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Studying nerve transfers: Searching for a consensus in nerve axons count

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Summary Axonal count is the base for efficient nerve transfer; despite its capital importance, few studies have been published on human material, most research approaches being performed on experimental animal models of nerve injury. Thus, standard analysis methods are still lacking.
Quantitative data obtained have to be reproducible and comparable with published data by other research groups. To share results with the scientific community, the standardization of quantitative analysis is a fundamental step. For this purpose, the experiences of the Italian, Austrian, German, Greek, and Iberian-Latin American groups have been compared with each other and with the existing literature to reach a consensus in the fiber count and draw up a protocol that can make future studies from different centers comparable.
The search for a standardization of the methodology was aimed to reduce all the factors that are associated with an increase in the variability of the results. All the preferential methods to be used have been suggested. On the other hand, alternative methods and different methods have been identified to achieve the same goal, which in our experience are completely comparable; therefore, they can be used indifferently by the different centers according to their experience and availability.

Introduction

Axonal count is the base for efficient nerve transfer; despite its capital importance, few studies have been published on human material, most research approaches being performed on experimental animal models of nerve injury.[1-3] Thus, standard analysis methods are still lacking.

Quantitative data obtained have to be reproducible and comparable with published data by other research groups. To share results with the scientific community, the standardization of quantitative analysis is a fundamental step.

The aim of this study is to compare different methods from several groups to achieve a consensus about fiber count.

For this purpose, the experiences of the Italian, Austrian, German, Greek, and Iberian-Latin American groups have been compared with each other and with the existing literature to reach a consensus in the fiber count and draw up a protocol that can make the studies coming from now on comparable from different centers.

Animal and human material

A first preliminary consideration must address the problem of the material to be studied. As previously stressed, most research studies are performed on animal experimental models, where the peripheral nerves are sampled and fixed immediately after sacrifice. A correct and rapid fixation is obviously pivotal to achieve good results and consistent axon counts. Regarding human nerve studies, in our experience, microscopic analyses of cadaver samples may still be adequate to achieve data about the nerve dimensions and neurotransmitters/neuromodulators involved, together with connective structures and eventual inflammatory elements.[4,5] Conversely, consistent and reliable axon counts may suffer from autolytic processes, such that it is necessary to maintain a short death-sampling interval, which could be possible in the context of a Body Donation Program.[6] Surgical samples (derived from demolitive operations) would surely be the best human material for morphometric and/or stereological approaches, which obviously stress upon the importance of patient consent for the donation of surgical samples for research purposes.[7]

In the sampling phase, it must also be stressed that oblique sections must be avoided as they can affect the surface measurements and as a consequence, the density estimates.[8]

Embedding and staining methods

The identification of the most appropriate histological staining method for axonal counting is the first step to standardize the results between the different study groups and to find a consensus in axonal counting.

We sought the best method, starting from both the literature and the experience of the individual centers.

Toluidine blue staining of resin-embedded semi-thin peripheral nerve sections fixed with osmium tetroxide is a traditional and a specific method for fiber counting as it provides detailed and well-contrasted images of the nervous structures.[9-15]

However, this method is not free from defects because tetroxide osmium is toxic, expensive, and requires long processing times; therefore, it has not always been used.

For this reason, various alternative methods are present in the literature that present advantages from time to time, but also numerous disadvantages, which limit their use.[16-26]

Paraffin, which allows to replace osmium tetroxide, does not allow to obtain equally thin sections in the range of 1-2 μm because it does not have a hardness such as to maintain the histological characteristics of the nerve at this thickness. It follows that high resolution images cannot be obtained for a more precise axonal count.

The use of cryogenic sections allows to obtain sections in the range of 1-2 μm, such as osmium tetroxide. With this method, however, both in literature and in our experience, it is not possible to obtain regular sections without a different and big crack.

The histological colors other than blue toluidine, such as hematoxylin eosin and Masson's trichrome, do not allow to obtain an adequate contrast of the myelin.[9,23]

All the defects of these alternative stains imply a reduction of the precision in the axonal count. It follows that despite its defects and limitations, the blue toluidine resin staining is to be considered the gold standard in the analysis of the morphology of the peripheral nerves.[12,13]

From the experience of our centers, we have drawn up a standard protocol for fabric processing:

Peripheral nerve tissue is removed atraumatically and fixed by immersion in a 2.5% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4-6 h at a temperature of 4 °C.

