Anat Embryol (2001) 204:1–10

REVIEW ARTICLE

Stefano Geuna · Pierluigi Tos · Renzo Guglielmone
Bruno Battiston · Maria G. Giacobini-Robecchi

Methodological issues in size estimation of myelinated nerve fibers in peripheral nerves

Accepted: 18 April 2001

Abstract Size estimation of myelinated nerve fibers in peripheral nerves is a very common task in neuromorphology and different dedicated morpho-quantitative procedures have been devised and used to date. Unfortunately, many reports on experimental nerve studies lack comprehensive information on the procedures that have been designed and applied for myelinated fiber size estimation. This paper addresses the issue in the light of the recent advances in quantitative morphology that have recognized the concept of unbiased estimates as the key methodological issue to be addressed in morpho-quantitative studies. The potential foundations of bias at various study levels are analysed together with indications on how to cope with them. In addition, the issue of the precision of size estimates is addressed and the various geometrical parameters that can be selected for myelinated nerve fiber size assessment are outlined. Taken together, information provided in this paper is expected to help investigators conduct an appropriate preliminary study design phase, the key step for setting up the most adequate morpho-quantitative procedure for any given research goal.

Keywords Nerve fibers · Quantitative morphology · Sampling · Bias · Precision

Introduction

Estimation of myelinated nerve fiber-size in peripheral nerves is a common goal of many studies that investigate peripheral nerves in various physiological, experimental and pathological conditions (e.g.: Brown et al. 1976; Pollock et al. 1984; Thomas et al. 1990; Bradley et al. 1995; Schionning and Larsen 1997; Schionning et al. 1998; Soong and Lin 1998; Ceballos et al. 1999; Geuna et al. 2000c; Tos et al. 2000). For example, this morpho-quantitative feature of nerve fibers represents (together with the total fiber number) the most commonly used morphological parameter for the experimental assessment of various surgical techniques for peripheral nerve repair (reviewed: Lundborg 1988; Ide 1996; Terzis et al. 1997; Battiston et al. 2000; Geuna et al. 2000b; Tos et al. 2000). Though it has been demonstrated that nerve function can not be assessed on the basis of one single parameter (Dellon and Mackinnon 1989; Kanaya et al. 1996; Mackinnon 1996), the correlation of some morphological parameters with functional recovery of a repaired nerve has recently been demonstrated (Kanaya et al. 1996), thus strengthening the importance of the morpho-quantitative assessment in the context of experimental nerve studies.

The size of a myelinated nerve fiber can be expressed by various geometrical parameters: the diameter (maximum, minimum, circle-equivalent), the perimeter and the cross-sectional area. If the quality of the histological material is good and allows high-resolution optical observation (as is usually obtained with resin-embedded nerves), the same parameters can also be measured for the axon, thus making it possible to calculate myelin thickness. Finally, some further valuable size parameters (namely the myelin-thickness/axon-diameter ratio, the fiber-diameter/axon-diameter ratio and the g-ratio), can be derived from the above-mentioned measurements by simple mathematical calculations.

In recent years, there has been a growing interest in a new generation of methods that have significantly improved morpho-quantitative data collection on various cellular and subcellular biological structures. The introduction of the design-based sampling strategies in quantitative morphology, that finds its roots more than twenty years ago (Gundersen 1977; Cruz-Orive 1980; Cruz-Orive and Weibel 1981; Sterio 1984), represents the

S. Geuna (✉) · R. Guglielmone · M.G. Giacobini-Robecchi
Dipartimento di Scienze Cliniche e Biologiche,
Università di Torino, Ospedale San Luigi,
Regione Gonzole 10, Orbassano (TO), 10043-Italy
e-mail: stefano.geuna@unito.it
Tel.: +39-011-6708135; Fax: +39-011-9038639

P. Tos · B. Battiston
Gruppo Interdivisionale di Microchirurgia (G.I.M.),
Ospedale C.T.O., Torino, Italy

main expression of such a trend (Mayhew and Gundersen 1996; Saper 1996; Hyman et al. 1998; Geuna 2000). Although this new approach has not made other quantitative methods totally obsolete (Hyman et al. 1998), and each particular scientific problem still has to be approached by choosing an evaluation method that takes into account various factors, its main merit is that it has emphasized the need to set up a system of rules that make it possible to overcome the various problems that can arise if one's aim is to quantify objects from biological samples. Number and size-estimation of peripheral nerve myelinated fibers often represents one such aim.

We have recently addressed the issue of the estimation of the total number of myelinated fibers in peripheral nerves in a paper describing the verification of a dedicated procedure (Geuna et al. 2000a). In addition, another paper has also outlined the rationale for the employment of design-based sampling strategies in quantitative morphology of the nervous system (Geuna 2000). In this paper, we will address the issue of the estimation of the size of myelinated nerve fibers from a theoretical and practical viewpoint, focusing on the study design steps for the selection and optimization of a morpho-quantitative procedure in relation to specific research resources and goals. In addition, the concepts of bias and precision, which are basic in quantitative morphology, are presented in appendix 1. Our main goal is to provide concrete help to the number of scientists (both morphologists and clinicians) involved in quantitative morphology of the peripheral nerves.

Potential foundations of bias in myelinated nerve fiber size estimation

There are various levels in a morpho-quantitative study where possible foundations of bias can exist. In the case for peripheral nerve fiber size estimation, the foundations of bias can be grouped as follows:

Level 1. The strain, gender and age of experimental animals (*strain-related, gender-related, age-related foundations of bias*)
Level 2. The point (level) along the nerve axis where sections are cut (*section-related foundations of bias*)
Level 3. The location of the sampling fields within the nerve cross-section profile (*location-related foundations of bias*)
Level 4. The inclusion-exclusion rules for sampling fiber profiles within the sampling fields (*morphology-related foundations of bias*)
Level 5. The method for measuring the selected size parameters (*measurement-related foundations bias*).

