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REVIEW

A review of recent advances in stereology for quantifying neural structure

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Summary

The science of stereology has undergone a revolution over the past decade with the introduction of design-based (assumption- or model-free) methods which are highly efficient and generally unbiased. No other morphometric approach currently offers these twin benefits. Stereology is ideal for extrapolating 3-D structural quantities (real volumes, surface areas, lengths and numbers) from simple counts made on 2-D slice images. The images may take various forms (e.g. physical or optical sections, MRI slices, CT scans) but they must be sampled so as to be random in orientation and/or position if valid estimates are to be made.

All the recent developments in stereology are applicable to problems in neuromorphometry. This review provides an account of major developments and the state of the art, emphasizes the importance of properly randomized sampling and identifies some applications to neural structure at different levels of organization. These include the counting and sizing of synapses, neurites, cells and whole brains.

Introduction

Much of modern neuroscience is concerned with abstracting information about the spatial content, arrangement and connectivity of organs, tissues, cells and cellular components. Often this must be gleaned from essentially flat (2-D) images of real (3-D) objects. The 2-D images may be (a) *physical slices* cut using a knife or microtome, (b) *optical slices* generated by focusing at different depths within a thick physical slice using a conventional optical microscope or (if the objects exhibit reflectance or fluorescence) a confocal microscope, (c) *slices obtained by medical imaging* such as CT, MRI and PET or (d) *projected images* of 3-D objects observed on a plane (e.g. cells growing on a matrix in a culture dish or contained within a thick tissue slice).

Regardless of their provenance and whether they are needed for qualitative or quantitative investigations, such images should always be collected by random sampling procedures, or their credentials will be suspect. This is particularly important given the wide range and accessibility of quantitative image analysis devices of the black-box variety. Only rigorously defined and executed random sampling, giving *every* item in the population the *same* chance of being selected, will provide an unbiased sample. Any other sampling scheme (e.g. deliberately choosing 'typical' or 'interesting' sections) will introduce bias, a source of error which cannot be eliminated merely by sampling more items, and which will serve only to frustrate the successful interpretation of apparent changes in structure.

In order to convert measurements made on 2-D slices into 3-D structural quantities, stereological methods must be exploited. Earlier methods relied heavily on the use of simplistic models of real objects and on assumptions about object shape, size and orientation. *The 'new stereology' does not.* Therefore, it is ideal for making unbiased estimates of 3-D structural quantities from counts performed on 2-D images (Weibel, 1979; Gundersen *et al.*, 1988a,b; Cruz-Orive & Weibel, 1990; Mayhew, 1991a). Its efficiency depends on generating chance encounters between randomly sampled sectional images and test probes superimposed on them. The probes may be points, lines or areas arranged in regular arrays (e.g. on a transparent test overlay or on an eyepiece graticule) or volumes (represented by the space between parallel section

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planes). Though developed in the context of physical slicing for histology and electron microscopy, stereology is applicable *wherever* random slices can be generated and ingredients within those slices can be recognized unequivocally.

The great strength and value of stereology resides in the two properties of unbiasedness and efficiency (Gundersen *et al.*, 1988b). A design-based approach to sampling satisfies the conditions necessary for maintaining the validity of relationships, giving the methods general applicability. By using systematic probes (see Gundersen & Jensen, 1987), sampling can also produce very efficient (i.e. high precision per unit cost) estimates of structural quantities. Moreover, the results of pilot analyses may be used to balance precision of estimation against the natural differences between animals (biological variation). By doing this, it is possible to optimize the overall efficiency of an experimental design (Shay, 1975; Cruz-Orive & Weibel, 1981; Gundersen & Østerby, 1981; Gupta *et al.*, 1983; McCance, 1989; Regeur & Pakkenberg, 1989; Mayhew & Olsen, 1991).

This review is concerned exclusively with stereological methods which could be employed to extrapolate 3-D data from 2-D images of neural components. The topics of serial sectioning reconstruction and morphometry of projected images (e.g. the analysis of neurite branching patterns) are not covered. Emphasis is placed on the fact that good stereology depends crucially on proper sampling. The necessary sampling conditions are given explicitly.

Elements and descriptors of neural structure

Neural structure spans many levels of organization from the subcellular to the systemic. Attention here will focus on methods which are appropriate for quantifying the following elements: organelle, synapse, neurite, cell and organ. In most cases, unambiguous recognition of these elements presents no difficulty. However, alternative definitions of what constitutes a synapse mean that different counting units are available, viz. boutons, apposition zones and paramembranous dense regions (Mayhew, 1979). Strict comparability of results from different groups will be sensitive to the choice of counting unit.

Alternative units for counting neurons are also permissible, viz. the cell, its nucleus and the nucleolus. If nuclei or nucleoli are the counting units, then an implicit assumption (that each cell contains one nucleus or nucleolus) *must* be satisfied before valid cell numbers can be obtained. Provided that unbiased number estimators are employed (see below), the nucleolus affords a very convenient and useful way of counting neurons (Nairn *et al.*, 1989; Mayhew *et al.*, 1990b; Mayhew, 1991b). However, the one cell-one nucleolus assumption must be justified or bias may be introduced (see Braendgaard & Gundersen, 1986; Campbell *et al.*, 1988). The nucleolus also offers a very useful way of estimating neuron size (see Gundersen, 1988; Møller *et al.*, 1990 and 'Particle size' below).

