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Numerical and areal density estimates of fibre type composition in a skeletal muscle (rat extensor digitorum longus)

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INTRODUCTION

Locomotory muscles of the rat hindlimb are composed of three major types of fibre. Although many schemes of classification have been published, these can be broadly identified as slow oxidative (SO or Type I), fast oxidative (FO or Type IIa) and fast glycolytic (FG or Type IIb) from their histochemical staining patterns. The relative contribution of these different fibre types in determining the functional characteristics of skeletal muscle is usually inferred from estimates of their numerical ratio. Typically SO fibres from fast muscles of the rat are around half the size of FG fibres (Egginton, 1987), consequently their physiological importance (e.g. in terms of total oxidative capacity) is overestimated. Occasionally reference is made to a qualitative estimate of their relative areas which, as tension development is proportional to fibre cross-section, may be more important in some circumstances. While these two indices of fibre composition are clearly different, there are few data regarding the magnitude of the discrepancy. This brief report quantifies the numerical and areal density of three major fibre types in a widely-studied mammalian hindlimb muscle, and illustrates the differing conclusions that may arise from an inappropriate choice of index or sampling regime.

MATERIAL AND METHODS

Male Sprague–Dawley rats of 290–350 g body weight were killed by an anaesthetic (sodium pentobarbitone) overdose. Extensor digitorum longus (EDL) muscles were chosen for histochemical analysis; whole muscles were taken at random from left or right legs, quickly removed and weighed. Mean weight was $177 \pm 5{\cdot}9$ mg ($\bar{x} \pm$ S.E.M.; $n = 7$). A segment of muscle from the mid-belly was then embedded in an inert mounting medium (OCT; Lamb) and frozen in isopentane (2-methylbutane) cooled in liquid nitrogen. Serial sections were cut (14 μm at -22 °C) and stained for succinic dehydrogenase (SDH; Nachlas *et al.* 1975), an indicator of oxidative capacity, and myosin ATPase (m. ATPase; Brooke & Kaiser, 1974) to differentiate between myosin isoforms and identify fibre types. Sections for Ca^{2+} m. ATPase were pre-incubated at pH 4·6 or fixed in 10% buffered formaldehyde and pre-incubated at pH 10·4. Incubation was at pH 9·6 using 5 mM ATP as substrate. The continuum of metabolic capacity among fast twitch fibres (Spamer & Pette, 1977) leads to an often arbitrary delineation between FO and FG fibres if classification is based solely on an oxidative marker. Myosin ATPase may provide a more objective method of identifying fibre types (Gollnick, Parsons & Oakley, 1983), although in our laboratory good agreement is shown between fibre classifications based on either m. ATPase or SDH histochemical stains (Egginton, 1987), allowing unambiguous categorisation of individual fibres.

