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Sampling schemes for estimating nerve fibre size. II. Methods for unifascicular nerve trunks

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INTRODUCTION

Mayhew & Sharma (1984) have compared the statistical qualities of alternative sampling regimes for estimating myelinated fibre diameters in a peripheral nerve, the tibial nerve of the rat, possessing one or more fascicles. From single biopsies at the same site in different animals, it has been noted that roughly 70% of animals produce unifascicular biopsies (Mayhew & Sharma, unpublished observations). Applying the binomial frequency distribution (Bailey, 1972), it can be predicted that a set of 6 animals gives a 1 in 8 chance that all biopsies will have one fascicle. Nerve trunks at other anatomical sites may be exclusively unifascicular. Therefore, the present paper extends the scope of the earlier study by assessing the merits of sampling schemes which have been employed to estimate the sizes of nerve fibres in such trunks.

In particular, systematic random square sampling (Mayhew & Sharma, 1984) has been compared with two methods which are permissible only on unifascicular trunks: axial strip sampling (Donovan, 1967) and sector sampling (Jakobsen, 1976; Diani *et al.* 1981; Bedi & Warren, 1983).

MATERIALS AND METHODS

Preparative stages

Full details of tissue preparation steps are provided in Mayhew & Sharma (1984). Briefly, biopsies of the right tibial nerve were obtained from adult male Wistar rats in which diabetes mellitus had been induced 10 weeks earlier by administration of streptozotocin. Specimens were fixed in paraformaldehyde/glutaraldehyde, washed in buffered sucrose and post-fixed in osmium tetroxide.

Out of the original group of six biopsies, four displayed but a single fascicle and formed the basis of this investigation. Semithin Araldite sections comprising entire transverse sections through biopsy specimens were stained with thionin and acridine orange. Montages of each transection were constructed from photomicrographs printed at a final magnification of $\times 800$.

Fibre measurement

Absolute values with which to assess the reliability, precision, cost in time, and efficiency of different sampling methods were obtained by measuring the external diameters of all myelinated fibres on each montage. Axons sectioned through paranodes and Schmidt–Lanterman incisures were excluded from measurement.

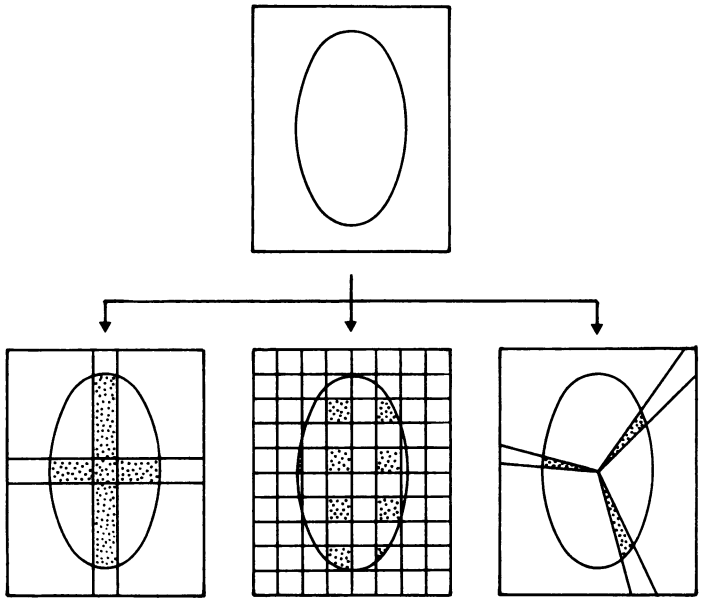

Fig. 1. Illustration of different sampling schemes. The upper Figure represents a transverse section through a unifascicular nerve trunk. The lower Figures illustrate axial strip sampling (left), systematic random square sampling (middle) and systematic random sector sampling (right). Myelinated fibres appearing in stippled areas are measured.

When all fibres on a given montage had been measured, the time taken to record the measurements was noted and a pre-recorded computer programme calculated the mean fibre diameter, fibre-to-fibre variance, standard deviation (S.D.) and coefficient of variation (CV %).