Stereological methods can be applied at all levels of organization from the organelle to the whole organ and can be employed to estimate:

(a) numbers of particles (whether organelles, nerve fibres, synapses, neurons or glial cells);

(b) lengths (e.g. of neurites) – although this usually refers to the length of a fibre, filament or tubule, under this general heading may be grouped inter-particle spacings (e.g. glia–neuron distances) and layer thicknesses (e.g. cortical depth);

(c) surface areas (e.g. of neuronal plasma membrane or cerebral/cerebellar cortex); and

(d) volumes (e.g. of mitochondria within neurons, of neurons themselves, of ventricles or subcortical grey matter or of whole brains).

A major area of morphometric interest is the counting and sizing of myelinated and unmyelinated fibres in cross-sections of peripheral nerve trunks or central tracts. Methods of sampling, sizing and counting in this context have been reviewed recently (Mayhew, 1988, 1990a) and are not covered here.

The stereological tools

Stereological estimators can be divided into two groups. The first comprises those (volume, number) for which it is necessary to randomize only the *location* of section planes. The second includes those (surface area, length, particle size, particle spacing, layer thickness) for which the *orientation* of sections must also be randomized.

ESTIMATING VOLUME, *V*

The volume of an object may be interesting in its own right or it may be required as an intermediate quantity for estimating a surface area, length or number. The latter quantities are often derived indirectly, by first estimating a component density. For instance, the packing density of cortical neurons might be expressed as a number per volume of cortex, N/V. In order to calculate absolute number, N, from N/V, the cortical volume needs to be determined. It is strongly recommended that component densities be converted to absolute quantities before biological interpretations are made. A potent illustration of the dangers of ignoring this advice is given elsewhere (Braendgaard & Gundersen, 1986).

If the object (say, the whole brain) is sufficiently big and can be isolated from surrounding structures, V can be obtained from mass and density or by fluid displacement (Scherle, 1970; Weibel, 1979; Regeur & Pakkenberg, 1989; Mayhew *et al.*, 1990c; Mayhew &

Olsen, 1991). When these techniques are impracticable, alternative methods must be sought. Such is the case for determining the volume of the brain (or brain regions) in a living individual, the cortex in a fresh or fixed brain, motoneuronal cell bodies in spinal cord or endoplasmic reticulum within neurons. These problems can be solved only by slicing and there are two basic approaches to estimating volumes from slices: direct and indirect methods.

The *direct* approach is to use the Cavalieri principle (see Gundersen & Jensen, 1987; Michel & Cruz-Orive, 1988) with which the volume of any arbitrary object can be estimated. The required sampling steps are (see Fig. 1):

(a) The object is cut into parallel slices. The location of the first slice must be uniform random in the interval 0–d where d is the mean distance between

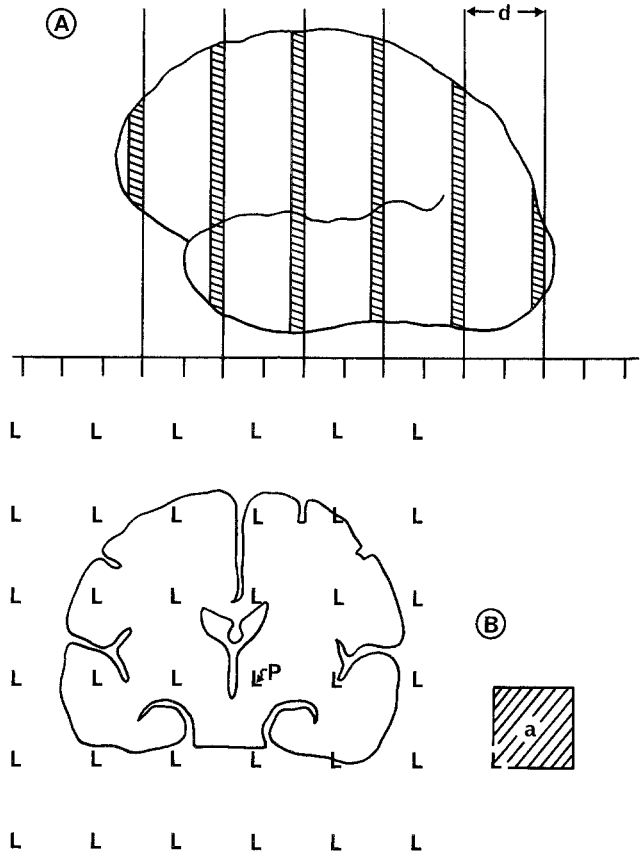


Fig. 1. The Cavalieri Principle. (A) A brain (frontal pole to left, occipital pole to right) is cut into a set of six systematic slices (hatched) separated by a distance d. The position of the first slice must be uniform random between 0 and d where 0 represents the position of a tangent to, say, the frontal pole. (B) The occipital face of one slice on which is randomly superimposed a systematic pattern of test points. Altogether, 11 points (of which one is indicated at P) fall on the cut face. Each point has an associated area (hatched square labelled a). By summing the points falling on the occipital faces of all six slices, an unbiased estimate of brain volume is obtained if d and a are known (see text).

designated slice faces. The unbiasedness of Cavalieri estimates does not depend on the orientation of the slices. However, orientation may well affect the precision (and, hence, efficiency) of estimation. Thus, a brain may be cut into coronal slices. Only one face of each slice (say, the more occipital) is measured and any slice which lacks this face is excluded from measurement.