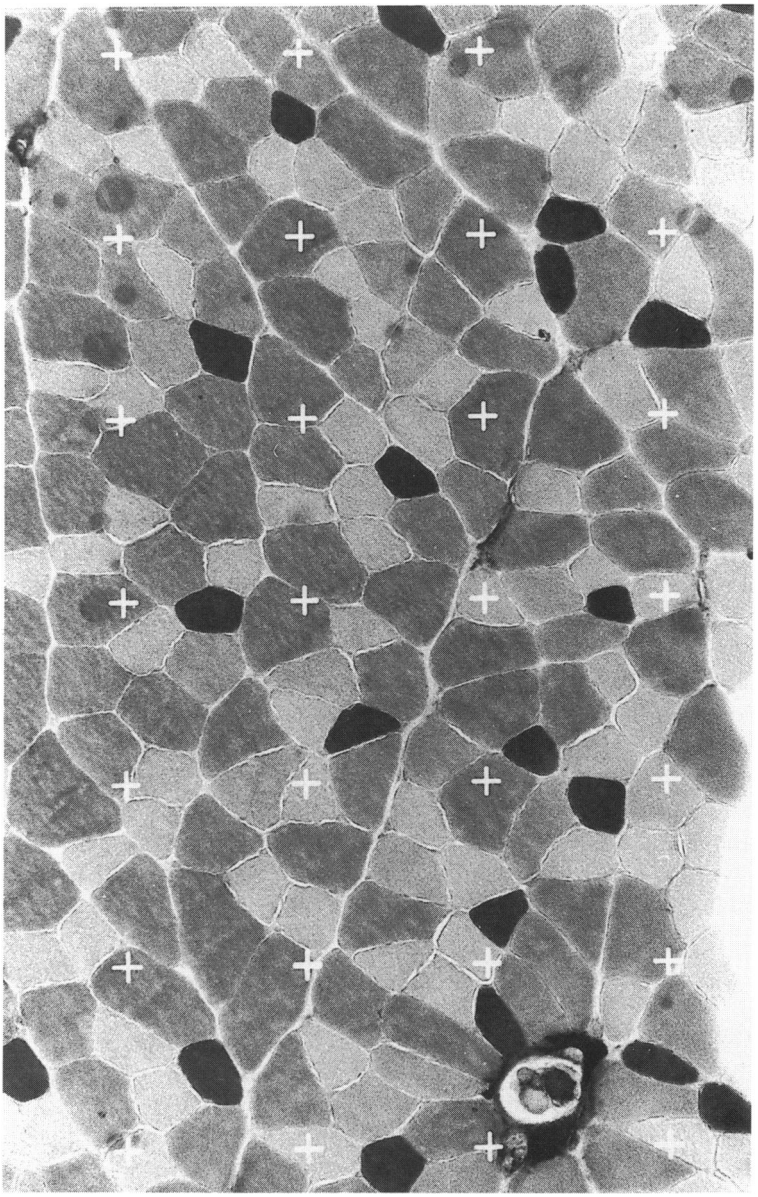

Fig. 1. Direct estimate of relative fibre area. A coherent square lattice counting frame, superimposed with random orientation, was used to determine the areal density of fibre types by standard point-counting methodology using grid line intersections as sample points (crosses). 12 μm frozen section; myosin ATPase stain, pH 4·6 pre-incubation.

A coherent square lattice counting frame was superimposed with a systematic, random position on the sections using a microscope drawing arm (Fig. 1). This provides a random sample of fibre content which is unbiased for their areal distribution, both within a muscle and among fibre types. The relative proportion of muscle cross-sectional area occupied by the three major fibre types was quantified using standard stereological point-counting methodology (e.g. Weibel, 1981), i.e. areal

density (area of object relative to area of reference) of individual fibre types is given as

$$A_A(f_k, m)\frac{\sum_{i-1}^{n} P_i(f_k)}{\sum_{i-1}^{n} P_i(m)}$$

where $P_i(f_k)$ = number of points (grid intersections) lying over fibre type k; $P_i(m)$ = total number of points lying over muscle tissue, summed over n fields (in this case 7 subsamples of tissue). Each field contained 400 points and covered 0·61 mm^2 (lattice spacing, d = 15·25 μm) at a magnification of × 50.

Total muscle cross-section (A) and number of fibres (N) are given as:

$$A = (n_i\,\bar{a}_i)+(n_j\,\bar{a}_j)+(n_k\,\bar{a}_k)$$

$$N = (n_i+n_j+n_k)$$

where n and $\bar{a}$ are number and mean fibre area of fibre types i, j and k. The overall mean fibre area is often quoted when fibre type is ignored:

$$\bar{a} = \frac{(n_i\,\bar{a}_i+n_j\,\bar{a}_j+n_k\,\bar{a}_k)}{N}$$

but produces a simple arithmetic error when used to calculate the numerical fraction of e.g. fibre type k as $n_k\,\bar{a}/N$, in the ratio $\bar{a}_k/\bar{a}$. A more common error is to ignore the difference between numerical and areal densities:

$$N_n(k) = \frac{n_k}{N}$$

$$A_A(k) = \frac{\Sigma a_k}{A} = \frac{(n_k\,\bar{a}_k)}{A}$$

Clearly if the mean area of a given fibre type can be determined, either directly from a subsample or taken from published data, then the numerical density can be used to estimate the areal density:

$$\text{est } A_A(k) = \frac{N_N(k)\,\bar{a}_k}{[(N_N(i)\,\bar{a}_i)+(N_N(j)\,\bar{a}_j)+(N_N(k)\,\bar{a}_K)]}$$