Since levels 2, 3 and 4 are all related to the sampling procedure, they can be grouped together under the term *sampling-related foundations of bias*.

Level 1. Strain-related, gender-related and age-related foundations of bias

This might seem too obvious a point to be worthy of discussion since it is well-known that any comparative study must be conducted on specimens from the same strain, gender and age. However, as regards the latter factor, quantitative assessment of peripheral nerve fibers in rats (Fraher et al. 1990) has demonstrated the existence of relevant age-related size changes over all of the animal's lifetime, thus making especially important the accuracy in dating the animal age. In particular, axon size in rat tibial nerve was shown to increase rapidly until 3 months (Fraher et al. 1990), i.e. the age range when rats are often used for experimental studies.

Level 2. Section-related foundations of bias

Unlike morpho-quantitative analysis in various other organs (Gundersen et al. 1988a,b; Mayhew 1992; West 1993; Mayhew and Gundersen 1996; Hyman et al. 1998), analysis of peripheral nerve fibers does not require the cutting of the all whole organ, since one section (perpendicular to the nerve main axis) is sufficient for the evaluation (for the theoretical rationale see: Geuna 2000 and Geuna et al. 2000a). Therefore, a question exists as to where to select the section for the analysis. The existence of a lateral asymmetry and a longitudinal variation in nerve fiber size parameters has been documented (Fraher 1992). Moreover, axon branching and nerve trunk collateral ramification can also modify, besides total fiber number, their mean size. To cope with lateral asymmetry one needs either to cut sections from nerves from the same body side (right or left) or to randomise sampling between the two sides. To cope with longitudinal variation (due to fiber-size variability, axon branching, and nerve ramification) one needs to cut sections either at a standardized level along the nerve (e.g. at the same distance from its origin or end) or to randomise sampling along the nerve segment.

Level 3. Location-related foundations of bias

Bias can originate from heterogeneity in the distribution of fibers throughout the nerve cross-sectional profile (Torch et al. 1989). In most papers reporting data on nerve fiber number, no information is given on the procedure used to select the sampling fields (often defined as "representative") where the fibers are measured (usually consisting in a total sample of about 100–200 myelinated nerve fiber profiles for each nerve cross-section). Figure 1 shows how heterogeneity in the distribution of fibers through the nerve can produce bias in their size estimation. If, as in the case of the drawing, larger fibers are preferentially located in the axial part of the nerve, size estimates would strongly depend on the position where the sampling fields are placed. If fibers are sam-

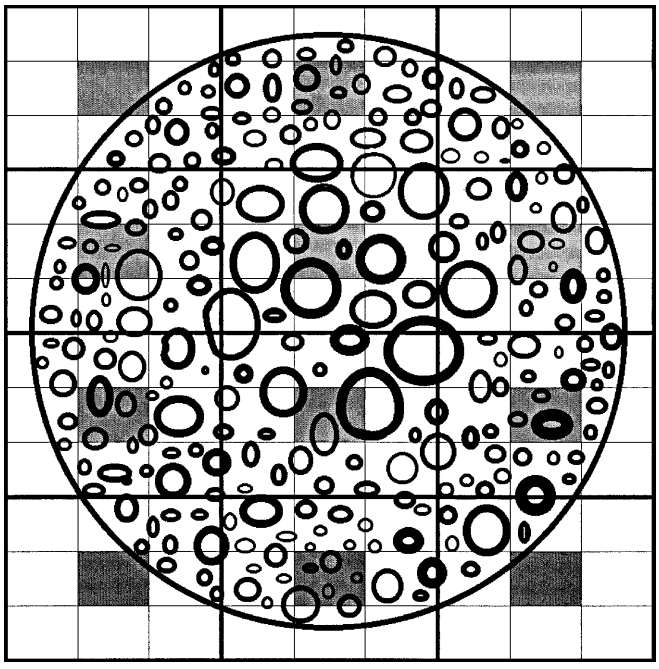

Fig. 1 Location-related bias. The schematic drawing shows a nerve cross-section with different-sized nerve fibers heterogeneously located throughout the nerve profile. A grid of rectangular sampling fields has been delineated on the nerve profile. Since larger fibers are mostly located in the axial part of the nerve, selecting the sampling boxes preferentially in this part of the nerve will result in a bias towards an overestimation of the mean size of that fiber population. The contrary is true if fibers are maily sampled in the nerve periphery. To assure that every part of the nerve has an equal chance of being sampled, a systematic random sampling procedure can be applied. In this drawing, the grey boxes have been systematically random selected by randomly choosing the first field among the sub-group of 9 boxes in the upper left (the field no. 5 in this case), and then "jumping" systematically to other sampling fields placed at fixed distances from the first one (modified from Geuna et al. 2000a)

pled mainly in the axial part of the nerve, fiber size estimation will be biased towards overestimation even if fibers are measured in a very careful and accurate way, and vice versa.

To cope with the bias originating at this study level, the investigator needs to respect a general golden rule of design-based sampling, i.e. every part of the structure (the nerve cross-section profile in this case) should have an equal chance of being sampled (Cruz-Orive and Weibel 1981; Larsen 1998; Geuna 2000). To reach this goal, in biological quantitative morphology systematic random sampling should be considered definitely more efficient than independent random sampling for both theoretical and experimental reasons (Mayhew and Sharma 1984a,b; Gundersen and Jensen 1987; Mayhew 1988; Torch et al. 1989; West 1993; Gundersen et al. 1999). Some of its advantages are that it reduces sampling variance, thus reducing the amount of sampling required to obtain a sufficient estimate precision (and thus a sufficient research workload), and that it tends to furnish smaller accidental errors (Gundersen and Jensen 1987). This sampling scheme consists in choosing a random starting sampling field and then systematically selecting the following sampling fields throughout the region by systematically "jumping" to the following fields placed automatically at a fixed distance. In this way three elements are accomplished that contribute to coping with the bias of quantitative data: (1) all parts of the structure have the same chance of being sampled; (2) fields are systematically distributed through various parts of the region (thus increasing the probability of detecting heterogeneity in the location of fibers); (3) subjectivity by the investigator in placing the fields is avoided because the position of the fields is automatically determined once the first field has been randomly selected.