(b) The areas of the designated slice faces are estimated by randomly superimposing a systematic array of test points on each face in turn. Given random positioning of the array on each slice, the sum of areas ΣA, is estimated unbiasedly by summing the points over all slices, ΣP, and multiplying by the planar area associated with each test point, a.

(c) Finally, the estimated volume (*est* V) of the object is calculated as

$$est\ V = \Sigma P \times a \times d$$

where the area, a, must be given on the scale of the object by taking account of the linear magnification. It is possible to select just a subset of slices by, for example, drawing a random sample of every third slice from a complete set.

It is *not* permitted to start slicing the object at some arbitrary distance (e.g. always at a distance of 1 cm) from one end. Such a scheme is biased because the region of the object lying between 0 cm and 1 cm *never* has a chance of being sampled. Similarly, any sampling scheme which fails to specify randomness of slice location cannot be regarded as generally unbiased.

A coefficient of error (CE) for volume estimates can be computed from the areas or point counts on the selected set of slices (see Gundersen & Jensen, 1987). It is known that efficient estimates (CE less than about 5%) can be obtained using just 5–6 systematic slices through an object (Gundersen & Jensen, 1987). Knowing this, the length of the object and the object-to-object variation, predictions about the best inter-slice distance and sample size can be made (for worked examples using human brains, see Regeur and Pakkenberg (1989) and Mayhew and Olsen (1991)).

Cavalieri volume estimates may be influenced by projection effects. Over-projection of slice areas can occur in thick microscopic slices when densely-stained or electron-opaque ingredients lie in a transparent or translucent matrix. Under-projection occurs when transparent/translucent ingredients are contained within an opaque matrix. The biases can be handled simply. With over-projection, the points falling on the largest slice in the set are subtracted from the point total for all slices (Gundersen & Jensen, 1987). By analogy, points falling on the largest slice must be added to the point total for all slices when there is under-projection (Gundersen, 1986). However, these corrections may not work well on objects which do not

have a smooth boundary. Therefore, in situations where it is not possible to view only the cut surface of each slice, it is preferable to minimize the impact of projection effects simply by reducing the thickness of slices.

The Cavalieri principle has provided estimates of ventricular, cortical and subcortical volumes in human and other mammalian brains. As well as macroscopic slices cut by brain-knife (Henery & Mayhew, 1989; Regeur & Pakkenberg, 1989; Mayhew, 1990b) or scalpel (Dantzer *et al.*, 1990; Mayhew *et al.*, 1990c), microtome slices (Pakkenberg & Gundersen, 1988; Bedi, 1991), CT scans (Pakkenberg *et al.*, 1989) and MRI slices (Mayhew & Olsen, 1991) have also been used. Provided the sampling rules are obeyed, the principle will also yield individual cell volumes from microscopic thin sections.

Studies on human brains have shown that efficient estimates of volumes can be obtained using only 4–5 slices (ventricles, Regeur & Pakkenberg, 1989), 5–6 slices (forebrain, Mayhew & Olsen, 1991) and 13 slices (cerebral cortex, Regeur & Pakkenberg, 1989) per brain.

Technical factors dictate that the Cavalieri principle would not provide direct estimates of, say, the volume of all mitochondria in thalamic neurons. For this purpose, an *indirect* approach based on multilevel sampling is necessary (Cruz-Orive & Weibel, 1981). The following protocol illustrates how this might be achieved. First, estimate the volume of the thalamus, $V_{(t)}$, by using the Cavalieri principle. Second, estimate the volume density of neurons within the thalamus, $V_{(n)}/V_{(t)}$, by taking systematic random samples of tissue blocks from the thalamus and then systematic samples of light microscopic fields of view from the blocks. This can be done simply by summing (over all fields of view and over all blocks) the test points which hit the neurons, $\Sigma P_{(n)}$, and those which hit the thalamus, $\Sigma P_{(t)}$. The point ratio $\Sigma P_{(n)}/\Sigma P_{(t)}$ is an unbiased estimator of $V_{(n)}/V_{(t)}$.

Third, estimate the volume density of the mitochondria within neurons, $V_{(m)}/V_{(n)}$, from systematic samples of electron microscopic fields of view drawn from tissue blocks. Again, this volume density is calculated from a point fraction, $\Sigma P_{(m)}/\Sigma P_{(n)}$, where $\Sigma P_{(m)}$ and $\Sigma P_{(n)}$ are summed over all fields and blocks. Finally, the volume of the chondriome, $V_{(m)}$, is estimated using the relation

$$est\ V_{(m)} = est\ V_{(t)} \times est\ V_{(n)}/V_{(t)} \times est\ V_{(m)}/V_{(n)}.$$

For reasons of efficiency, it is sensible to restrict linear magnification at each sampling level to the *minimum* which allows recognition of the compartments concerned. It is also important to ensure the consistent definition of compartments in moving from one sampling level to the next (Cruz-Orive & Weibel, 1981). If this definition is prejudiced by technical factors such as differences in fixation, image resolution or specimen contrast, then bias will arise.