Subsequently, the group mean diameter was computed, together with its variance (S^2) and standard error (S.E.M.). The total measurement time was also noted.

Sampling schemes

Three sampling schemes were compared: axial strip sampling, systematic random square sampling and systematic random sector sampling (Fig. 1). Other possible schemes were not included since earlier analyses had demonstrated that they were, at best, only as efficient as systematic selection of squares. Where appropriate, each scheme was applied in a series of ten independent trials, to allow for sampling variation in estimated fibre means and variances.

(*a*) *Axial strip sampling*

As described by Donovan (1967), fibres in near-circular transverse sections of the cat optic nerve were sampled in complete strips across two diameters at right angles to each other. In the present study, her design has been modified to allow for the fact that transverse sections of rat tibial nerve biopsies with only one fascicle are elliptical rather than circular in outline. Therefore the strips were taken across the long and short axes of each biopsy. Such sampling is not strictly random since it is dependent upon the quality of the specimen. For this reason, only one trial was undertaken with this design. To ensure freedom from selection bias, only those fibres

were measured whose centres lay within the boundaries of the strips (Miles, 1978). This design captured roughly 24% of the fibre complement of each nerve.

(*b*) *Systematic random square sampling*

Full details are given in Mayhew & Sharma (1984). Fibres were selected in a systematic random pattern of squares on each montage. Two types of selection were made, a 1:9 and a 1:16 set of squares. In both cases, the location of the first square was decided by chance. These schemes selected about 11% and 6% of fibres respectively.

(*c*) *Sector sampling*

This approach has been described in several studies on the measurement of nerve fibre size (Donovan, 1967; Sima, 1974; Jakobsen, 1976; Diani *et al.* 1981; Bedi & Warren, 1983). Most are based on simple random selections of sectors but the scheme has been modified here by taking a systematic random arrangement. For this purpose, a set of three sectors sharing a common vertex was drawn on white card. Each sector subtended an angle of 10° and was separated from its neighbour by an angle of 110°. This drawing was photocopied on transparent sheeting and superimposed on each montage in turn so that its vertex was approximately coincident with the centre of the nerve fascicle. In each trial, sector orientation was independent of fascicular quality and content. Fibres were measured if their centres fell inside the sectors. About 8% of all fibres were sampled using this design.

Data handling

After every sampling trial, calculations were made of the group mean fibre diameter, variance (S^2) and S.E.M., and the time (t) involved in taking the measurements recorded. The efficiency (E) was estimated using the expression $E = 1/(S^2.t)$ and individual sampling efficiencies (E_s) were divided by the absolute value (E_e) in order to compare different schemes (Mayhew & Sharma, 1984).

RESULTS

Measurement of every fibre

A total of 11823 fibres was measured. The smallest recorded diameter was less than 1 μm and the diameter of the largest fibre was 14·5 μm.

The number of fibres per nerve varied from 2784 to 3108 (Table 1) and the group mean ± S.E.M. was 2956 ± 67. Group mean fibre diameter was 5·43 ± 0·21 μm, demonstrating a variability between animals which was small in comparison to the fibre-to-fibre differences within each animal (coefficient of variation = 33–38% of corresponding animal means). Distributions of fibre size were essentially similar in the four rats.

The time taken to measure all biopsies was just under 20 hours (Table 1). With an observed variance between animals of 0·178 μm^2, the index of efficiency (E_e) was 0·287 $\mu m^{-2}\ hr^{-1}$.

Fibre sampling schemes

All schemes produced reliable trial estimates of the group mean fibre diameter which were consistently within 5% of the absolute value of 5·43 μm (Tables 2–4). Axial strip sampling gave a group mean diameter of 5·50 μm, within 2% of the absolute value.