Practical guidelines to performing systematic random sampling on nerve cross-section profiles are given in detail elsewhere (Mayhew and Sharma 1984a; Larsen 1998; Geuna et al. 2000a). Figure 1 gives a practical example of a method for systematically random placing the sampling fields (represented by gray boxes in the drawing) on a nerve section. The whole nerve cross-section profile is preliminarily divided into several large subgroups of sampling fields (in this example twelve subgroups of nine boxes each were created). One of the nine boxes (the field no. 5 in this case) is then randomly selected in one of the subgroups (the upper left in this case). Finally, the same box (no. 5) is systematically selected in all other sub-groups of sampling fields.

The total number of fibers sampled can be modified by changing the size and the number of the sampling fields. The amount of sampling is related to the required precision of estimates. It should be here emphasized that, as a general rule, in order to cope with location related bias it is better to select a higher number of small-sized sampling fields rather than using few and large fields (Schmitz 1997,1998; Larsen 1998).

Level 4. Morphology-related foundations of bias

Morphological foundations of bias can originate from variability in fiber size, shape and orientation and are linked to the related differences in the probability of fiber profiles being intersected by the sampling frame edges and appearing in more than one sampling field: the so called "edge effect" (Gundersen 1977,1978; Larsen 1998; Geuna et al. 2000a). Most studies including data on peripheral nerve quantitative morphology report that fibers are selected within sampling boxes without providing any information on "what happens" when a fiber profile intersects the box edges. In fact, a larger fiber profile has a higher chance of appearing in more than one sampling field than a smaller fiber and therefore of intersecting the frame edges. Including all edging fiber profiles is biased towards larger profiles and will systematically lead to overestimate of both fiber number and mean size in a nerve trunk, whilst excluding any edging profile is biased towards smaller profiles and will systematically lead to an underestimation of fiber number and mean size.

This foundation of bias can be coped with by adopting an unbiased set of inclusion/exclusion rules for sampling fiber profiles within the sampling fields that assures that the probability of selecting an object solely depends on the presence of the particle, not on any measure of its size, shape and curvature (Gundersen 1986). There are several methods currently available for coping with the morphology-related foundation of bias of nerve fiber profiles in cross sections: the associated-point techniques (Miles 1978; Gundersen 1986; Geuna et al 2000a), the unbiased counting frame (Gundersen 1977,1978, 1986; Schionning and Larsen 1997; Larsen 1998; Schionning et al. 1998) and the two-dimensional (2-D) disector (Gundersen 1986; Larsen 1998; Larsen and Gundersen 1999; Geuna et al. 2000a).

Level 5. Measurement-related foundations of bias

The procedures commonly used for size assessment of biological structures fall under the category of local estimators (Tandrup et al. 1997). This means that two steps are required: the first step is the sampling of objects by an appropriate method (see previous paragraph), whilst the second step is the local measurement of the sampled objects.

The most commonly used method for the local measurement of nerve fiber size is based on the employment of point grids and line grids placed on micrographs of the nerve cross section. Though very simple, this method is unbiased and efficient and allows to appropriately carrying on almost all investigations regarding nerve fiber size assessment (Gundersen 1988; Larsen 1998). As an alternative, a new design-based technique, named the two-dimensional (2-D) nucleator (Gundersen 1988; Larsen 1998), has been recently deveoloped and fruitfully applied to nerve fibers profiles (Schionning and Larsen 1997; Larsen 1998; Schionning et al. 1998). This method is based on a strong theoretical basis (Gundersen 1988) and has proved to be very efficient and accurate (Gundersen 1988; Larsen 1998).

Independently of the method used for measurements, bias in nerve fiber measurements can orignate from two main elements: (1) the obliquity of the plane of section, (2) the shrinkage of fiber borders:

1. To cope with obliquity of the plane of section, particular attention should be paid to straightening and correctly orientating the nerve trunk before sectioning. If perfusion fixation is used, straight extension of the nerve can be obtained by stretching the animal's limbs during perfusion. A possible alternative to animal perfusion is dipping and then immersion of the nerve in the fixative solution, extending it straight for the first 10–15 min of fixation. In our experience, this procedure, besides allowing a good straight extension of nerves, preserves very well the ultrastructure of nerve fibers (Geuna et al. 2000b). In addition, it is the method of choice for processing human nerves obtained at biopsy or autopsy. In any case, it should be noted that this potential foundation of bias has been shown to introduce a high degree of uncertainty in nerve fiber size assessment only in case of an obliquity of the plane of section higher than 25° (Fraher 1980; Larsen 1998), a degree that can be usually easily avoided.
2. Tissue shrinkage. Shrinkage of the tissues due to fixative procedure is almost impossible to avoid and represents a major problem for comparing data from the literature. As regards peripheral nerve morphometry, it has been shown that duration of glutaraldehyde fixation (Onishi et al. 1974a), time elapsed between death and fixation (Onishi et al., 1974b), and osmolarity of the fixative solution (Onishi et al. 1976) can significantly influence axonal area. To cope with this unavoidable foundation of bias particular attention should be paid to processing all the nerves following precisely the same protocol. In addition, if comparison between one's own results and data from previously published studies is sought, this element should be taken into great consideration.

Assessment of precision of size estimates

In quantitative morphology the term precision refers to the statistical variance of an estimation procedure (West 1999), which is mainly related to the dispersion of the size values of the population of objects and the amount of objects sampled. However, this term in its broad sense can also refer to the precision of the single measurements (a meaning that is more in line with the literal definition of precision) that is mainly related to the technical equipment used for the measurements and the quality of the histological material. Since the latter meaning can be effectively replaced by the synonym "accuracy", we will in this paper, in order to avoid confusion, use the term "precision" to refer to estimate precision whilst the term "accuracy" will be used to refer to measurement precision.

