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Basic stereological relationships for quantitative microscopical anatomy – a simple systematic approach

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INTRODUCTION

Stereology is concerned with the mathematical reconstruction of three dimensional structure based on the mensuration of (ideally) two dimensional images. In biomedical research, this usually means thin histological sections prepared for light and electron microscopy. Measurements of recognizable components in the sections are made by superimposing a test lattice bearing a pattern of areas, lines and points, referred to as test probes. By evaluating the chance encounters between the components and the test probes, quantitative information characterizing organelles, cells and tissues may be derived (Weibel, 1969, 1974; Elias, Hennig & Schwartz, 1971; Weibel & Bolender, 1973).

The majority of stereological parameters are ratios, and amongst these there exists a core of fundamental relationships known as 'the component densities'. The best known and most frequently employed component densities are those which refer component volume (V), surface area (S), length (M) and number (N) to a specified containing *volume*, for example that of the average cell in a population. By convention, component densities in a volume are given the symbols V_V, S_V, M_V and N_V respectively (Weibel, 1969).

A synopsis of these basic relationships is given in Table 1. The list is not intended to be complete; for instance, component numerical density in a volume (N_V) may be estimated in other ways, but these are equivalent to the formulation shown, particularly when the particles being counted are spherical. Moreover, not all component densities can be estimated with each type of test probe: V_V can be estimated from the ratios A_A, L_L and P_P (that is, using any test probe) but S_V can only be calculated with the aid of areal or linear probes, and not with test points, since the probability of a test point hitting a profile boundary trace is effectively zero.

Component densities in a volume are undoubtedly valuable descriptors of biological structure. However, cell surfaces and their specialisations (e.g. synapses, desmosomes and microvilli) are often of greater interest than volumes. Consequently, there is scope for relating component dimensions to a containing *surface* at least, since such parameters may be more pertinent to studies attempting to correlate structure with function. In fact, by an elementary and systematic approach it is possible to extend the basic relations to embrace the component densities S_S, M_S and N_S (on a surface), M_M and N_M (along a length) and N_N (in a number). Unfortunately, the newcomer to this field will not find a comprehensive list of these possibilities in the relevant literature.

This report provides such a list. It includes comparatively simple derivations of principles for estimating the additional component densities, and presents them in

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Table 1. *'Basic stereological parameters' and principles for their estimation. Principles for use with alternative test probes (areas, lines, points) are indicated in sequence*

Component parameter	Reference parameter V
Volume, V	$V_V = \quad A_A = L_L = P_P$
Surface, S	$S_V = (4/\pi)B_A = 2I_L$
Length, M	$M_V = 2Q_A$*
Number, N	$N_V = N_A/\overline{D}$
	$\uparrow$
Component dimension	$\overline{D}$ In 3-dimensional case

* Note: The symbols M (length of structure) and Q (transections of lineal structure by a test area) follow recommendations in Weibel & Bolender (1973). They maintain consistency in the meanings of L and P, which are reserved for test lines and test points respectively. The formula $M_V = 2Q_A$ is therefore equivalent to $L_V = 2P_A$, which is often used in the literature.

a form which makes them easier to understand and apply. Useful relations between parameters are emphasised, and some practical applications are discussed. A preliminary communication of this work has been presented already (Mayhew, 1978*a*). For those with the necessary mathematical expertise, a generalised treatment of most of the relationships will be found in Miles & Davy (1976).

The system of parameter indexing adopted in the ensuing text is modelled on suggestions made by Weibel (1969, 1976). On this system, the descriptor $S_{Vc,e}$ indicates the surface density of a component surface c in a containing reference volume e, and $S_{Sa,c}$ the surface density of a surface feature a on the surface of c. The same rationale is employed for indexing the principles by which the parameters are estimated.

COMPONENT DENSITIES ON A SURFACE

Principles can be established for estimating the parameters S_S, M_S and N_S, component densities per reference surface.