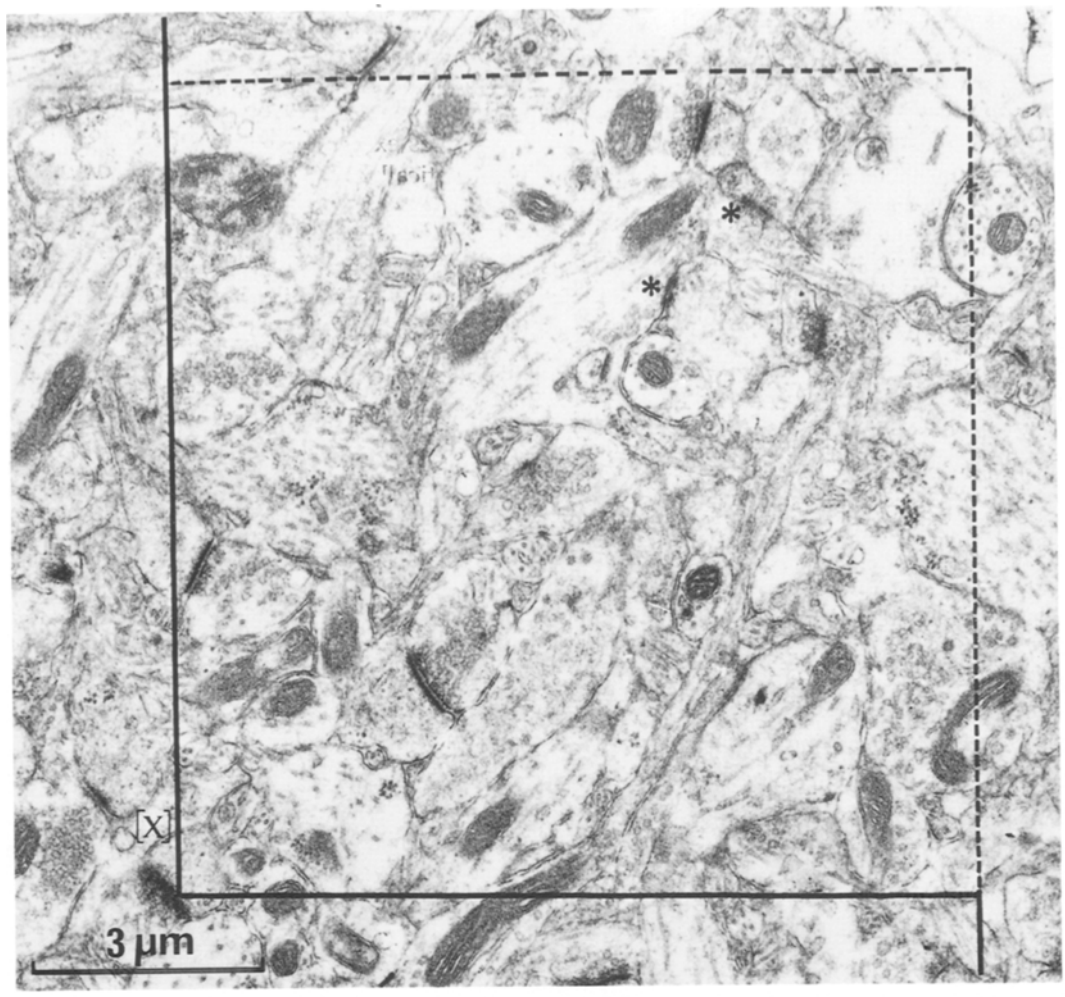
ESTIMATING NUMBER, *N*

In the past, counting particles in 3-D was exclusively assumption-based. Neurons and synapses were counted by making assumptions about their shape and spatial orientation. Although often compromised by inadequacies of sampling as well, the methods were the best available at the time and their use acknowledged the fact that the probability of sectioning depends not only on the section thickness but also on the particles themselves. Larger neurons (e.g. Purkinje neurons in the cerebellum) have more chance of being cut than smaller neurons (e.g. granule cells). Similarly, fusiform cells have more chance of being cut if they are sectioned transversely rather than longitudinally. Finally, irregular particles (e.g. mitochondria) may be hit by a plane more than once leading to difficulties in deciding whether or not the several profiles appearing on the section plane belong to only one particle.

To overcome these problems, and obtain unbiased estimates of particle number, it is necessary to sample them with a volume probe (Gundersen, 1986; Cruz-Orive, 1987). The use of lower-dimensional probes introduces errors governed by size, shape and spatial orientation. Thus, single sections (2-D probes) will sample particles with probabilities determined by their largest linear dimension in a direction perpendicular to the section plane.

A major methodological advance, independence of particle shape, size distribution or orientation, was made by using a volume probe which consisted of a stack of serial sections of arbitrary direction but