The corresponding numerical density (relative number) of fibre type i is given as

$$N_N(i) = \frac{n_i}{(n_i+n_j+n_k)}$$

where n_i, n_j and n_k are the number of fibres of type i, j and k (e.g. type I, II a and II b), respectively. This deceptively simple index may produce quite erroneous values if an inappropriate sampling protocol is used. For example, counting all fibres within a given field of view or sample frame will bias the data in inverse proportion to fibre size; hence small oxidative fibres will be overestimated while large glycolytic fibres will be underestimated. Application of the unbiased sampling rule used in this study (one of many possible, see Gundersen, 1977) for numerical distribution of fibres is illustrated in Figure 2.

Statistical comparisons were made with Student's t test.

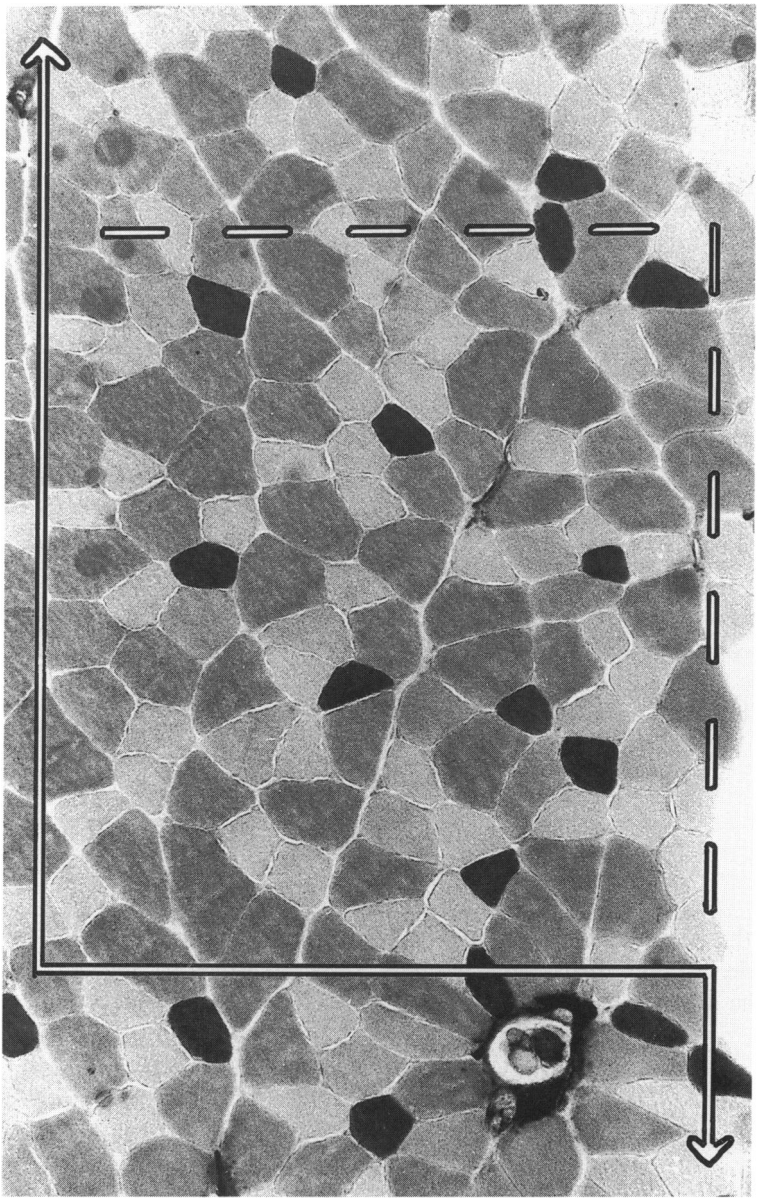

Fig. 2. Unbiased estimate of fibre number for the same field as Fig. 1. Only those fibres that lie entirely within the sampling frame, and those that exclusively intersect either of the pair of previously designated adjacent inclusion edges of the frame (dotted lines), are counted. Determining the size of these fibres then also provides an unbiased estimate of mean area for each fibre type. In this example ten SO fibres (darkly staining) are included and one excluded from the analysis.