Imagine a three dimensional structure (c) on the reference surface of which lie two structural differentiations (a and b), a being in the form of a number of discrete patches or discs and b in the form of a curved continuous line or filament. Let c be embedded in a containing volume (e). Now, let e be transected by random planes. By dimensional reduction (*vide infra*), the volume e will be represented on each section by an area (A); the surfaces of c and a will appear as boundary trace lengths (B), and the thin filament b will be represented by transections (Q). Linear test probes applied to the sections will make intersections (I) with the boundary traces of c and a (see Fig. 1).

(A) *Surface density on a surface* (S_S)

From the principles shown in Table 1 we obtain

$$S_{Vc,e} = (4/\pi)B_{Ac,e} = 2I_{Lc,e} \tag{1}$$

$$S_{Va,e} = (4/\pi)B_{Aa,e} = 2I_{La,e} \tag{2}$$

from which it follows, dividing (2) by (1), that

$$S_{Sa,c} = B_a/B_c = I_a/I_c.$$

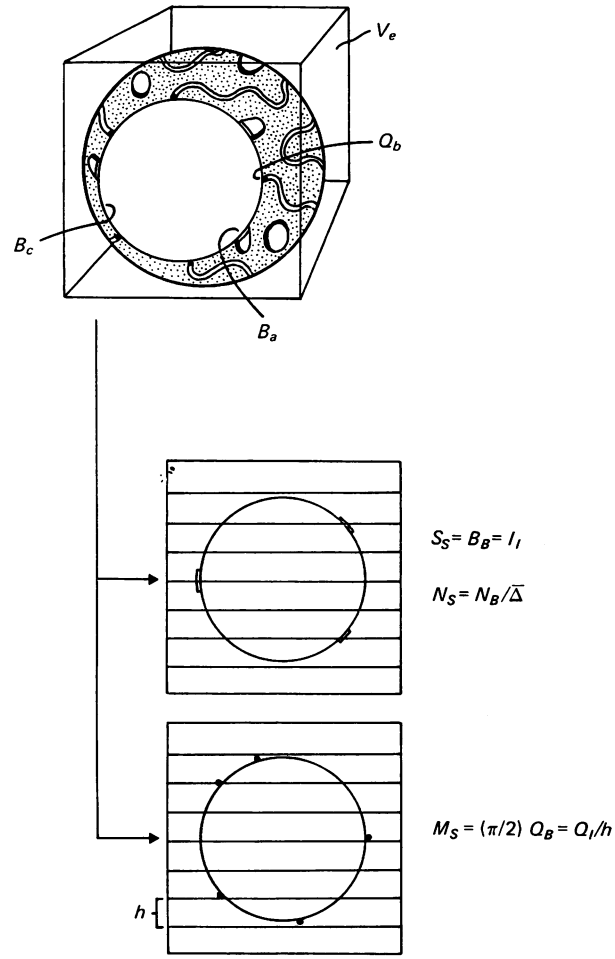

Fig. 1. A simple model to illustrate principles for estimating component densities on a surface. The containing volume (V_e) encloses a sphere on the surface of which lie two structural types, a surface feature in the form of 'patches' or 'discs' and a lineal feature. On sectioning, the sphere is represented by a circular profile of trace length B_c, the discs by traces of total length B_a and the filament by a number of transections (Q_b). For clarity, disc traces and filament transections are shown separately on the sections. See text for further details.

The total reference area (A) and total line scan (L) are common and cancel out, since $S_{Vc,e}$ and $S_{Va,e}$ are estimated from the same sample of sections (or micrographs). The relation can be written in abbreviation as $S_S = B_B = I_I$, and we observe the analogy with $V_V = A_A = L_L = P_P$.

Thus, the surface density of a specialised feature a on surface c may be estimated in two ways:

(i) *on a test area by relating the total trace length of a to the total trace length of c*, and

(ii) *using linear probes by relating the total intersections of trace a with the test lines to the total intersections of trace c by those same lines* (Fig. 1).