Table 1. *Estimates of number, external diameter and total measurement time per animal for fibres in four unifascicular tibial nerve trunks*

Animal serial number	Fibre number	Diameter (μm)	Time (hours)
3	2977	5·33 ± 2·00 (37·5)*	4·63
4	2784	5·80 ± 2·06 (35·5)	4·77
5	2954	4·87 ± 1·82 (37·3)	4·87
6	3108	5·71 ± 2·12 (37·1)	5·30

* Mean ± standard deviation (coefficient of variation per cent)

Table 2. *Systematic square sampling* (1:9) *estimates of fibre number, group mean diameter and measurement time for* 10 *trials on four animals*

Trial serial number	Fibre number	Diameter (μm) group mean ± S.E.M.	Time (hours)
1	1240	5·41 ± 0·18	2·10
2	1292	5·49 ± 0·24	2·18
3	1398	5·36 ± 0·17	2·30
4	1332	5·36 ± 0·18	2·21
5	1313	5·37 ± 0·16	2·19
6	1391	5·52 ± 0·24	2·33
7	1275	5·32 ± 0·19	2·13
8	1296	5·40 ± 0·22	2·16
9	1298	5·45 ± 0·23	2·13
10	1411	5·45 ± 0·25	2·33

Table 3. *Systematic square sampling* (1:16) *estimates of fibre number, group mean diameter and measurement time for* 10 *trials on four animals*

Trial serial number	Fibre number	Diameter (μm) group mean ± S.E.M.	Time (hours)
1	710	5·59 ± 0·17	1·15
2	836	5·36 ± 0·15	1·38
3	693	5·17 ± 0·25	1·14
4	739	5·46 ± 0·30	1·23
5	690	5·44 ± 0·12	1·16
6	780	5·35 ± 0·09	1·30
7	694	5·58 ± 0·25	1·14
8	779	5·32 ± 0·14	1·27
9	681	5·43 ± 0·13	1·14
10	808	5·50 ± 0·23	1·33

The precision of individual trial estimates, expressed as S.E.M., varied from 0·09 to 0·30 μm (Tables 2–4), compared with the reference value of 0·21 μm. With axial strip sampling, the estimated S.E.M. was 0·25 μm. Thus, all sampling schemes yielded reasonably precise estimates of their group mean diameters.

The measurement of fibre diameters in two axial strips on all four biopsies took

Table 4. *Systematic sector sampling estimates of fibre number, group mean diameter and measurement time for* 10 *trials on four animals*

Trial serial number	Fibre number	Diameter (μm) group mean ± S.E.M.	Time (hours)
1	1088	5·48 ± 0·20	1·77
2	1020	5·50 ± 0·23	1·68
3	1063	5·43 ± 0·28	1·76
4	967	5·39 ± 0·25	1·61
5	944	5·46 ± 0·16	1·58
6	914	5·56 ± 0·28	1·52
7	951	5·40 ± 0·28	1·58
8	947	5·40 ± 0·23	1·57
9	950	5·57 ± 0·24	1·56
10	922	5·37 ± 0·21	1·55

Table 5. *Relative efficiencies (E_s/E_e) of different sampling schemes in each set of trials*

Trial serial number	Sampling schemes: Systematic square 1:9	Systematic square 1:16	Systematic sector
1	12·3	27·1	12·3
2	7·1	29·1	9·5
3	13·8	11·8	6·4
4	11·9	7·9	8·8
5	15·3	51·8	21·9
6	6·3	83·2	7·2
7	11·3	12·2	7·1
8	8·1	33·5	10·0
9	7·5	44·8	9·5
10	6·0	12·3	13·2

about 4·6 hours. Measurement times for the other schemes were 2·1–2·3 hours using 1:9 square sampling, 1·1–1·4 hours for 1:16 square sampling and 1·5–1·8 hours with sector sampling.

On the basis of observed costs in time and the variances, strip sampling was found to be three times more efficient than measuring every fibre. For other schemes, the *minimum* relative efficiencies (Table 5) were six times (1:9 square sampling and sector sampling) and eight times (1:16 square sampling) the reference value.

DISCUSSION

The present findings confirm the authors' earlier conclusions that sampling produces cost-beneficial, unbiased, estimates of nerve fibre diameter and is highly preferable to measuring every fibre (Mayhew, 1983; Mayhew & Sharma, 1984) in nerve trunks.

