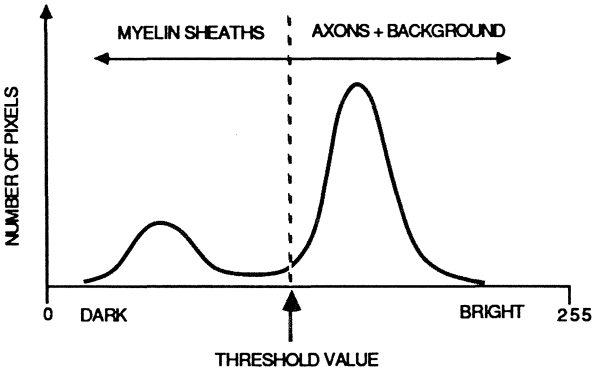

Fig. 2. Segmentation threshold is determined by the operator. Each picture point has a gray level ranged between O (black) and 255 (white). Picture points whose gray-level is over the threshold value are considered as myelin sheaths, while those with a gray level below the threshold value are considered as axons or background.

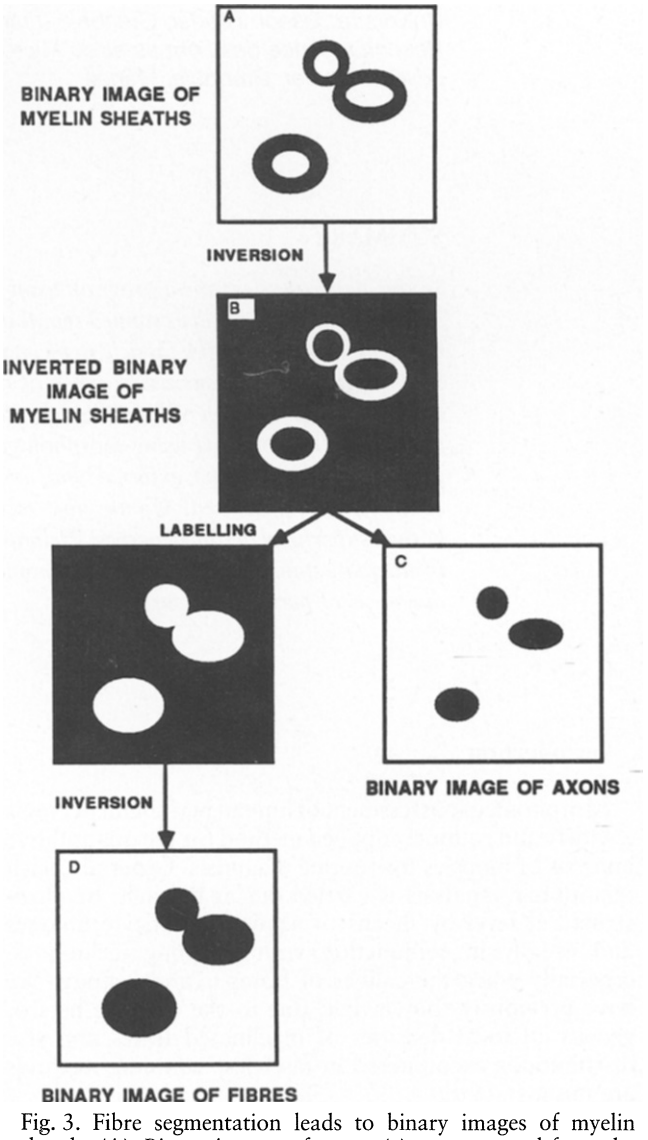

Fig. 3. Fibre segmentation leads to binary images of myelin sheaths (A). Binary images of axons (c) are extracted from the inverted binary images of myelin sheaths (B). From the difference between the two images B and C, an inverted image of fibres is extracted, and its inversion represents the binary image of fibres (D). Axonal and fibre diameters are measured from binary image of axons and binary image of fibres respectively.