The relation $S_S = B_B$ has been invoked repeatedly by neurohistologists interested in quantifying synaptic apposition zones, trace lengths being measured directly by

means of an opisometer (odometer, map-measurer's wheel) in order to estimate S_S (e.g. Blackstad & Dahl, 1962). The numerical equivalence of ratios B_B and I_I is determined by $B = (\pi/2).I.h$, where h is the spacing between the test lines (Weibel, 1969).

The relation $S_S = I_I$ will also be found in Weibel & Bolender (1973). Weibel (1976) has provided an expression for calculating the relative standard error (S.E.) of S_S estimates:

$$\text{S.E.} = \sqrt{(1\text{-}S_S)/(S_S.I_c)}$$

where I_c is the total number of intersections of c counted. The formula allows a prediction of minimal sample size required to achieve a certain precision for S_S.

(B) *Length density on a surface* (M_S)

The basic formulations also indicate that

$$M_{Vb,e} = 2Q_{Ab,e} \tag{3}$$

and dividing (3) by (1) we obtain

$$M_{Sb,c} = (2Q_{Ab,e})/((4/\pi)B_{Ac,e}) = (2Q_{Ab,e})/(2I_{Lc,e}).$$

Since the test area (A) is common and related to the test line length (L) by the spacing (h) between test lines, this yields

$$M_{Sb,c} = (Q_b/B_c)/(2/\pi) = (Q_b/I_c)/h$$

or, again as an abbreviation, $M_S = (\pi/2)\,Q_B = Q_I/h$.

Thus, the length density of feature b on surface c may be estimated

(i) *on a test area by relating the number of transections of b to the total trace length of c*, and

(ii) *using linear probes by relating the number of transections of b to the total intersections of trace c and the spacing between those probes* (Fig. 1).

This new relation, $M_S = Q_I/h$, may also be derived from absolute dimensions of the component and its containing surface using formulae cited in Elias *et al.* (1971). If the containing volume e is serially sectioned into slices of uniform thickness T, the absolute length of filament b is given by the expression

$$M_b = 2Q_b.T$$

and the absolute surface area of c by

$$S_c = 2I_c.h.T$$

where h is the spacing of test lines superposed on each serial section.

(C) *Numerical density on a surface* (N_S)

From Table 1, we have

$$N_{Va,e} = N_{Aa,e}/\overline{D}_a \tag{4}$$

where $\overline{D}_a$ represents the mean caliper diameter of the patches (a) on the surface of c.

Let us now impose some restrictions on the shape and size distribution of these patches such that each can be described by a thin flat circular disc of diameter Δ_a. Then the mean diameter of the total monodisperse population will be $\overline{\Delta}_a$ and for such a population $\overline{D}_a = (\pi/4)\overline{\Delta}_a$ (Hilliard, 1967).

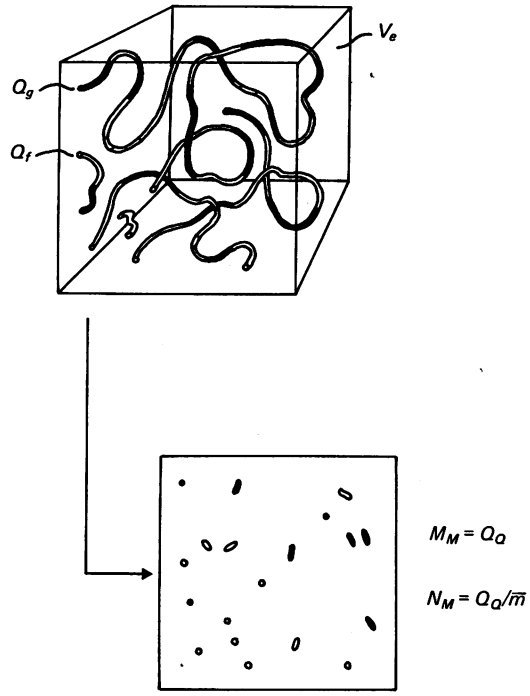

Fig. 2. A simple model to illustrate principles for estimating component densities along a length. The containing volume (V_e) encloses a long filament (f) which bears regions of specialisation (g). On sectioning, these lineal features give rise to transections, totals Q_f and Q_g, shown on the section. See text for further details.