The comparatively low efficiency of axial strip sampling is related to the larger proportion of fibres selected. This type of scheme has potentially serious drawbacks. As applied in this study, the scheme was not independent of the tissue analysed because of the ellipticity of the nerve transections. Even in the cat optic nerve, which

is almost circular in transverse section (Donovan, 1967), positioning is not independent if strips are placed across diameters. There is always the danger of introducing systematic error (bias) due to the fact that central regions of the specimen are preferentially oversampled compared with peripheral regions. This bias has been observed in morphometric analyses of the cat optic nerve, where the spatial distribution of fibres is heterogeneous in terms of both size and number: the fibres tend to be smaller and more numerous peripherally (Van Crevel & Verhaart, 1963; Donovan, 1967). Applied to the cat optic nerve, therefore, this scheme tends to systematically overestimate fibre size and underestimate fibre number (Donovan, 1967). This source of bias is probably less significant in the present context because fibres in the rat tibial nerve are distributed rather uniformly. But the axial scheme certainly lacks the general applicability of the other procedures employed in this and its companion investigation (Mayhew & Sharma, 1984).

In the present study, systematic sector sampling is at least as efficient as 1:9 square sampling, though less efficient than selecting a 1:16 pattern of squares: the latter captures fewer fibres than sector sampling. It is possible that the efficiency of sector analysis could be improved by designing more but smaller-angled sectors capturing fewer fibres. Therefore, it may be feasible to devise a systematic sector sampling scheme which has all the advantages of square sampling (in evenly covering all regions of the specimen) and is comparable to it in terms of overall efficiency for an equivalent number of fibres. It is evident that any differences in sampling precision will be diminished in proportion to the *final* precision of estimation, which is determined primarily by the *number of animals* examined (Shay, 1975; Nicholson, 1978; Gundersen & Østerby, 1981; Mayhew, 1983).

These investigations (see also Mayhew & Sharma, 1984) have restricted attention to random sampling errors involved in measuring peripheral nerve fibres cut transversely. Such measurements are also susceptible to biases arising from technical considerations, notably shrinkage, distortion, resolution and method of measurement, but these factors have been discussed elsewhere (Duncan, 1934; Williams & Wendell-Smith, 1960; Forrester & Peters, 1967; Donovan, 1967; Fraher, 1980). Such errors influence the accuracy of estimation and no corrections have been made for them in the present study because absolute values have been used to draw purely internal comparisons. Only in this sense must the 'absolute' values be regarded as accurate.

One source of bias which is especially pertinent to internal comparisons is selection bias; every effort has been made to minimise this factor (Mayhew & Sharma, 1984). In particular, an unbiased selection and counting convention has been adopted which involves measuring only fibres whose centres fall within the borders of the sampling unit, whether square, strip or sector. In a recent study of the rat tibial nerve, an automated flying-spot microscope was used to measure selected fibres (Ellis, Rosen & Cavanagh, 1980). In this system, fibres are selected if they fall entirely within the sampling unit but not if they touch its boundaries. Unfortunately, this convention tends to favour the inclusion of smaller fibres since these have a better chance of falling completely within the sampling frame. Fibre size measurements performed on this basis are therefore likely to be underestimated systematically and this could lead to problems when trying, for example, to correlate morphometric estimates with electrophysiological values of impulse conduction velocities. In this context, it is interesting to note that the mean fibre diameter of the experimental diabetic rats is about 5·4 μm. For mammalian myelinated fibres at 37 °C, the conduction velocity in

m sec^{-1} is approximately six times the external diameter measured in μm (Rushton, 1951). On this basis, the corresponding conduction velocity of rat tibial nerves would be about 33 m sec^{-1}.

SUMMARY

The total number of myelinated fibres in four unifascicular tibial nerves from diabetic rats has been counted and measured in order to assess the merits of various schemes for estimating group mean fibre diameter.