Fig. 2. The disector and particle number. The pair of micrographs [X,Y] is sampled from layers II and III of rat visual cortex and represents a disector pair of two parallel section planes separated by a distance of 64 nm. Shown are some synaptic densities (s) and mitochondrial profiles (m). To count synaptic densities, we may use X as the reference and Y as the look-up section (and/or vice versa). In the unbiased counting frame on reference X there are two synaptic densities (indicated by asterisks (*) on X) which do *not* touch the forbidden lines (solid) and which do *not* appear on look-up Y. In the reverse direction, again, there are two (different) synapses which appear on reference Y which do not appear on look-up X (see asterisks on Y). For these two directions, the average number of synapses is two (= 4/2) and these synapses occur in a disector volume given by the product of frame area and the distance between section planes.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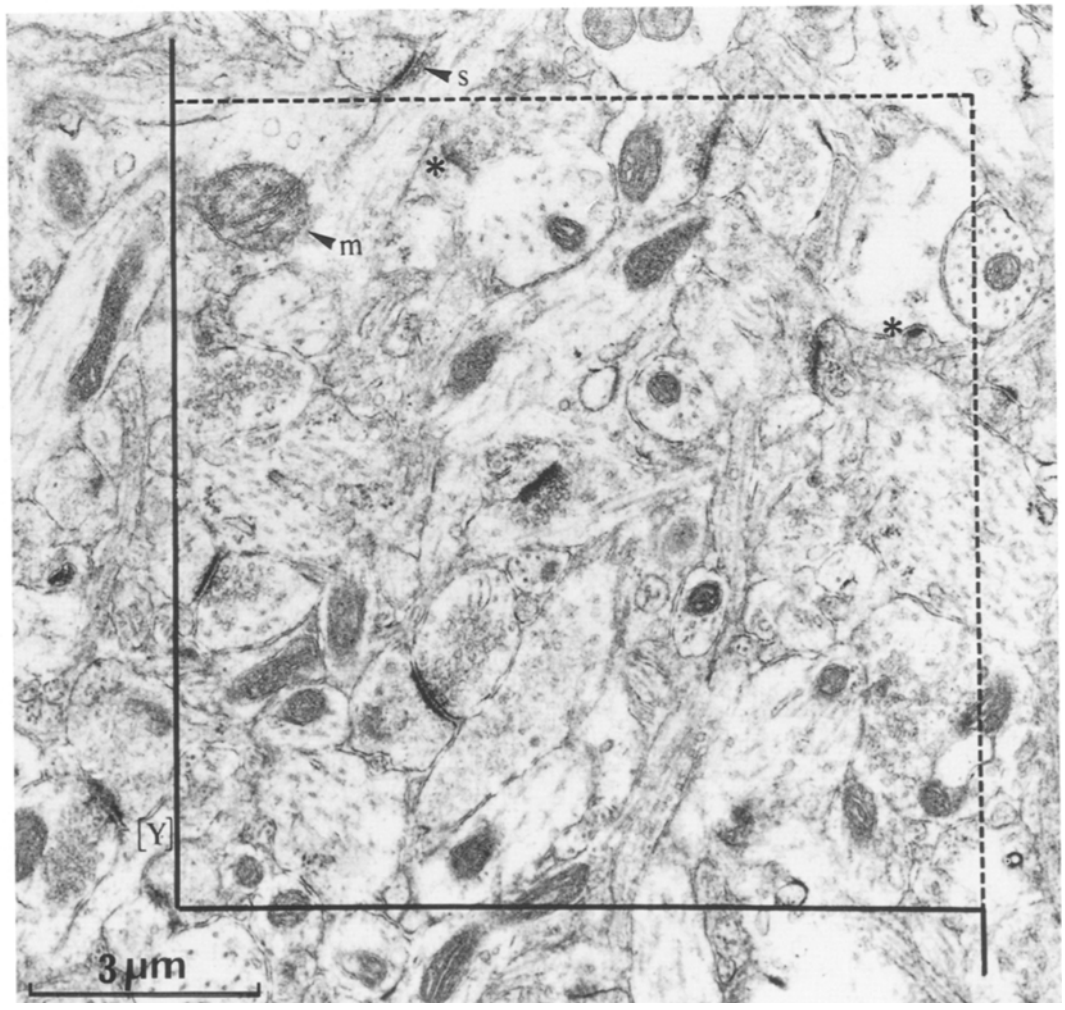
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random position (Cruz-Orive, 1980; de Groot, 1988a). However, this method has been succeeded by the 'disector' (Sterio, 1984) and the 'fractionator' (Gundersen, 1986), methods of superior efficiency which require but pairs of parallel sections.

The disector

This device (Fig. 2) counts particles between randomly positioned pairs of parallel slice planes separated by a known distance. If it is not possible to view between these slice planes (e.g. by tracking through optical section planes), the distance should be less than the minimum linear dimension of the particles and, for reasons of efficiency, preferably about 1/3 to 1/4 of particle size. Using an unbiased 3-D rule, particles are counted if they appear in an unbiased counting frame on one slice plane (the 'reference') but not on its partner (the 'look-up', see Fig. 2). In effect, the number of particles so counted, by convention designated Q^-, is contained within a volume equal to the area of the counting frame, A, multiplied by the distance between the upper (or lower) faces of the slices, d. In short,

$$est\ N/V = Q^-/(A \times d).$$

Since section orientation is not critical for achieving unbiasedness, convenient directions of sectioning can be selected so as to further improve efficiency. In addition, the same pair of sections may be used in forward (reference to look-up) and reverse (look-up to reference) directions and the average of the two counts can be taken (see Fig. 2). Notice that the disector is unaffected by sectioning artefacts (image overprojection and truncation). However, implemented in its basic form, the disector demands an accurate knowledge of slice separation. If this corresponds to section thickness, t, or to some multiple thereof, then it can be determined by using Small's fold method, microinterferometry, re-sectioning or some other technique (see Small, 1968; Goldstein & Hartmann-Goldstein, 1974; Bedi, 1987; de Groot, 1988b; Evans & Howard, 1989).

An alternative to cutting physical disectors is optical sectioning. Two possibilities exist but both require the z-distance of the microscope to be measured accurately, e.g. with a microcator (Braendgaard *et al.*, 1990). For transmitted light microscopy, thick sections can be cut and focused at different planes (Gundersen, 1986). Particles are counted in optical disectors when they (or some part of them, such as the nucleus of a neuron) first come into focus within the counting frame. It is essential to use high numerical aperture, oil-immersion objectives so that movements in the z-direction reflect true movements of the focal plane through the thick section. The use of glycol methacrylate as an embedding medium permits optical sectioning in thick (*ca* 25 μm or greater) sections to be performed with minimal shrinkage effects. If the particles exhibit fluorescence or reflectance, they can be optically sectioned by confocal microscopy and counted with an unbiased counting brick (Howard *et al.*, 1985).