Dividing formula (4) by (1), we obtain

$$N_{Sa,c} = (N_{Aa,e}/\overline{D}_a)/((4/\pi)B_{Ac,e}).$$

Now since the reference area (A) is constant, substituting for $\overline{D}_a$ yields

$$N_{Sa,c} = N_{Ba,c}/\overline{\Delta}_a$$

or, more simply, $N_S = N_B/\overline{\Delta}$. Observe the similarity to $N_V = N_A/D$.

Thus, the numerical density of a feature a on a surface c may be estimated *on a test area by relating the number of observed traces of a to the total trace length of c and the mean diameter of a* (Fig. 1).

In fact, the derivation presented above is for a very restricted model of known feature (disc) size and shape. However, the basic formulation was derived first by Cruz Orive (personal communication, 1975) who adopted a more rigorous approach of much wider validity and applicability. Therefore, it may be possible in practice to relax the present constraints provided $\overline{\Delta}$ can be adequately defined in terms of feature size and shape, a situation that also pertains with $\overline{D}$ (Weibel, 1974).

An alternative formulation, $N_S = S_S/\overline{s}$ where $\overline{s}$ is mean disc surface area, has been proposed by Kaiserman-Abramof & Peters (1972). For circular discs of uniform size, mean disc surface is calculated from $\overline{s} = (\pi\overline{\Delta}^2)/4$.

In practice of course, mean disc diameter must be estimated from measured trace lengths appearing on the section. Where the discs are all of the same size, mean trace

length $\overline{B} = (\pi/4)\overline{\Delta}$ (Kaiserman-Abramof & Peters, 1972). Hence, for such discs $\overline{B} = \overline{D}$. For polydisperse populations of circular discs a more flexible approach is required and it is necessary to reconstruct the true size distribution of the discs from the size distribution of measured trace lengths (see, for example, Anker & Cragg, 1974).

COMPONENT DENSITIES ALONG A LENGTH

In this section, principles for establishing M_M and N_M are proposed. For this purpose, imagine a thin curved filament (f) randomly embedded in a reference containing volume (e) and bearing a number of discrete regions of specialisation (g) scattered along its length. If e is transected by random planes, then on each plane the volume will be represented by an area (A) and the lineal structures f and g will appear as transections (Q), as illustrated in Figure 2.

(A) *Length density along a length* (M_M)

From basic principles we obtain the following length densities in the common containing volume

$$M_{Vf,e} = 2Q_{Af,e} \tag{5}$$

$$M_{Vg,e} = 2Q_{Ag,e} \tag{6}$$

and dividing (6) by (5) we have

$$M_{Mg,f} = Q_{Ag}/Q_{Af}$$

which, since A is common to both, is a new basic principle $M_M = Q_Q$. Once again, observe the analogy with other stereological principles which have consistency in the component and reference dimensions (i.e. principles for estimating V_V and S_S).

The formulation indicates that the length density of a feature g along a reference length f is estimated *using areal probes by relating the total transections of g to those of f* (Fig. 2).