Accuracy of measurements

Although it has been shown that, in quantitative morphology, accuracy in each single measurement is less relevant than estimation unbiasedness (Fraher 1980; Gundersen 1992; Larsen 1998; West 1999), nonetheless, information on this measurement feature must be given in any morpho-quantitative report to allow data interpretation in relation to the study aims.

Accuracy refers to two factors: (1) the inherent ability of the morphometric equipment (both manual or computerized) to detect size differences within the magnitude range of the objects under observation; (2) the reproducibility of the measurements. The first factor, which mainly depends on the magnitude range of the objects under study, can be assessed by measuring an appropriate test

profile before using the method on the histological material (Fraher 1980). The magnification of observation should be then adapted to the magnitude of the objects. In our experience, a minimal magnification of at least x3000 times is needed for peripheral nerve myelinated fiber size assessment. The second factor can be well-assessed by quantifying the variance of repeated measurements on the tissue under investigation. Being strictly dependent on the type and quality of the material under investigation, accuracy on repeated measurements should be re-tested for any specific research context even if a previously used procedure is employed.

Precision of estimates: the coefficient of error

The coefficient of error (CE) is an estimator of the precision of the quantitative estimates (Larsen 1998). This coefficient, which represents the variance in statistical terms (West 1999), depends on the number of objects sampled and can be thus adjusted to match the investigator's needs just by changing the amount of sampling (and by consequently adjusting the sampling scheme design).

Two coefficients provide us with useful information about the components of the observed variance and can be used to optimize the sampling scheme: the coefficient of error of the estimates, *CE(est)*, and the observed coefficient of variation, *CV(obs)*. The two coefficents are related by the following formula:

$$CV(obs)^2 = CV(biol)^2 + CE(est)^2$$

where *CV(biol)* is the biological coefficient of variation, i.e. the ordinary inter-individual variability of a given parameter.

For size estimates there is a further level of variance besides the inter-individual one, namely the CV of the sizes within an individual. This coefficient is related to the biological CV of individual fiber sizes within an animal and the CE of the individual estimate in the same manner as for inter-individual CV.

The *CV(z)* within an animal is estimated by the following formula (Larsen 1998):

$$CV(z) = SD(z) / \bar{z}$$

where *z* is the size estimate (e.g. the cross-sectional area of fibers) and $\bar{z}$ is the mean estimate from a particular animal.

The formula for estimating the *CE(z)* for the mean size is estimated as (Larsen 1998):

$$CE(\bar{z}) = SEM(z) / \bar{z}$$

where *z* is again the size estimate and $\bar{z}$ is the mean size estimate from that particular nerve.

The inter-individual $CV(\bar{\bar{z}})$ is estimated as (Larsen 1998):

$$CV(\bar{\bar{z}}) = SD(\bar{\bar{z}}) / \bar{\bar{z}}$$

where $\bar{\bar{z}}$ is group average of the mean sizes.

The precision that is required to avoid an unnecessary workload in any specific study (e.g. avoid keeping the CE under too low a level by increasing the amount of sampling more than necessary) depends on two elements: the biological variability among subjects and the overall demands of the study (Larsen 1998). If a high degree of variability is found among subjects, it is not useful to look for high estimate precision in analysing each subject, it would be more useful to evaluate more subjects since one of the guiding rules of sampling is that one should put the most effort into the sampling level that contributes most to the overall variance. This point is well summarized by Gundersen and Østerby (1980): "Count more, less well!". As regards the second element that influences the required precision (i.e. the overall demands of the study), a high precision (i.e. a CE <0.05) might be sought only when particularly little size changes are investigated (e.g. if one wants to detect differences lower than 10%). However, if this is the case, the CV has to be concurrently reduced by adequately increasing the number of subjects analysed.

Parameters for size assessment

Table 1 lists the various geometrical parameters that can be used for the assessment myelinated nerve fibers (and/or axons).

The diameter together with the conduction velocity are the classical parameters for nerve fiber size assessment (Erlanger and Gasser 1937; Lloyd 1943; Rexed 1944; Schalow et al. 1995). They are strictly related since axon diameter has proved to be the main determinant of conduction velocity (Hoffman 1995). Various types of diameters of nerve fibers and/or axons have been used to assess their size: the maximum diameter (which is strongly biased by obliquity of cross-sectional fiber profiles), the minimum diameter (which is strongly biased by fiber shrinkage), and the circle-equivalent diameter which represents the diameter of a circle the area of which corresponds to the cross-sectional area of the fiber and/or axon (Karnes et al. 1977).

Cross-sectional area is another commonly used size-estimation parameter for myelinated nerve fibers. However, in our opinion, data on the cross-sectional area of nerve fibers are less easily and immediately to interpreted by readers (both basic scientists and clinicians), in comparison with diameter data, because the latter is the

Table 1 Size estimation parameters for myelinated nerve fibers

1. Diameter (fiber or axon)	Maximum
	Minimum
	Circle-equivalent
2. Cross-sectional area (fiber or axon)	
3. Perimeter (fiber or axon)	
4. Myelin thickness	
5. Myelin-thickness/axon-diameter ratio	
6. Fiber-diameter/axon-diameter ratio or axon-diameter/fiber-diameter (g-ratio)	

classical parameter used to classify nerve fibers (Erlanger and Gasser 1937; Lloyd 1943; Rexed 1944; Hoffman 1995; Schalow et al. 1995).

There are four more size parameters, achievable by simple mathematical calculations if data on both fiber and axon size are available, that deserve mention. These parameters, that are important when the development (Fraher et al. 1990) or regeneration (Kanaya et al. 1996) of nerve fibers is investigated, are: the myelin thickness; the myelin-thickness/axon-diameter ratio; the fiber-diameter/axon-diameter ratio and its opposite, the axon-diameter/fiber-diameter ratio (g-ratio).