Post-fixed in a 1% solution of Osmium tetroxide for 2 h, dehydrated in an ascending alcohol series and embedded in araldite CY212 + 2-dodecenyl succinic acid anhydride + benzyl dimethylamine and dibutyl phthalate mixture. Semi-thin transverse sections with a thickness of one micron are obtained with an ultramicrotome and colored with a 1% staining of blue toluene.

The choice of the method for axon counting: morphometric vs stereological

Stereology may be defined as a methodology, combining statistical sampling principles and stochastic geometry, to derive three-dimensional data from two-dimensional cross-sections of different structures. Stereology represents an important step forward in the morphological and quantitative evaluation of neuronal tissue, as the direct microscopic evaluation of each single axon for each nerve is an inefficient method, not applicable to routine clinical practice and is in limited use in some conditions to set a gold standard on which to validate other methods.[27-34]

A real alternative to stereology, on the other hand, is represented, albeit with its limitations by histomorphometry. Histomorphometry uses image processing programs and binary imaging for the study of nerve sections providing a complete description of the characteristics of the tissue, based on the shape and morphology. However, it requires the subjective setting in the program of a threshold, a condition that can increase interloper variability and, moreover, histomorphometry cannot quantify the glia or inflammatory cells and therefore, cannot provide a complete analysis of a damaged nerve. This severely limits its possibilities of use and prevents its adoption as a standard method.[35-38]

Standardization of the stereological method to reduce variability

Design-based sampling is usually considered the preferable approach to ensure that morphological variables of biological objects do not influence the probability of each object being sampled.

To standardize the design-based sampling of the stereological method, the various sources of variability on which it is possible to intervene were analyzed:

- sampling frame used (2-D dissector vs unbiased counting frame)
- simple random sampling versus systematic random sampling
- size of the frames adopted and relative sampling scheme
- different instrumental availability in workstations in the different centers
- recognition of fibers by the various operators

Sampling frame

We assessed the impact of the sampling frame (unbiased counting frame vs 2D-dissector) on the variability of the results obtained in the fiber count and from our experience as well as from the study of the literature, there is no statistically significant difference. Even the use of a circular or square shape does not affect the accuracy of the measurement.[39] For this reason, the use of one frame rather than another does not affect the value obtained by individual studies, and it is, therefore, possible to use the method with which you have more experience in each center according to the following protocols aimed at limiting the bias.[39]

- Unbiased counting frame

This methodology involves the use of a square frame; all fibers that are entirely within the frame or that touch the upper or right margin (inclusion lines) are considered and counted, while the fibers that touch the lower or left margin (exclusion lines) are excluded. In the event that a fiber crosses both an inclusion and an exclusion line (even in its infinite extensions), it is excluded.

- Two-dimensional (2D) dissector

This methodology identifies a single unambiguous point for each nerve fiber. It takes account of the nerve fiber in the counting frame simply if the unambiguous point is included in the frame independently from the whole nerve. This unambiguous point is identified as the "top" or "upper" edge of a fiber profile of each of these nerve sections.

If the "top" is inside the counting frame, it is considered inside and therefore counted, otherwise not. In the event that the "top" falls precisely on the edge of the frame, a criterion based on an upper inclusion half-frame and a lower exclusion half-frame is used. In the rare case where the "top" falls to the limit between the inclusion and exclusion frame, if it is on the right it is included, the opposite if it is on the left.

Once the data with the two different stereological protocols have been obtained, it is possible to estimate the total number of fibers through two different approaches, which in our experience and in the literature have proven to be equivalent.[39]

One method involves calculating the average axonal density from the sampling frames and multiplying it by the cross-sectional area of the investigated nerve.[39]

The other method is the fractionator approach that does not need the calculation of the whole nerve area and is based on the knowledge of the size of the sampling frames and the distance between the frames along the x and y axis. Starting from these, the sampling area is calculated and is used to obtain the total number of axons.[39]

Simple random sampling versus systematic random sampling

It is obviously widely shared that sampling must be performed randomly to give the same probability to be sampled to each object. However, two different approaches can be followed. In simple random sampling, each sample frame is randomly put on the section and each sampling is independent from the other. The spatial localization of each sample frame may be performed with the help of a table of random numbers or an electronic random number generator. Conversely, systematic and uniformly random sampling involves a first random localization of the sampling frame and then, the uniform sampling of the remaining structure through fixed sampling intervals (usually an XY step).[3,40,41] This means that every nth data sample is chosen. Systematic uniformly random sampling should be preferred as it better ensures the same probability to be chosen for each object.