To avoid the estimation of slice separation entirely, a design-based approach can be adopted. In the sampling regime used by Pakkenberg and Gundersen (1988), which is tantamount to a fractionator scheme (see below and Geiser *et al.*, 1990), estimates of the reference volume (determined using the Cavalieri principle) and numerical density (determined using the disector) were made on sections from the same set so that section thickness, t, was common to both estimations. A similar trick (the 'double disector') can be employed to count small objects (e.g. synapses) in ultrathin sections. The idea is to determine N/V of both synapses and neurons in the same sets of sections to obtain a synapse-to-neuron ratio. By estimating the number of neurons from semithin sections, the absolute number of synapses can be found (see Braendgaard & Gundersen, 1986; Gundersen *et al.*, 1988a).

Notice that the disector yields numerical density, N/V, rather than N itself. In consequence, estimates are sensitive to preparation artefacts such as fixation shrinkage. However, when combined with Cavalieri estimates of volume, these difficulties can also be circumvented (Gundersen, 1986; Pakkenberg & Gundersen, 1988).

Various modifications of the disector have been used to count neurons and other neural cells (Braendgaard & Gundersen, 1986; Gundersen *et al.*, 1988a; Pakkenberg & Gundersen, 1988, 1989; West *et al.*, 1988; Bjugn *et al.*, 1989; Korbo *et al.*, 1990; Pakkenberg, 1990; West & Gundersen, 1990; Bedi, 1991; Bjugn, 1991; Fukui & Bedi, 1991; Pakkenberg *et al.*, 1991; Tandrup, unpublished). The disector has also been used to count perforated and non-perforated synapses (Braendgaard & Gundersen, 1986; de Groot & Bierman, 1986, 1987; Calverley *et al.*, 1988; Gundersen *et al.*, 1988a; Hunter & Stewart, 1989; Braendgaard *et al.*, 1990; Calverley & Jones, 1990; Siklos *et al.*, 1990; Fukui & Bedi, 1991). It use with a novel definition of what constitutes a capillary allows the estimation of capillary number (for an application to renal glomeruli, see Nyengaard and colleagues (1988)).

The fractionator

The fractionator principle side-steps the residual problems of processing distortion and section thickness by yielding direct, unbiased and highly efficient estimates of N. It is not necessary to know the final linear magnification, the area of the sampling/counting frame, section separation or the volume (fixed, fresh

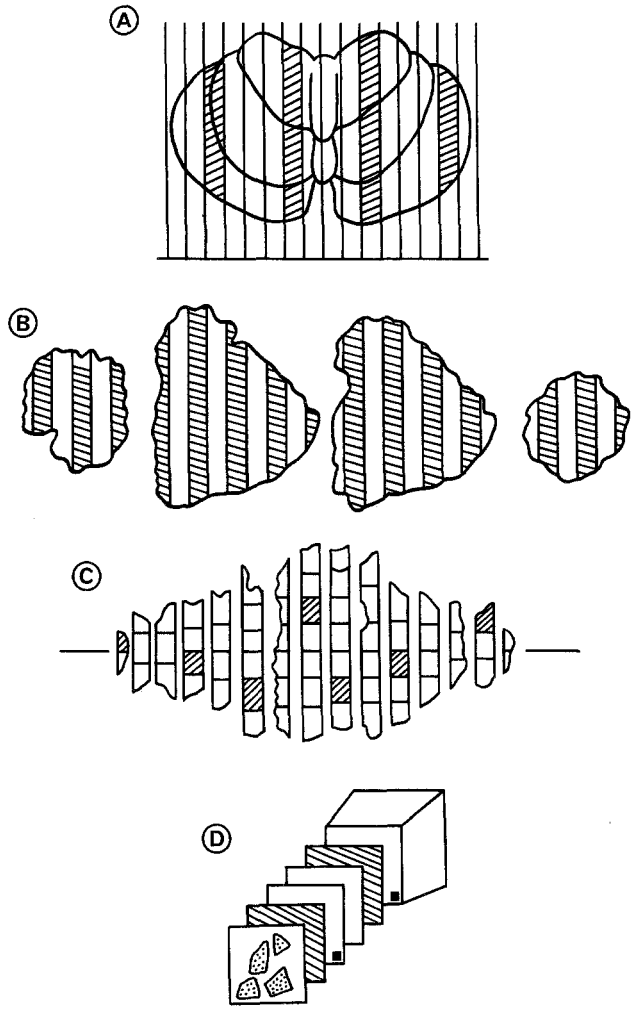

Fig. 3. Fractionator sampling. (A) A cerebellum is cut into a set of systematic slices of which a fraction, 1 in 4, is selected (hatched slices) with a uniform random start. (B) The chosen slices form naturally a 'dome' distribution (smaller slices are peripheral, bigger slices are central) and are cut into strips from which a systematic sample of 1 in 2 is drawn. (C) The chosen strips have been arranged in an artificial 'dome' pattern and cut into pieces from which a further fraction, 1 in 10, is drawn. (D) The pieces are embedded together in a block which is exhaustively serially sectioned. A systematic selection of 1 in 3 tissue sections is taken in which particles (cells, nuclei or nucleoli) are counted using either single sections (hatched) or disector pairs of sections (hatched plus look-up sections identified by solid squares). In this example, the number so counted would be multiplied by 240 (= 4 × 2 × 10 × 3) to obtain an unbiased estimate of total particle number in the cerebellum (see text for further details).

or otherwise) of the reference space. All that is required is random sampling in 3-D space with known sampling fractions.