(B) *Numerical density along a length* (N_M)

Let g be composed of individual filaments of mean length $\overline{m}$. From Table 1, we see that $N_{Vg,e}$ may be determined by invoking the relation

$$N_{Vg,e} = N_{Ag,e}/\overline{D}_g \tag{7}$$

and that

$$M_{Vf,e} = 2Q_{Af,e}. \tag{8}$$

For thin filaments of mean length $\overline{m}$ randomly oriented within a containing volume, $\overline{D} = \overline{m}/2$ (Hilliard, 1967). Therefore, dividing (7) by (8)

$$N_{Mg,f} = (2N_{Ag,e}/\overline{m})/(2Q_{Af,e})$$

$$= (N_{Ag,e}/Q_{Af,e})/\overline{m}$$

or, since A is constant, $N_M = N_Q/\overline{m}$ which is equivalent in practice to $Q_Q/\overline{m}$ and also to $M_M/\overline{m}$.

Thus, the numerical density of a lineal feature g along a reference length f is estimated *using areal probes by the relationship between the numbers of transections of g and f and the mean length of g* (Fig. 2).

From these five classes of basic principles it is now possible to extend Table 1 by systematically filling in the gaps. A summary of present findings is therefore offered in Table 2.

Table 2. *An extended list of basic stereological relations for comparison with Table* 1

Component parameter	Reference parameter			
	V	S	M	N
Volume, V	$V_V = A_A = L_L = P_P$	—	—	—
Surface, S	$S_V = (4/\pi)B_A = 2_{IL}$	$S_S = B_B = I_I$	—	—
Length, M	$M_V = 2Q_A$	$M_S = (\pi/2)Q_B = Q_I/h$	$M_M = Q_Q$	—
Number, N	$N_V = N_A/\overline{D}$ ↑	$N_S = N_B/\bar{\Delta}$ ↑	$N_M = N_Q/\overline{m}$ ↑	see text
Component dimension	$\overline{D}$ In 3-dim. case	$\bar{\Delta}$ In 2-dim. case	$\overline{m}$ In 1-dim. case	

The symmetries evident in these relationships are more extensive than indicated. For example, $N_M = M_M/\overline{m}$ and $N_S = S_S/\bar{s}$ are reminiscent also of the Loud (1968) formulation $N_V = V_V/\bar{v}$, where $\bar{v}$ is mean particle volume. As Weibel & Bolender (1973) have pointed out, these symmetries reflect an underlying and fundamental law of dimensional reduction which, for the three dimensional case, takes the form

$$d_t = d_o + d_p - 3,$$

where d_o, d_p and d_t refer to the dimensions of the object (component, feature), the test probe and the image presented by the probe respectively. Thus, a volume ($d_o = 3$) sectioned by an area probe ($d_p = 2$) is represented by an area ($d_t = 2$) but a surface ($d_o = 2$) sectioned by the same probe appears as a trace length ($d_t = 1$). So we see $V_V = A_A$ and $S_S = B_B$ in this context.

OTHER POSSIBILITIES

The obvious lateral extension of Table 2 would be to derive expressions for estimating the parameter N_N, the number of components contained by a single reference stucture (e.g. nuclei per average cell). Like the corresponding densities V_N, S_N and M_N this may be derived quite simply by multiplying an appropriate component density by its corresponding absolute reference parameter. For instance, V_N may be calculated from $V_V.\bar{v}_r$ where $\bar{v}_r$ is the absolute reference volume; S_N by $S_V.\bar{v}_r$ or $S_S.\bar{s}_r$ and so on. On this regime, N_N could be estimated in several ways (e.g. by $N_V.\bar{v}_r$).

In addition, for two convex bodies a and b (a being contained by b) of profile numerical densities N_{Aa} and N_{Ab}, with mean caliper diameters $\overline{D}_a$ and $\overline{D}_b$, the number of structures a per structure b would be

$$N_{Na,b} = (N_{Aa}/\overline{D}_a)/(N_{Ab}/\overline{D}_b).$$

Relations between the basic parameters often prove useful for deriving other information which, for various reasons, is difficult to obtain by direct measurement on the sections. They also allow one to economise on analytical effort. As two illustrations, consider the following.