Sampling sizes and frame

We compared the sampling scheme to "small and frequent" vs "large and few" frames. In our experience, the use of the method with small and frequent frames each of which investigates about 0.2—0.3% of the total surface, associated with an overall sampling of 2—3% of the nerve, gives a better representation of the heterogeneity of the nerve.[39] The results obtained with this method return an estimate of the total number of fibers with statistically not relevant differences from the real value. This method is also more efficient than the "large and few" sampling scheme because although it investigates, a smaller total surface area give results with significantly lower variability, even between different centers. For these reasons, it is the method recommended by us to obtain reproducible and comparable results.

Instrumental availability in the workstation

The stereological method can be performed with optical microscopy (100x with immersion objective) without the support of digital images, monitors, and dedicated software. In our experience, the measurements performed by different centers with different technological possibilities did not show statistically significant variations. It is possible to conclude that, at present, an advanced workstation does not translate into measurements that are more accurate and is therefore not mandatory to consider the reliability of a study. This is therefore in contrast with some old studies in literature that considered the stereological method possible only with expensive equipment such as integrated cameras and dedicated instruments.[39]

A specific consideration may be made about instrumentation needed for a correct random sampling on the microscope. Both for simple and systematic random sampling (but mainly for the latter), a microscope equipped with a motorized object table would be necessary to permit standardized XY steps. It must be considered, however, that at least for small nerves, the quite low areas of transverse sections could allow a quite rigorous random sampling also with manual object table.

Nerve fiber recognition

The correct recognition of the fibers is an essential point of the stereological method, particularly when looking for a standardization that allows comparing studies from different centers. For this reason, we recommend that these studies be conducted by researchers with extensive experience in the evaluation of peripheral nerve tissue.

The errors in this case are related to the ambiguity of certain figures under microscopy rather than to gross observer errors. Therefore, experience alone is not enough, and we recommend to perform a calibration of the method before a study of this kind is carried out by a group for the first time. For this reason, we suggest a test called Nerve Fiber Recognition, which can be performed by experimenters to practice to distinguish ambiguous images. In this test, the observer is asked to define several ambiguous images reminiscent of myelin fibers and to indicate them with the colors green (axon), yellow (doubtful image), and red (other tissues). The test obviously follows the solution of the proposed images with the visualization of the same by electron microscopy.[39]

The experimenter therefore has the opportunity not only to evaluate his degree of precision but also to learn from mistakes and improve his skills. In our opinion, this is an excellent method to reduce interoperator variability. The possible solution to bring the analysis to electron microscopy, on the other hand, is not feasible as it is not possible to view a nerve in its entirety, particularly if it is large, and consequently, it is not possible to perform random samplings on the entire surface, a necessary condition to correctly perform the stereological method.[42]

Further considerations with regard to the possibility to differentially count myelinated and unmyelinated axons. In our experience, this may be possible on semi-thin sections, but it may be quite difficult such that most papers do not distinguish between them in morphometric/stereological analyses. A detailed differentiation for quantification is preferable on TEM. However, some myelinization parameters may also be derived on semi-thin sections such as the mean myelin thickness and the ratios between external and internal diameters of myelinated fibers (D/d).[3]

Conclusion

Achieving a consensus in fiber counting to make studies in literature comparable is possible and has been discussed in this manuscript. The search for a standardization of the methodology was aimed to reduce all the factors that are associated with an increase in the variability of the results. All the preferential methods to be used have been suggested.

On the other hand, alternative methods and different methods have been identified to achieve the same goal, which in our experience are completely comparable; therefore, they can be used indifferently by the different centers according to their experience and availability.

Ethical approval

N/A.

Declaration of Competing Interest

The authors report no conflicts of interest to disclose.

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