RESULTS

The relationship between numerical proportion and areal density of fibre types clearly illustrates the effect of relative fibre size; although FO fibres comprise 36% of the total number of fibres, their combined area represents only 19% of the muscle cross-section (Fig. 3). Relative number is also misleading when comparing distribution of fibre types; although some regions of the muscle have nearly equal numbers of both

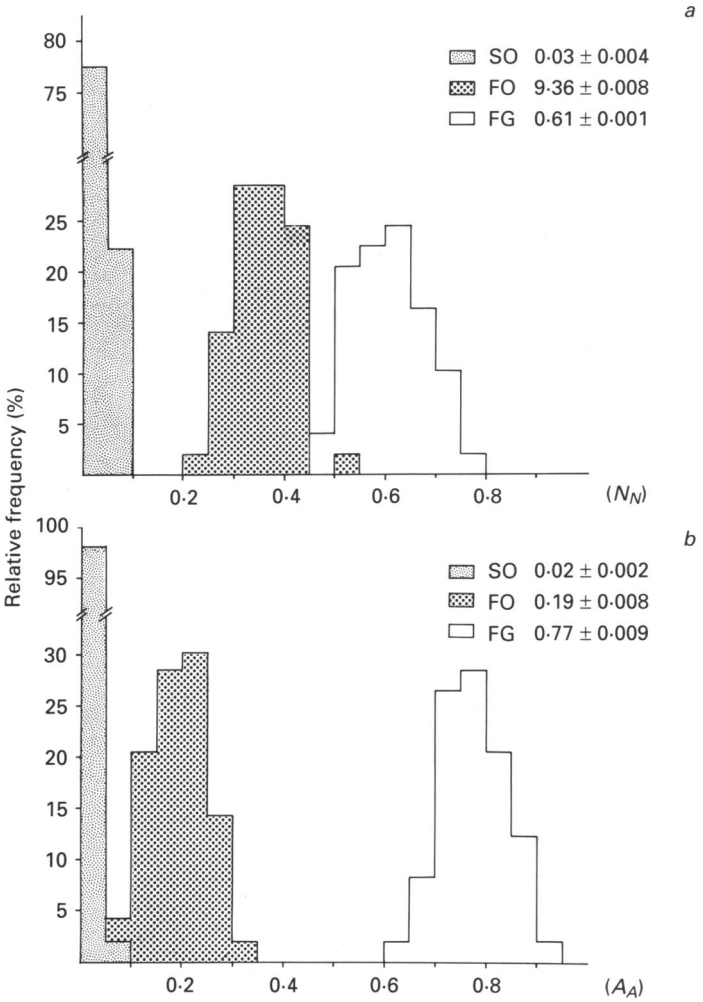

Fig. 3(*a–b*). Comparison of numerical and areal densities of fibre types in rat EDL. Proportion of slow oxidative (SO), fast oxidative (FO) and fast glycolytic (FG) fibres are given as mean ± S.E.M. (n = 49 fields). (*a*) Numerical density, N_N. Over 20% of fields contain 5–10% SO fibres, while < 5% have 75–80% FG fibres. The proportion of FO and FG fibres are distributed loosely around the 50% category, with a few fields having even numbers of both fibre types. (*b*) Areal density, A_A, Fibre size markedly affects the contribution of fibre number on a per muscle area basis. In this case only 2% of fields contain 5–10% SO fibres, while around 30% are covered by 75–80% FG fibres. Given that FG fibres are approximately twice the size of FO fibres, a distinct segregation of distributions appears between oxidative (stippled blocks) and glycolytic (open blocks) fibres.