The volume in which the particles are contained can be cut into uniform random slices of arbitrary size, shape and number but the slice planes should not intersect inside the object if the slices are used directly to make disector pairs (e.g. see Pakkenberg & Gundersen, 1988). The fraction, $1/f$, of slices sampled in this way will contain $n \times f$ particles where n indicates the number counted in the sample. Often, the sample will comprise sets of disector pairs (when n is equivalent to Q^-) but, for certain neurons, it is possible to use the nucleolus as a point-like inclusion for counting in sets of single sections (Braendgaard & Gundersen, 1986; Nairn *et al.*, 1989; Mayhew, 1991b).

In many cases, the fractionator will be applied as a multi-stage sampling scheme with systematic random sampling at each stage (see Fig. 3). The estimate of number N is then obtained using

$$est\ N = n \times f_1 \times f_2 \times f_3 \ldots \times f_k$$

where f_k is the reciprocal of the sampling fraction at the lowest sampling stage. The number of stages and the fraction chosen in each can be adjusted to suit the demands of a particular experiment so that, eventually, a reasonable number (say, less than 200 neurons (Mayhew *et al.*, 1990b)) is counted. The object may be fragmented quite arbitrarily at any stage but the last one in which parallel slices are adviseable for technical reasons.

Efficiency may be improved by cutting so that pieces contain roughly equal numbers of particles. For an organ which is homogeneous in terms of the 3-D spatial distribution of particles, this is tantamount to cutting into pieces of roughly equal size. In general, however, it is preferable to arrange the tissue fragments obtained at a given sampling stage into a series with a 'dome' pattern (Fig. 3). On this pattern, the fragments at the ends of the series would be the smallest and successive fragments would gradually increase in size in passing from each end and towards the middle (Ogbuihi & Cruz-Orive, 1990). Often, this pattern will be approximated in practice, at least at the first sampling stage, e.g. if the organ is convex and its slices are preserved in their natural order after cutting. To further improve efficiency, without introducing bias, portions of the organ which do not contain the particles (e.g. white matter does not contain cortical neurons) can be regarded as superfluous and they can be trimmed and discarded.

As an example, Nairn and colleagues (1989) employed a four-stage scheme to count the nucleoli of Purkinje neurons in human cerebella. In this case, N is an unbiased estimate of Purkinje nucleoli and yields an unbiased estimate of neuron number *only* if the underlying assumption, viz. that each neuron contains a single nucleolus, is correct (see Braendgaard & Gundersen, 1986; Campbell *et al.*, 1988 for instances where this assumption is invalid). The cerebellum was first divided into uniform random slices which were cut, in turn, into strips. Superfluous white matter (which does not contain any Purkinje neurons) was

identified, excised and discarded. A systematic selection of $1/f_1$ of these strips was then taken. At stage 2, the chosen strips were cut into smaller pieces and a fraction, $1/f_2$, of these pieces was drawn. At stage 3, chosen pieces were cut into blocks from which a systematic selection of $1/f_3$ was drawn. Finally, the blocks were cut into a complete set of serial sections from which a systematic sample of $1/f_4$ was chosen. These sections were stained and the number of nucleoli seen within them (n) was counted.

Recently, a method for predicting the variance of a fractionator estimate of number has appeared (Cruz-Orive, 1990). The method is an improvement on that proposed by Gundersen (1986). At the first sampling stage, the specimen is divided into two systematic samples. The empirical variance of the pooled estimate of N is then calculated using the pair of observations made on these two stage 1 samples.

Illustrations of the use of the fractionator to count neurons in physical and in optical sections can be found elsewhere (Braendgaard & Gundersen, 1986; Pakkenberg & Gundersen, 1988; Nairn *et al.*, 1989; Mayhew *et al.*, 1990b; Mayhew, 1991b; West *et al.*, 1991). For counts of particles in other tissues, see Geiser and colleagues (1990) and Ogbuihi & Cruz-Orive (1990).

ESTIMATING SURFACE AREA, S

Unbiased estimates of the surface area of arbitrary objects can be obtained *only* by stereological analysis of slice images. Surface area is calculated as the product of a surface density, S/V, and the reference volume. Earlier methods for estimating the cortical surface area of the cerebrum from parallel slices were biased because they depended on assumptions or did not randomize orientation sufficiently. At least some were also inefficient because very many thin sections were required in order to reduce the biases (see Henery & Mayhew, 1989).

Imagine an object (or collection of objects) embedded in a reference volume which is sliced. When test lines of total length L are superimposed on the slices, the boundary traces of the object surface(s) make chance intersections, I, with the lines. In fact, the density I/L is directly proportional to the surface density of the object in its containing volume (Weibel, 1979). The relation is

$$est\ S/V = 2 \times \Sigma I/\Sigma L$$

(where ΣI and ΣL are summed over all slices) and its validity requires isotropy of the chance encounters between the overall surface and the test lines. The test lines must be isotropic uniform random (IUR) in 3-D space. Isotropic means that all directions in space are equally likely whilst uniform signifies that all positions are equally likely. Thus, IUR implies that intersections must cover all possible directions in 3-D space with equal probability density (imagine them being distributed uniformly as points on the surface of a sphere).