The final decision on the selection of one or more of these size parameters for a specific research should represent one of the main steps of the study-design phase and should be done on the basis of the quality of the histological material (which might be poor, especially in case of the precious human nerves), the equipment available, and, eventually, the demands of the morpho-quantitative data within the overall research goals.

Discussion

In recent times, the prevailing relevance of the issue of unbiasedness of morpho-quantitative data in the light of their interpretation and evaluation, has been more and more recognized by morphologists, and procedures mainly aimed at coping with bias have been given a growing interest by the scientific community (Mayhew and Gundersen 1996; Saper 1996; Hyman et al. 1998; West 1999). Although peripheral nerves are often quantitatively investigated, the methodological issue of data unbiasedness has so far been almost ignored in most research papers reporting the results of morpho-quantitative nerve fiber analysis. Nowadays, comprehensive theoretical and practical demonstrations of the relevance of this issue, and the description of the methodological devices to cope with bias of quantitative data on peripheral nerve fibers, can be found in the literature and have been reviewed in this paper (Gundersen 1977, 1978, 1992; Mayhew and Sharma 1984a,b; Gundersen and Østerby 1980; Gundersen et al. 1988a,b; West and Gundersen 1990; Mayhew 1992: West 1993, 1999; Mayhew and Gundersen 1996; Larsen 1998; Schionning and Larsen 1997; Schmitz 1997,1998; Hyman et al. 1998; Schionning et al. 1998; Saper 1999; Geuna 2000; Geuna et al. 2000a). Identifying and coping with the possible foundations of bias, as well as taking into consideration the related limitations in quantitative data interpretation, is especially important when comparative studies are concerned.

The use of the term unbiased in morpho-quantitative reports deserves particular discussion. To claim that a method is free of bias is utopian! More realistically, the term unbiasedness should be used (and is used here) to refer to an “ideal goal” towards which the scientist must tend to by systematically analysing the possible foundations of bias, selecting the most appropriate method in order to cope with them, noting carefully each method’s unavoidable limitations and finally interpreting results within those limitations to address important scientific issues (Saper 1999; Geuna 2000). With this in view, this paper was written with the main goal of facilitating dissemination of this analytical approach to those scientists (both morphologists and clinicians) involved in peripheral nerve quantitative morphology.

Though the introduction of design-based sampling strategies in quantitative morphology is still under debate (Hyman et al. 1998; Saper 1999; Geuna 2000), it has undoubtedly focused the attention of researchers on the need for a more rigorous study of the design phase when morpho-quantitative data are sought. This phase, besides being theoretical, also needs to be practical, i.e. based on a preliminary pilot study that should be conducted when a method is applied for the first time to investigate a given biological issue by a particular research group, and must take into consideration various factors such as the quality of the histological material under analysis, the equipment available in each laboratory and the ultimate use of quantitative data within the overall study demands. Table 2 summarizes the main steps that the investigator needs to follow to optimize a method for the estimation of myelinated nerve fiber size in peripheral nerves.

It has been observed that the spread of the design-based morpho-quantitative approach in the scientific community is rather slow (Mayhew and Gundersen 1996; Saper 1996). The reasons why many scientists resist taking into consideration the new generation of methods are both theoretical and practical (Hyman et al. 1998; Geuna 2000; Geuna et al. 2000a). From a theoretical viewpoint, the relative complexity of some of the concepts and principles that underlie the rules that need to be respected for quantitative estimation can lead to the lack of their full comprehension, especially by scientists who are not already confident with similar issues, with the consequence of an a priori rejection of considering the new approaches. As regards the practical impediments, these are mainly related to difficulties in correctly applying theoretical issues to the practical procedures. To cope with these impediments, this paper was meant to provide clear and (as far as possible) simple information on both theoretical issues that are relevant to quantitative estimation of myelinated fibers in peripheral nerves and on their trasfer into practice.

Automated approaches deserves a particular mention. Recently, fully computerized devices for the automated measurement of nerve fibers have been developed (Fok et al. 1996; Campadelli et al. 1999; Dolapchieva et al. 2000; Romero et al. 2000). These new types of automated approach to nerve fiber morphometry are characterized by a high accuracy and a remarkable efficiency (it takes some seconds to process images with hundreds of axons profiles). In addition, automated devices also allow the measurement of other important spatial parameters of nerve fibers, such as their position within a nerve. Since cost-effectiveness represents a major advantage of

Table 2 Practical steps for designing a procedure for myelinated nerve fiber size estimation