Table 1. *Relative number and area of fibre types in EDL of Sprague–Dawley rats Mean ± S.E.M.* (N = *7 animals*)

	Numerical density (N_N)	Areal density (A_A)	*P*
SO	0·030 ± 0·0067	0·016 ± 010039	NS
FO	0·362 ± 0·0128	0·193 ± 0·0094	< ·001
FG	0·608 ± 0·0121	0·771 ± 0·0090	< ·001
Interstitium	—	0·021 ± 0·0016	—

Note: Interstitium represents the interstitial space and component tissue such as nerve bundles, blood vessels etc. Seven fields per muscle were examined. Numerical density is estimated as number of fibres of a given type/total number of fibres; areal density as mean fibre area of a given type/total fibre area.

Table 2. *Calculated and measured areal density*

		Fibre type		
	Overall	SO	FO	FG
$\bar{a}$ (μm^2)	155·3	648	1059	1892
A_A	—	0·016	0·193	0·771
est. A_A	—	0·013	0·247	0·741
error (%)	—	18·8	28·0	3·9

Mean areas for different fibre types were taken from an earlier (planimetric) study using comparable animals of the same breeding stock (Egginton, 1987), and used to estimate areal density (est. A_A) from numerical density as described under Methods.

types of fast fibres, the areal density of FG fibres is consistently > 60% while that of FO fibres is < 40%. This discrepancy between indices leads to a 20–30% error in estimated areal density of oxidative fibre types, compared to measured values, although glycolytic fibre content may be quantified either directly or interpolated from fibre number and mean fibre area (Table 1).

DISCUSSION

Conclusions based on apparent changes in muscle composition clearly need to take into account the manner in which data are expressed. Random fields sampling a mixed muscle will give quite different estimates of relative fibre content according to whether the number or area distribution is considered to be important. The most appropriate index will, of course, depend on the nature of the investigation. For example in a histochemical analysis of glycogen utilisation during muscular activity (Egginton & Hudlická, 1987) the differential in size between FG fibres, which show depletion, and oxidative fibres, which do not, would lead to an underestimation of the extent of glycogen utilisation by nearly a factor of 2, if numerical density of depleted fibres were to be used. Although this is a rather extreme example, significant errors may still exist when the differential in fibre size is much less, particularly where changes occur within a specific fibre type. For example it is known that the maximum tension a fibre can develop is proportional to its cross-sectional area, and that fibre size is very plastic with increased usage causing relative hypertrophy and disuse causing relative atrophy (Burke, 1981). While the numerical fibre type proportion (N_N) of a muscle is usually fairly constant among animals the relative fatiguability during exercise often varies quite markedly, a fact that may simply reflect a variation in A_A of oxidative fibre types.

Other workers have recognised that a numerical proportion carries little information about the relative mass of fibre types within a muscle. In a survey of the rat hindlimb musculature Armstrong & Phelps (1984) compared the percentage fibre composition with an indirect estimate of the relative area, and hence mass, of muscle for the three major fibre types. It is worth considering the relative merits of a direct and indirect estimate of A_A. Point-counting stereology provides a rather quick estimate of A_A such that the analytic efficiency (accuracy per unit effort) far outweighs the indirect approach, allowing the option of increasing resolution and for investigating further the spatial heterogeneity in composition. More importantly, indirect estimates of A_A are subject to significant errors (Table 2). This is always likely to be a problem with compound indices, as the errors involved in estimating N_N and mean fibre cross-sectional area are multiplicative, although these may be minimised with very large

samples. Furthermore, where delineation of fibres is a problem, e.g. when using histochemical sections stained for oxidative enzyme activity or using inaccurate digitiser algorithms (an often undetected error; see Cornelisse & van den Berg, 1984), the robust statistical nature of point-counting makes this the method of choice. Of course for many applications such accuracy may be unnecessary. For example, Armstrong & Laughlin (1983) observed that patterns of blood flow distribution within and among hindlimb muscles during exercise are closely related to the fibre type population. This relationship was not significantly altered when an indirect estimate of fibre type A_A was substituted for N_N (Armstrong & Phelps, 1984), suggesting that when investigating gross changes such methodological limitations may be acceptable.