This was the case when a fibre was cut at a Schmidt Lantermann incisure level, or when two fibres were in close contact (Fig. 4). The measure of the shape of the fibre profile expressed as the index of circularity (IC) was used as a criterion to distinguish between these two situations. If the external profile measured had a low value of the index of circularity (IC <0.5), then the detected inner contours corresponded to axons of fibres in close contact and their separation was achieved by reconstructing them from the enclosed axons as described further. Otherwise, the fibre was considered as a Schmidt-Lantermann incisure, with two surrounding myelin sheaths, and was indexed in the file as a "double-sheathed" fibre.

From the axon logic mask, the process of image dilatation was conducted in an iterative manner and the dilatated image was superimposed after each step on the mask of the fibre. Dilatation will be repeated until the logical intersection of the two superimposed masks remains equal to the dilated mask. Dilatation was stopped when the area of the dilatation spread out of the fibre mask, that is when the former intersection falls under a threshold value of 98% of the area of the dilatated axon mask (Fig. 4).

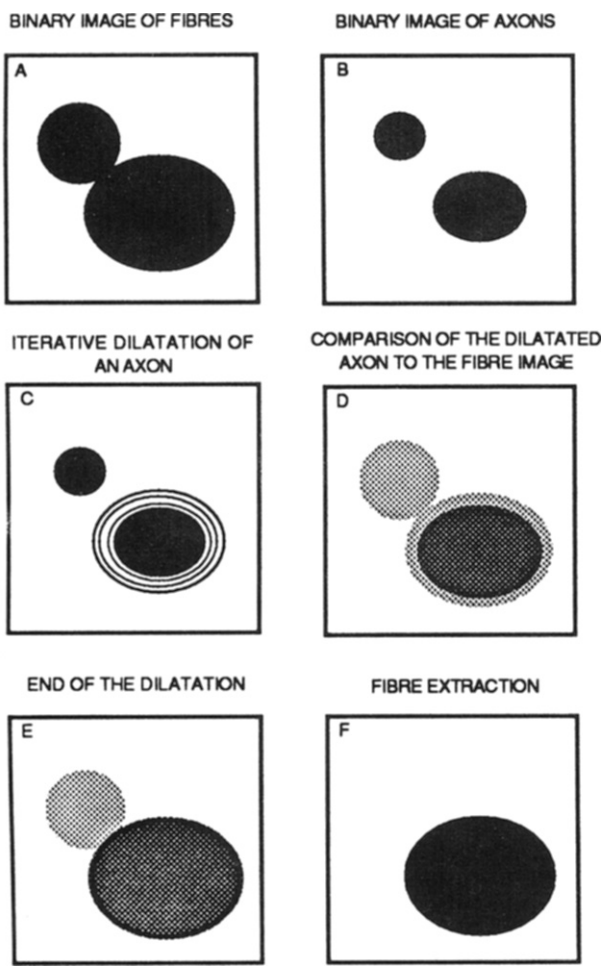

Fig. 4. If two axons are in close contact, then a special subroutine using mathematical procedures such as dilatation is employed. The binary image of the axon is dilated in an iterative manner (C), and the dilated image (D, dark gray) is compared to the fibre image (D, light gray). The dilatation is stopped when the common surface area of the two superimposed images represents 95% (E), and the fibre image is extracted. The extraction of the next axon is conducted in the same way.

Statistical Analysis of the Data

Multiparametric classification of myelinated fibres: Statistical analysis of morphometric data collected from human superficial peroneal nerves is not easy since the myelinated fibre population is a mixture of two groups of nerve fibres, large and small.

We have previously developed a computer programm for their automatic classification[9,10]. The separate examination of the parameters and of the relationships obtained for each isolated large and small fibre group was used for a comparative study of normal and pathological nerves. This method led to a good separation of the two fibre subpopulations and then allowed a comparison of the different morphometric parameters of each subpopulation between individuals or groups of individuals (unpublished data).