The average nerve trunk contained some 2960 fibres which were measured in just under five hours. With different sampling designs, the average measurement time per nerve was reduced to between 17 and 69 minutes, with little consequent loss of reliability or precision of estimated mean fibre size. The most efficient schemes were those taking systematic samples of squares or sectors. A modification of a method relying on complete strips across two diameters of each nerve was the least efficient sampling approach. It had the additional disadvantage of introducing systematic errors which could affect the accuracy of measurements made on nerve trunks with heterogeneous spatial distributions of fibre size and number.

This paper completes an investigation into random sampling errors influencing morphometric estimates of fibre size based on uni- or multifascicular nerve trunks.

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REFERENCES

BAILEY, N. T. J. (1972). *Statistical Methods in Biology*. London: English Universities Press.

BEDI, K. S. & WARREN, M. A. (1983). The effects of undernutrition during early life on the rat optic nerve fibre number and size-frequency distribution. *Journal of Comparative Neurology* **219**, 125–132.

DIANI, A. R., DAVIS, D. E., FIX, J. D., SWARTZMAN, J. & GERRITSEN, G. C. (1981). Morphometric analysis of autonomic neuropathology in the abdominal sympathetic trunk of the ketonuric diabetic chinese hamster. *Acta neuropathologica* **53**, 293–298.

DONOVAN, A. (1967). The nerve fibre composition of the cat optic nerve. *Journal of Anatomy* **101**, 1–11.

DUNCAN, D. (1934). A relation between axone diameter and myelination determined by measurement of myelinated spinal root fibres. *Journal of Comparative Neurology* **60**, 437–471.

ELLIS, T. J., ROSEN, D. & CAVANAGH, J. B. (1980). Automated measurement of peripheral nerve fibres in transverse sections. *Journal of Biomedical Engineering* **2**, 272–280.

FORRESTER, J. & PETERS, A. (1967). Nerve fibres in optic nerve of the rat. *Nature* **214**, 245–247.

FRAHER, J. P. (1980). On methods for measuring nerve fibres. *Journal of Anatomy* **130**, 139–151.

GUNDERSEN, H. J. G. & ØSTERBY, R. (1981). Optimizing sampling efficiency of stereological studies in biology: Or 'Do more less well!'. *Journal of Microscopy* **121**, 65–73.

JAKOBSEN, J. (1976). Axonal dwindling in early experimental diabetes. I. A study of cross sectioned nerves. *Diabetologia* **12**, 539–546.

MAYHEW, T. M. (1983). Stereology: progress in quantitative microscopical anatomy. In *Progress in Anatomy*, Vol. 3 (ed. V. Navaratnam & R. J. Harrison), pp. 81–112. Cambridge University Press.

MAYHEW, T. M. & SHARMA, A. K. (1984). Sampling schemes for estimating nerve fibre size. I. Methods for nerve trunks of mixed fascicularity. *Journal of Anatomy* **139**, 45–58.

MILES, R. E. (1978). The sampling, by quadrats, of planar aggregates. *Journal of Microscopy* **113**, 257–267.

NICHOLSON, W. L. (1978). Application of statistical methods in quantitative microscopy. *Journal of Microscopy* **113**, 223–240.

RUSHTON, W. A. H. (1951). A theory of the effects of fibre size in medullated nerve. *Journal of Physiology* **115**, 101–122.

SHAY, J. (1975). Economy of effort in electron microscope morphometry. *American Journal of Pathology* **81**, 503–512.

SIMA, A. (1974). Relation between the numbers of myelin lamellae and axon circumference in fibres of ventral and dorsal roots and optic nerve of normal, underfed and rehabilitated rats: An ultrastructural morphometric study. *Acta physiologica scandinavica, Suppl.* **410**, 5–55.

VAN CREVEL, H. & VERHAART, W. J. C. (1963). The rate of secondary degeneration in the central nervous system. II. The optic nerve of the cat. *Journal of Anatomy* **97**, 451–464.

WILLIAMS, P. L. & WENDELL-SMITH, C. P. (1960). The use of fixed and stained sections in quantitative studies of peripheral nerve. *Quarterly Journal of Microscopical Sciences* **101**, 43–54.