STEP 1 Selection of strain, gender and age of experimental animals
Besides the usual rules for organizing the various experimental and control groups of animals with regard to strain and gender, particular attention should be paid to the age of animals. In particular, if rats are selected the age between 3 and 6 months has to be preferred because of the lower age-related variability in this lifeperiod (Fraher et al. 1990)
STEP 2 Histology and selection of the level of sectioning
Particular attention should be paid in respecting a rigid fixation protocol in order to avoid fixation-related bias (Onishi et al. 1974a, b, 1976) An adequate straight extension of the nerve should be obtained either by stretching the animal's limbs during perfusion or by dipping the nerve in the fixation solution, extending it straight for the first 10–15 minutes of fixation Resin embedding, semi-thin sectioning and Nissl toluidine-blue staining (Richardson method) is a very effective tissue processing for myelinated nerve fiber quantitative analysis
STEP 3 Sampling of fibers
Ideally, each fiber profile must have the same chance of being sampled irrespectively of: (1) its location throughout the nerve cross-sectional profile, (2) its size, shape and orientation. The first foundation of bias can be efficiently coped with by a systematic random sampling procedure (Larsen 1998; Geuna et al. 2000a) so that any part of the nerve cross-sectional profile has the same chance of being sampled. The other foundations of bias can be efficiently coped with by the unbiased counting methods (Gundersen 1977, 1978; Miles 1978; Larsen 1998; Geuna et al. 2000a), so that any fiber inside a sampling field has the same chance of being measured independently of its morphology In any case, detailed information on how the sampling fields have been sampled and how the fibers are sampled within the fields need always to be clearly reported in any dedicated paper in order to allow the reader to correctly interpret quantitative data
STEP 4 Selection of size estimation parameters
The size estimation parameter(s) must be selected on the basis of the quality of the histological material, the equipment available and the overall research goals Linear parameters (diameter, perimeter) are less biased by obliquity of sectioning than 2-D parameters (cross-sectional area; Larsen 1998) As a general rule, the diameter is of most immediate interpretation by readers in relation to classical nerve fiber classifications Independently of the parameter selected, it is of fundamental importance for data interpretation to clearly specify which size estimation parameter was used and why
S*TEP 5 Selection and assessment of the method for size measurement*
Detailed information on the method used to measure sizes has to be given, and an evaluation of the accuracy of the method reported
STEP 6 - Assessment of the precision of estimates (estimation of the CE)
The precision of quantitative estimates has to be assessed by estimating the coefficient of error (CE; Larsen 1998) The degree of estimate precision should be related to inter-individual and intra-individual variability assessed by the coefficient of variation (CV; West and Gundersen 1990)

a morphometric approach, it can be foreseen that the high efficiency of the new automated devices, together with the present availability of high-power personal computers at relatively low costs will probably make them the methods of choice for peripheral nerve quantitative morphology in the near future.

One of the main practical differences between the new and the traditional morpho-quantitative approach is that, in the former, much effort is spent in the design phase of the study rather than in the counting and measuring work. This fact, which is in contrast with older mophometrical habits of many scientists, might seem to increase the workload, but it does not since the workload is just shifted towards the study-design phase. On the contrary, once a procedure has been correctly designed and set up, the measuring workload is usually reduced, a great advantage in quantitative morphology.

Appendix 1 The concepts of bias and precision in quantitative morphology

In quantitative morphology, the terms bias and precision are used to refer to two separate features of quantitative estimates obtained by any method devised to quantify a morphological parameter. Though these two terms refer to two clearly separate aspects of an estimate, they have been often confused (Gundersen 1992; Larsen 1998; West 1999). Since this is not just a matter of terminological confusion, but hides a conceptual misunderstanding that can be at the basis of the erroneous design of morpho-quantitative studies and/or the misinterpretation of quantitative data, it is important to provide a clear explanation of these two concepts and the related practical issues in quantitative morphology. The term biasedness refers to an estimation procedure for which the mean of repeated estimates deviates systematically from the true value regardless of the amount of sampling (West 1999). On the other hand, the term precision is used in a conceptually different meaning: in fact, it is a feature of the estimates (as the term bias) designating the statistical variance (West 1999). Figure 2 illustrates the concepts of

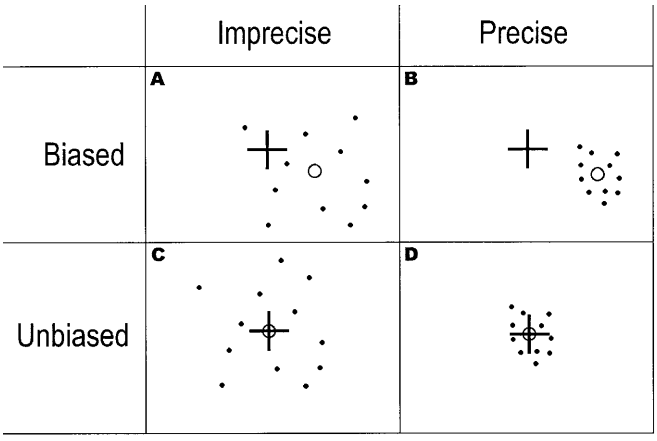

Fig. 2A–D The concepts of bias and precision of estimates. Small black dots represent mean size repeated estimates made on independent sets of size measurements on a given population of objects; the bigger white dot represents the mean of the repeated estimates; the centre of the black cross represents the true mean size value of that population of objects. If a method is biased (**A, B**), the mean of the repeated estimates deviates systematically from the true value regardless of the estimate precision, whilst with an unbiased method (**C, D**) the two values coincide. Increasing the precision of the single estimates (by increasing the amount of sampling), when a methodological bias exists (**B**) will only result in making more precise an estimate that still remains biased (modified from Gundersen 1992)

bias and precision. From a practical viewpoint, one of the main differences between the two concepts is that the increase in the amount of sampling, whilst it increases the precision of an estimate, does not reduce the biasedness of that estimate! Therefore, if an estimating procedure is biased, increasing the amount of sampling (e.g. the number of nerve fibers measured) will increase the precision of an estimate that, on average, will systematically, and in the same direction (over- under-estimation) deviate from the true value. If statistical comparisons are sought, this may produce a misleading interpretation of data since statistically significant numerical differences might be the result of the original bias rather than true differences among various groups of objects. In addition, while estimate precision can be detected from the data themselves (by estimating the coefficient of error as described earlier in this paper), the estimate bias cannot, and should be dealt with by designing a sampling strategy aimed at coping with any possible foundation of bias (West 1999). For these reasons, a great deal of effort should be dedicated to the preliminary study-design phase of any study with the aim of detecting and coping with any possible foundation of bias.