Graphic representation of the morphometric results: Measurements of morphometric parameters are represented as histogram frequency distributions for the following parameters:

– external diameter (outer myelin sheath contour)
– axonal diameter (inner myelin sheath contour)

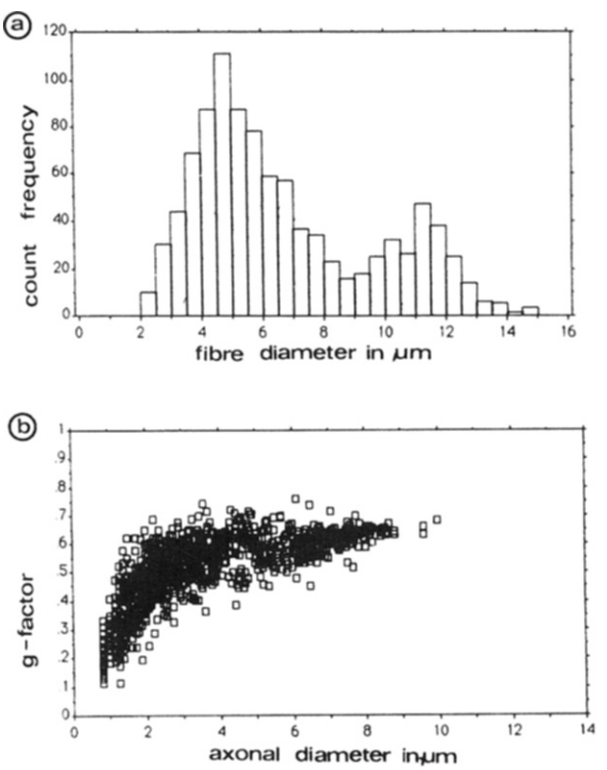

Fig. 5. Distribution of myelinated fibre sizes (a) reveals that the myelinated fibre population is compound of two normally distributed subpopulations. Scattergram of the relationship of the g-factor versus the axonal diameter shows two clusters of fibres corresponding to the large and small myelinated fibre subpopulations.

– myelin sheath thickness
– g-factor (ratio of the axonal diameter and the external diameter)

Correlations between pairs of these parameters were also made. Figure 5 illustrates a histogram obtained from the analysis of a human superficial peroneal nerve of a control subject aged 24 years, and a graph of relationship between the g-factor versus axonal diameter. Scattergrams of g-factor and axonal diameter are the most informative representation of fibre structure as has been shown[3].

Discussion

In the assessment of peripheral nerves, problems that the morphologist has to face include the accuracy and the reproducibility of the measurements, the accuracy and the representativeness of the sample to be evaluated, an adequate mode of analysis of data obtained, and their comparison to an appropriate data base. For human nerve biopsies, morphometric assessment is generally carried out with routinely and widely applied semi-automatic ultrastructural techniques based on morphometric analysis of an assumed representative sample. However, there are only few studies in which the accuracy of sampling methods is evaluated by means of adequate testing procedures[1]. Morphometric analyses of human nerve biopsies are complicated by the heterogeneity of the myelinated fibre populations, and their bimodal size distributions[9,10]. Thus, in such situations, the representativeness of a sample analysed at the ultrastructural level must be considered in terms of good reproducibility of the proportions between the two subpopulations (large and small myelinated fibres), accuracy of the mean values and standard deviations of the morphometric parameters within each fibre subpopulation. In a previous study, we described a method for the automatic classification of myelinated fibres[10]. This way, the usual parametric tests can be used for the comparative analysis of nerve biopsies, by considering each fibre subpopulation separately. If bimodally distributed parameters are considered, their comparison must be undertaken by means of non-parametric testing procedures[1]. However, the morphometric analyses of human peripheral nerves performed on electron micrographs are time consuming and require the analysis of a sample which is assumed to be representative of the whole nerve fascicle. Consequently, in most studies, the distributions are assumed to be Gaussian, but these assumptions were not tested. In a previous study, we showed that the spatial distribution of myelinated fibres within a nerve was often non-uniform, and therefore it was not possible to define a statistically valid sampling method for the accurate evaluation of myelinated fibre density[5]. Similar difficulties arose for the study of myelinated fibre size distribution. We showed that in the human superficial peroneal nerve, myelinated fibres are not randomly distributed according to their sizes[8].