For a long cylindrical structure, the relation between V_V and M_V can be used to assess the mean cross sectional area ($\bar{a}$) of that structure, since

$$V_V/M_V = V/M = \bar{a}.$$

This equation has been employed (Mayhew & Momoh, 1974) to approximate the

mean calibre (cross sectional) diameter of blood vessels in the ventral horn of rat spinal cord. In the same study, S_V was calculated from the same micrographs and used to estimate blood vessel surface-to-volume ratio since

$$S_V/V_V = S/V.$$

A third relationship also has interesting potential, particularly for determining synaptic population densities. For a monodisperse population of flat circular discs, $\overline{B} = \overline{D}$ (*vide supra*). Thus, their numerical density per unit volume, estimated from plane sections, is given by $N_V = N_A/\overline{B}$ (see Mayhew, 1978*b*). Note that $\overline{D}$ is *not* the same as $\overline{\Delta}$ (cf. Anker & Cragg, 1974).

The newcomer should try to appreciate the potential of such inter-relations of stereological parameters as they are often overlooked.

PRACTICAL APPLICATIONS OF THE PRINCIPLES

It is not the intention here to give an exhaustive list of possibilities for applying the fundamental stereological relations to biological material. Their potential is better appreciated by referring to the wealth of published applications, most of which rely solely on methods shown in Table 1. Nevertheless, the neophyte might find a general guide, to which he can compare his own model system, helpful.

At least some of the principles described herein have been applied already, albeit to a very limited extent. Let us consider first component densities on a surface.

Many cell surface specialisations are of great functional interest to biologists. Intercellular adhesion complexes and neuronal synapses provide two useful examples for present purposes, both being of some physiological importance. In fact, the relation $S_S = B_B$ is a familiar one to neurologists: Kaiserman-Abramof & Peters (1972), for example, used an opisometer to evaluate the relative surface area of Betz cell perikarya occupied by axon terminals in cat cerebral cortex. More recently, Gabella (1976) has adopted the same approach to assess the coverage of smooth muscle cells by caveolae in the guinea-pig taenia coli.

Sometimes intersections are much easier to quantify than trace lengths and a more efficient method is then offered by the relation $S_S = I_I$. This method has been adopted by Mayhew & Tring (1977, unpublished observations) to assess the relative surface area of stratum corneum cells covered by modified desmosomes in normal human skin and in psoriasis. The same approach has been used by White & Gohari (personal communication, 1977) to study desmosomes and hemidesmosomes in hamster cheek-pouch epithelium.

In the above applications, synapses, desmosomes and caveolae were regarded as regions of differentiation within the cell plasmalemma. About 23% of the Betz cell perikaryon is covered by axon terminals and, by coincidence, roughly 23 % of the surface of an average stratum corneum cell in normal skin is occupied by modified desmosomes. About 40 % of the basal plasmalemma of stratum basale cells in normal cheek-pouch epithelium makes hemidesmosomal contact with the underlying basal lamina, compared with only 13 % in a carcinoma.

Exactly the same basic relations can be invoked to estimate the S_S values for different types of synaptic contact or the glial cell investment of neurons (Blackstad & Dahl, 1962; Conradi, 1969); the relative surface of nuclear envelope occupied by nuclear pores; the relative surface of plasmalemma labelled by an immunocytochemical marker; the relative surface of capillary endothelium broken by fenestrations,

and so on. Blouin, Bolender & Weibel (1977) have used the relationship to compare the distribution of membranes between different cell types in liver and other possibilities are mentioned by Weibel (1976) for estimating surface complexity factors introduced by microvilli, membrane ruffles and such.

To-date, I know of no published application of stereological principles for estimating M_S. However, they could be used for instance to determine the length densities of zonulae occludentes (tight junctions) which bind adjoining epithelial cells such as intestinal absorptive cells and cells in the stratum corneum of amphibian skin (e.g. Farquhar & Palade, 1963, 1965).