Appendix 2 Notes on equipment requirements and on size estimation of unmyelinated nerve fibers

Though the use of the procedures and methods described in this paper can be facilitated by computerized image-analyzers that reproduce microscope images on a monitor and make it possible to delineate frames of various sizes on the monitor and measure objects and fields, they can also be applied working on photographic prints. Photographs are taken by jumping systematically at random through the nerve profile and then printed at a sufficient final magnification (at least x 3,000 times). Sampling fields are then randomly selected within a frame of boxes (of the designed sampling size) that are drawn on the photographs. Nerve fiber profiles are then sampled using the inclusion/exclusion rules of an unbiased sampling method such as the 2-D disector. Finally, fiber profiles are measured by the point-counting technique using printed transparencies. This photographic procedure (as well as all the principles and guidelines outlined in the present paper) can work quite well also for unmyelinated fiber-size estimation. Electron-microscope photographs are systematically random taken through the nerve profile and then printed at a sufficient final magnification (at least x 10,000). Sampling fields and fibers are then sampled and measured in the same way as described above.

Acknowledgements This work has been financed by grants from the MURST (Ministero della Ricerca Scientifica e Tecnologica). The authors are grateful to two anonymous reviewers who significantly contributed to the improvement of the quality of the original submitted manuscript with their critical comments and suggestions. The authors whish also to thank Prof. A. Nixon for the English language revision.

References

Battiston B, Tos P, Geuna S, Giacobini-Robecchi MG, Guglielmone R (2000) Nerve repair by means of vein filled with muscle grafts. II. Morphological analysis of regeneration. Microsurgery 20:37–41

Bradley JL, Thomas PK, King RH, Muddle JR, Ward JD, Tesfaye S, Boulton AJ, Tsigos C, Young RJ (1995) Myelinated nerve fibre regeneration in diabetic sensory polyneuropathy: correlation with type of diabetes. Acta Neuropathol 90:403– 410

Brown HG, Martin JR, Asbury AK (1976) Painful diabetic neuropathy. A morphometric study. Arch Neurol 33:164–171

Campadelli P, Gangai C, Pasquale F (1999) Automated morphometric analysis in peripheral neuropathies. Comp Biol Med 29:147–156

Ceballos D, Cuadras J, Verdu E, Navarro X (1999) Morphometric and ultrastructural changes with ageing in mouse peripheral nerve. J Anat 195:563–576

Cruz-Orive LM (1980) On the estimation of particle number. J Microsc 120:15–27

Cruz-Orive LM, Weibel ER (1981) Sampling designs for stereology. J Microsc 122:235–257

Dellon AL, Mackinnon SE (1989) Selection of the appropriate parameter to measure neural regeneration. Ann Plast Surg 23: 197–202

Dolapchieva S, Eggers R, Kühnel W (2000) Automatic image analysis of the postnatal growth of axons and myelin sheats in the tibial and peroneal nerves of the rabbit. Ann Anat 182: 133–142

Erlanger J, Gasser HS (1937) Electrical signs of nervous activity. University of Pensylvania Press, Philadelphia

Fok YL, Chan JCK, Chin RT (1996) Automated analysis of nerve-cell images using active contour models. IEEE Trans Med Imag 15:353–368

Fraher JP (1980) On methods of measuring nerve fibres. J Anat 130:139–151

Fraher JP (1992) Myelin-axon relationships in the rat phrenic nerve: longitudinal variation and lateral asymmetry. J Comp Neurol 323:551–557

Fraher JP, O'Leary D, Moran MA, Cole M, King RH, Thomas PK (1990) Relative growth and maturation of axon size and myelin thickness in the tibial nerve of the rat. I. Normal animals. Acta Neuropathol 79:364–374

Geuna S (2000) Appreciating the difference between design-based and model-based sampling strategies in quantitative morphology of the nervous system. J Comp Neurol 427:333– 339

Geuna S, Tos P, Battiston B, Guglielmone R (2000a) Verification of the two-dimensional disector, a method for the unbiased estimation of density and number of myelinated nerve fibers in peripheral nerves. Ann Anat 182:23–34

Geuna S, Tos P, Battiston B, Guglielmone R, Giacobini-Robecchi MG (2000b) Morphological analysis of peripheral nerve regenerated by means of vein grafts filled with fresh skeletal muscle. Anat Embryol 201:475–482

Geuna S, Tos P, Battiston B, Guglielmone R, Giacobini-Robecchi MG (2000c) A stereological study of long-term regeneration of rat severed sciatic nerve repaired by means of muscle-vein-combined grafts. Ital J Anat Embryol 105: 67–73

Gundersen HJG (1977) Notes on the estimation of the numerical density of arbitrary profiles: the edge effect. J Microsc 111: 219–223

Gundersen HJG (1978) Estimators of the number of objects per area unbiased by edge effects. Microsc Acta 81:107–117

Gundersen HJG (1986) Stereology of arbitrary particles. J Microsc 143:3–45

Gundersen HJG (1988) The nucleator. J Microsc 151:3–21

Gundersen HJC (1992) Stereology: The fast lane between neuroanatomy and brain function – or still only a tightrope? Acta Neurol Scand [Suppl] 137:8–13

Gundersen HJG, Jensen EB (1987) The efficiency of systematic sampling in stereology and its prediction. J Microsc 147: 229–263

Gundersen HJG, Østerby R (1980) Optimizing sampling efficiency of stereological studies in biology: or "Do more, less well!". J Microsc 121:65–73

Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB, Vesterby A, West MJ (1988a) Some new, simple and efficient stereological methods and their use in pathological research. APMIS 96:379–394

Gundersen HJG, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB, Vesterby A, West MJ (1988b) The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96:857–881

Gundersen HJG, Jensen EBV, Kieu K, Nielsen J (1999) The efficiency of systematic sampling in stereology – reconsidered. J Microsc 193:199–211

Hoffman PN (1995) The synthesis, axonal transport, and phosphorilation of neurofilaments determine axonal caliber in myelinated nerve fibers. Neuroscientist 1:76–84

Hyman BT, Gomez-Isla T, Irizarry MC (1998) Stereology: a practical primer for neuropathology. J Neuropathol Exp Neurol 57:305–310

Ide C (1996) Peripheral nerve regeneration. Neurosci Res 25: 101–121

Kanaya F, Firrel JC, Breidenbach WC (1996) Sciatic function index, nerve conduction tests, muscle contraction, and axon mophometry as indicators of regeneration. Plast Reconstr Surg 98:1264–1271