An important additional consideration is the requirement of an unbiased sampling regime, particularly when one wishes to estimate fibre number. This is particularly important where a large size differential exists between fibre types, and requires the unambiguous and unique association of a fibre profile with only one out of a possible series of contiguous, but non-overlapping counting frames (see Gundersen, 1977). Without strict adherence to these simple counting rules it is difficult to distinguish, for example, between true fibre hypertrophy and fibre type transformation occurring within a given size category (Egginton, 1987). Although widely accepted by stereologists, application of these simple principles appears to be insufficiently employed in morphometric analyses of muscle.

SUMMARY

The composition of a mixed fast skeletal muscle (rat extensor digitorum longus) was examined to quantify the difference between the relative number of the three major fibre types in a representative muscle and their relative contribution to muscle cross-section, i.e. numerical (N_N) and areal (A_A) densities, respectively. These two indices clearly differ in their physiological relevance. While the former may be useful in describing hyperplasia, the latter allows for differences in size among fibre types.

When estimated as N_N, over 20% of fields contained 5–10% SO fibres and < 5% had 75–80% FG fibres. In contrast, only 2% of fields had an A_A of 5–10% for SO fibres while around 30% contained 75–80% FG fibres.

The importance of a direct method for estimating A_A is emphasised, as an indirect approach may have an error of 20–30% when used for oxidative fibre types. The use of an unbiased sampling regime to minimise error in determining both numerical and areal densities of different fibre types is illustrated.

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REFERENCES

ARMSTRONG, R. B. & LAUGHLIN, M. H. (1983). Blood flows within and among rat muscles as a function of time during high speed treadmill exercise. *Journal of Physiology* **344**, 189–208.

ARMSTRONG, R. B. & PHELPS, R. O. (1984). Muscle fibre type composition of the rat hindlimb. *American Journal of Physiology* **171**, 259–272.

BROOKE, M. H. & KAISER, K. K. (1974). The use of abuse of muscle histochemistry. *Annals of the New York Academy of Sciences* **228**, 121–144.

BURKE, R. E. (1981). Motor units: anatomy, physiology and functional organisation. In *Handbook of Physiology. The Nervous System* (ed. V. B. Brooks), section 1, Chapter 10, pp. 345–422. Bethesda, MD: American Physiological Society.

CORNELISSE, J. T. W. A. & VAN DEN BERG, T. J. T. P. (1984). Profile boundary length can be overestimated by as much as 41% when using a digitizer tablet. *Journal of Microscopy* **136**, 341–344.

EGGINTON, S. (1987). Effect of an anabolic hormone on aerobic capacity of rat striated muscle. *Pflügers Archiv* **410**, 356–361.

EGGINTON, S. & HUDLICKÁ, O. (1987). Development of fatigue and relative fibre glycogen content in rat skeletal muscle. *Journal of Physiology* **390**, 147P.

GOLLNICK, P. D., PARSONS, D. & OAKLEY, C. R. (1983). Differentiation of fiber types in skeletal muscle from the sequential inactivation of myofibrillar actomyosin ATPase during acid preincubation. *Histochemistry* **77**, 543–555.

GUNDERSEN, H. J. G. (1977). Notes on the estimation of the numerical profiles: the edge effect. *Journal of Microscopy* **111**, 219–223.

NACHLAS, M. M., TSOU, K., DESONSA, G., CHENG, C. & SELIGMAN, A. (1957). Cytochemical demonstration of SDH by the use of a new p-nitrophenol substituted derivative. *Journal of Histochemistry and Cytochemistry* **5**, 420–436.

SPAMER, C. & PETTE, D. (1977). Activity pattern of phosphofructokinase, glyceraldehydephosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase in microdissected fast and slow fibers from rabbit psoas and soleus muscle. *Histochemistry* **52**, 201–216.

WEIBEL, E. R. (1981). *Stereological Methods*, Vol 1, *Practical Methods*. London: Academic Press.