Estimators of N_S have also been applied to synapses and desmosomes: each 100 μm^2 of Betz cell perikaryon forms symmetrical synapses with some 13 axon terminals (Kaiserman-Abramof & Peters, 1972). An equivalent surface of rat spinal motoneuron perikaryon bears roughly 10 synapses, its proximal dendrites about 28 and its distal 26 (Momoh, 1976). These figures compare favourably with densities of 5 and 8 per 100 μm^2, calculated for two neurons in the lateral geniculate nucleus of the rat by reconstruction of serial sections (Karlsson, 1966). Each 100 μm^2 of stratum corneum cell membrane in human epidermis has about 250 desmosomal contact sites (Mayhew & Tring).

In these N_S studies, contact sites were assumed to be flat circular discs for the sake of calculation, and mean disc diameter was estimated from feature trace length (measured either directly with an opisometer or estimated from numbers of intersections made with linear test probes).

As a descriptor of cell morphology, N_S could be used also to estimate the number of nuclear pores per unit surface of nuclear envelope; the number of microvilli, cilia or pinosomes per unit cell surface; the number of caveolae per unit surface of smooth muscle cell; the relative numbers of ribosomes per unit surface of rough endoplasmic reticulum and so on.

Applications for component densities along a length are less obvious since suitable biological mimics of the theoretical models are harder to find. A further problem with $N_M = N_Q/\overline{m}$ may be the difficulty of establishing $\overline{m}$ from random transections. However, $M_M = Q_Q$ could be employed to establish the relative lengths of uriniferous tubule segments in kidney (perhaps also different functional segments along secretory ducts) and for comparing the lengths of different structures in a common containing volume (e.g. different types of filament in skeletal muscle; myelinated and unmyelinated axons, or axons and dendrites in nervous tissue; tubules in axons in nerve fibres).

N_N estimators are of great value for defining numbers of nuclei/cell, nucleoli/nucleus, liver cells/lobule, secretory cells/acinus, synapses/neuron and so on.

CONCLUDING REMARKS

Stereological analysis of tissue sections rests on certain assumptions about the design and representativeness of sampling, thickness of section, and unambiguity of definition of components (for more details consult Weibel, 1969; Elias *et al.* 1971; Miles & Davy, 1976). These constraints apply equally to the additional component densities described here.

A particular bugbear is section thickness: the above principles assume true plane sectioning but histological sections have a finite thickness. This tends to an overestimation of component densities, the magnitude of error depending on the relative

size of component and section thickness, and it is not always possible to correct for this. It should be borne in mind that numerical densities of relatively small structures will be over-estimated, and it is to be hoped that correction procedures for dealing with inflated N_S and N_M estimates will become available as they are for N_V data.

Whilst section thickness over-estimates S_V values, Weibel (1976) has suggested this error may be less important for S_S.

It is emphasised that the derivations offered here are not proofs. Their validities depend in turn on those of the principles appearing in Table 1. However, they have been proved in general by Miles & Davy (1976).

In conclusion, a systematic attempt has been made to extend the list of stereological principles in order to include component densities on a surface (which seem to have important biological potential) but also component densities along a length. Recent work, particularly within the last 6 years, has made increasing use of these new possibilities but the newcomer has not, until now, been able to find a more comprehensive list than those which refer to component densities in a volume.

SUMMARY

There exists in the literature a core of formulations regarded as 'the basic stereological principles' for quantifying cell and tissue morphology. They may be used to obtain information relating component volume, surface area, length and number to a specified containing volume (the so-called component densities in a volume: V_V, S_V, M_V and N_V). However, principles may also be formulated for relating these component dimensions to a containing *surface* (S_S, M_S and N_S), containing *length* (M_M and N_M) and a containing *number* (N_N).

Methods for estimating these previously neglected stereological relations are presented. Possible biological applications of the principles are also discussed.

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