Different sampling methods have been evaluated, and it was not possible to define an adequate sampling scheme for evaluating myelinated fibre size distribution[8]. Consequently, we developed a programm of morphometric analysis of transverse semi-thin sections, on a commercially available image analyzer SAMBA[2]. This method offers a number of advantages: it allows us to avoid the sampling problems since it permits the measurement of myelinated fibres to be restricted to a given area. Indeed, the interactive computer assisted image analysis allows the measurement of all myelinated fibres in the zone defined by the operator. The analysis of 1000 myelinated fibres took about one hour.

In other automatic morphometric studies of the human superficial peroneal nerve, the authors proposed the measurement of the "density of myelin" within each frame analysed, and the measurement of morphometric parameters of single fibres in samples of 200 myelinated fibres per nerve[4]. Finally the method we propose allowed a fully automatic scanning of the preparation analysed, while in other studies, the changes of microscopic fields were performed manually[7]. The method described in this paper will be adapted for the upgraded model of the SAMBA system (SAMBA 2005), and will then allow a spatial resolution of 0.125 μm which will greatly improve the accuracy of the measurements. The study of more cases will be suitable for compiling a large number of "normal data", and will be helpful for a biological and quantitative characterization of human peripheral neuropathies by means of the multivariate analysis (discriminant analysis) available on the statistical analysis program of the morphometric data.

Acknowledgements

We thank Prof. P. Stœbner and the Service de Microscopie Electronique et Pathologie Ultrastructurale, C.H.R. de Grenoble for providing us with the tissue samples and Mrs V. Von Hagen for reading the manuscript.

This work was supported by a grant from the Association Française de lutte contre les Myopathies (AFM 1988).

References

[1] Bronson RT, Bishop Y, Hedley-Whyte (1978) A contribution to the electron microscopic morphometric analysis of peripheral nerve. J Comp Neur *178:* 177–186

[2] Brugal G (1984) Image analysis of microscopic preparations. Meth Achiev exp Pathol *11:* 1–33

[3] Friede RL (1986) Computer editing of morphometric data on nerve fibers. An improved computer program. Acta Neuropath *72:* 74–81

[4] Grosse R, Burlet C, Floquet A, Floquet J (1981) Morphometric study of the post-natal maturation of human superficial peroneal nerve sampled by biopsy. Achieved using automatic image analysis. Stereol lugosl *1:* 533–538

[5] Saxod R, Torch S, Vila A, Laurent A, Stœbner P (1985) The density of myelinated fibres is related to the fascicle diameter in human superficial peroneal nerve. Statistical study of 41 normal samples. J Neurol Sci *71:* 49–65

[6] Selva J, Schoëvaërt-Brossault D, Said G (1981) Automated morphometric analysis of cross sections of normal and pathological sural nerve biopsy specimens. Bio Cell *42:* 57–64

[7] Singh I (1964) A modification of Masson-Hamperl method for staining of argentaffin cells. Anat Anz *15:* 81–82

[8] Torch S, Stœbner P, Usson Y, Drouet d'Aubigny G, Saxod R (1988) There is no simple adequate sampling scheme for estimating myelinated fibre size distribution in human peripheral nerve. A statistical ultrastructural study. J Neurosci Meth *27:* 149–164

[9] Usson Y, Drouet d'Aubigny (1985) Multimodal frequency distribution of peripheral nerves. Anal Quant Cytol Histol *7:* 294–298

[10] Usson Y, Torch S, Drouet d'Aubigny G (1987) A method for automatic classification of large and small myelinated fibre populations in peripheral nerves. J Neurosci Meth *20:* 237–248

Received March 31, 1989 · Accepted in revised form July 19, 1989

Key Words: Peripheral nerve – Nerve fibers – Peripheral neuropathies

R. Saxod, Laboratoire de Biologie Animale, Equipe de Neurobiologie du Developpement, Université Joseph Fourier, BP 53X, 38041 Grenoble, Cedex France