Karnes J, Robb R, O'Brien PC, Lambert EH, Dyck PJ (1977) Computerized image recognition for morphometry of nerve attribute of shape of sampled transverse sections of myelinated fibers which best estimates their average diameter. J Neurol Sci 34:43–51

Larsen JO (1998) Stereology of nerve cross sections. J Neurosci Methods 85:107–118

Larsen JO, Gundersen HJG (1999) Virtual test systems for global spatial sampling in thick, arbitrarily oriented uniform, random section. Acta Stereol 18:381–388

Lloyd DPC (1943) Reflex action in relation to pattern and peripheral source of afferent stimulation. J Neurophysiol 6: 111–119

Lundborg G (1988) Nerve injury and repair. Churchill Livingstone, Edinburgh

Mackinnon SE (1996) Sciatic function index, nerve conduction tests, muscle contraction, and axon mophometry as indicators of regeneration [Discussion]. Plast Reconstr Surg 98:1272–1273

Mayhew TM (1988) An efficient sampling scheme for estimating fibre number from nerve cross sections: the fractionator. J Anat 157:127–134

Mayhew TM (1992) A review of recent advances in stereology for quantifying neural structure. J Neurocytol 21:313–328

Mayhew TM, Gundersen HJG (1996) 'If you assume, you can make an ass out of u and me': a decade of the disector for stereological counting of particles in 3D space. J Anat 188: 1–15

Mayhew TM, Sharma AK (1984a) Sampling schemes for estimating nerve fibre size. I. Methods for nerve trunks of mixed fascicularity. J Anat 139:45–48

Mayhew TM, Sharma AK (1984b) Sampling schemes for estimating nerve fibre size. II. Methods for unifascicular nerve trunks. J Anat 139:59–66

Miles RE (1978) The sampling, by quadrats, of planar aggregates. J Microsc 113:257–276

Ohnishi A, Offord K, Dyck PJ (1974a) Studies to improve fixation of human nerves. Part 1. Effect of duration of glutaraldehyde fixation on peripheral nerve morphometry. J Neurol Sci 23:223–226

Ohnishi A, O'Brien PC, Dyck PJ (1974b) Studies to improve fixation of human nerves. Part 2. Effect of time elapsed between death and glutaraldehyde fixation on relationship of axonal area to number of mylein lamellae. J Neurol Sci 23: 387–390

Ohnishi A, O'Brien PC, Dyck PJ (1976) Studies to improve fixation of human nerves. Part 3. Effect of osmolality of glutaraldehyde solutions on relationship of axonal area to number of myelin lamellae. J Neurol Sci 27:193–199

Pollock M, Nukada H, Allpress S, Calder C, Mackinnon M (1984) Peripheral nerve morphometry in stroke patients. J Neurol Sci 65:341–352

Rexed B (1944) Contributions to knowledge of the postnatal development of the peripheral nervous system in man. Acta Psychiatr Neurol Suppl 33:1–206

Romero E, Cuisenaire O, Denef JF, Delbeke J, Macq B, Veraart C (2000) Automatic morphometry of nerve histological sections. J Neurosci Methods 97:111–122

Saper CB (1996) Any way you cut it: a new journal policy for the use of unbiased counting methods. J Comp Neurol 364:5

Saper CB (1999) Unbiased stereology: three-dimensional measurement in microscopy [Book review]. Trends Neurosci 22:94–96

Schalow G, Zach GA, Warzok R (1995) Classification of human peripheral nerve fibre groups by conduction velocity and nerve fibre diameter is preserved following spinal cord injury. J Auton Nerv Syst 8:125–150

Schiønning JD, Larsen JO (1997) A stereological study of dorsal root ganglion cells and nerve root fibers from rats treated with inorganic mercury. Acta Neuropathol 94:280–286

Schiønning JD, Larsen JO, Eide R (1998) A stereological study of dorsal root ganglion cells and nerve root fibers from rats exposed to mercury vapor. Acta Neuropathol 96:185–190

Schmitz C (1997) Towards more readily comprehensible procedures in disector stereology. J Neurocytol 26:707–710

Schmitz C (1998) Variation of fractionator estimates and its prediction. Anat Embryol 198:371–397

Soong BW, Lin KP (1998) Correlation of peripheral nerve fiber loss and trinucleotide repeats in Machado-Joseph disease. Can J Neurol Sci 25:59–63

Sterio DC (1984) The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 134:127–136

Tandrup T, Gundersen HJC, Vedel-Jensen EB (1997) The optical rotator. J Microsc 186:108–120

Terzis JK, Sun DD, Thanos PK (1997) Historical and basic science review: past, present and future of nerve repair. J Reconstr Microsurg 13:215–225

Thomas PK, Fraher JP, O'Leary D, Moran MA, Cole M, King RH (1990) Relative growth and maturation of axon size and myelin thickness in the tibial nerve of the rat. II. Effect of streptozotocin-induced diabetes. Acta Neuropathol 79:375– 386

Torch S, Stoeber P, Usson Y, D'Aubigny GD, Saxod R (1989) There is no simple adequate sampling scheme for estimating the myelinated fibre size distribution in human peripheral nerve: a statistical ultrastructural study. J Neurosci Methods 27:149–164

Tos P, Battiston B, Geuna S, Giacobini-Robecchi MG, Hill MA, Lanzetta M, Owen ER (2000) Tissue specificity in rat peripheral nerve regeneration through combined skeletal muscle and vein conduit grafts. Microsurgery 20:65–71

West MJ (1993) New stereological methods for counting neurons. Neurobiol Aging 14: 275–286

West MJ (1999) Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. Trends Neurosci 22:51–61

West MJ, Gundersen HJG (1990) Unbiased stereological estimation of the number of neurons in the human hippocampus. J Comp Neurol 296:1–22