By associating each line element, l, with a test point P, an unbiased estimate of ΣL is derived from $\Sigma P \times l$. Again, l must be corrected by the final linear magnification factor. The estimation of S/V now reduces to counting two types of chance event, viz. test intersections hitting the surface and test points hitting the volume. The absolute surface area (in cm^2) is derived by multiplying the estimated S/V (in cm^2/cm^3) by the volume of the reference space (in cm^3).

Isotropic uniform random lines in 3-D space may be generated in different ways (see Baddeley *et al.*, 1986; Gundersen *et al.*, 1988b; Mattfeldt *et al.*, 1990; Nyengaard & Gundersen, 1992). One possibility is to aim for IUR sectioning by physically randomizing tissues within blocks prior to sectioning (Stringer *et al.*, 1982). However, this approach is not rigorous enough and design-based solutions which guarantee isotropy of sectioning are preferable.

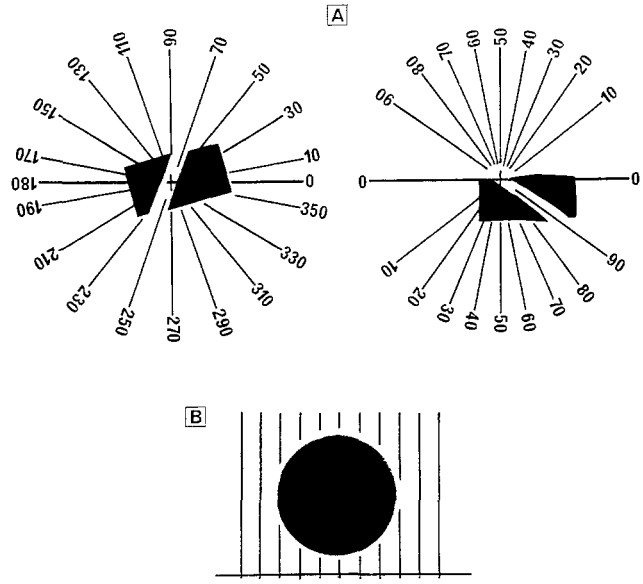

Fig. 4. Cutting IUR planes in 3-D space. (A) An orientator is used to choose two angles at random. A specimen is placed on an arbitrary surface near the centre of a compass rose, an angle is chosen from a table of random numbers and the specimen is cut normal to the rose in this direction (here 70–250 axis). The face just exposed is placed on a second (cosine-weighted) rose so that it lies parallel to the 0–0 axis. Now a second direction is chosen (here the 90–90 axis) and another orthogonal slice made along it. The newly created plane is IUR. (B) An isector is used to cut a sphere in which the specimen has been embedded. The sphere is rolled around a lattice of lines and cut normal to the lattice. Cutting along a given line produces an isotropic plane and cutting along them all produces a uniform random sample of planes. The planes must be independent of the specimen within the sphere.

The isector

The 'isector' (see Fig. 4) is the most recent solution to the problem of generating section planes which are IUR in 3-D and involves embedding samples of tissue in spherical moulds (Nyengaard & Gundersen, 1992). It relies on the simple fact that, once the embedding medium has hardened, rolling the resulting sphere randomizes orientation in 3-D space and guarantees isotropy of a section plane cut through it. If this plane is independent of the specimen contained within the sphere, then IUR planes through the tissue will be produced. This can be achieved easily by mounting the rolled sphere on fresh embedding medium so that it can be cut on the microtome in the chosen direction or by cutting the sphere directly on a substrate of systematic guide lines (Fig. 4). This simple and direct method is suitable for small specimens such as those required for transmission electron microscopy. Amongst other purposes (see below), the IUR section planes may be used to estimate S/V, in which case test lines are applied to the planes and intersections between the test lines and surface boundary traces are counted.

The orientator

Another solution is to cut directionally-independent isotropic sections using pairs of random numbers. To improve efficiency, the use of 'ortrips' (sets of three mutually perpendicular slices) has been devised. Lately, this approach has been refined as the 'orientator' (Mattfeldt *et al.*, 1990).

The orientator (Fig. 4) is a device for generating slice planes which are IUR in 3-D space (for some applications, see Mattfeldt *et al.*, 1990 and Gundersen *et al.*, 1988a). Briefly, uniform random samples of tissue are taken from within the object and an IUR slice plane is cut from each sample. Again, these planes can be used in order to estimate S/V as indicated above. Since a *pair* of angular coordinates is needed in order to specify the location of any given point on the surface of a sphere, IUR planes are generated with the orientator in two steps using a pair of compass roses (see Fig. 4):

(a) Cut the object by an arbitrary plane and place this plane on the first compass rose. Next, choose a random angle of longitude and cut the object perpendicular to the original arbitrary plane.

(b) Place the new plane on the second rose and choose a cosine-weighted random angle. The angle must be cosine-weighted because it is the normal to the section plane which must be sine-weighted. This compensates for the fact that angles nearer the equator delimit areas on a sphere's surface which are greater (and therefore include more points) than those delimited by angles nearer the poles. Cutting the object at this angle provides the final isotropic plane.

Vertical sectioning

Vertical sectioning is more efficient than the orientator when the surfaces of interest invest the object (e.g. the cortex investing the cerebrum) or are highly ordered (e.g. layered). Here, vertical signifies perpendicular to some convenient and recogniseable 'horizontal' reference which may be a natural feature of the object (e.g. the medial aspect of a cerebral hemisphere, see Fig. 5)

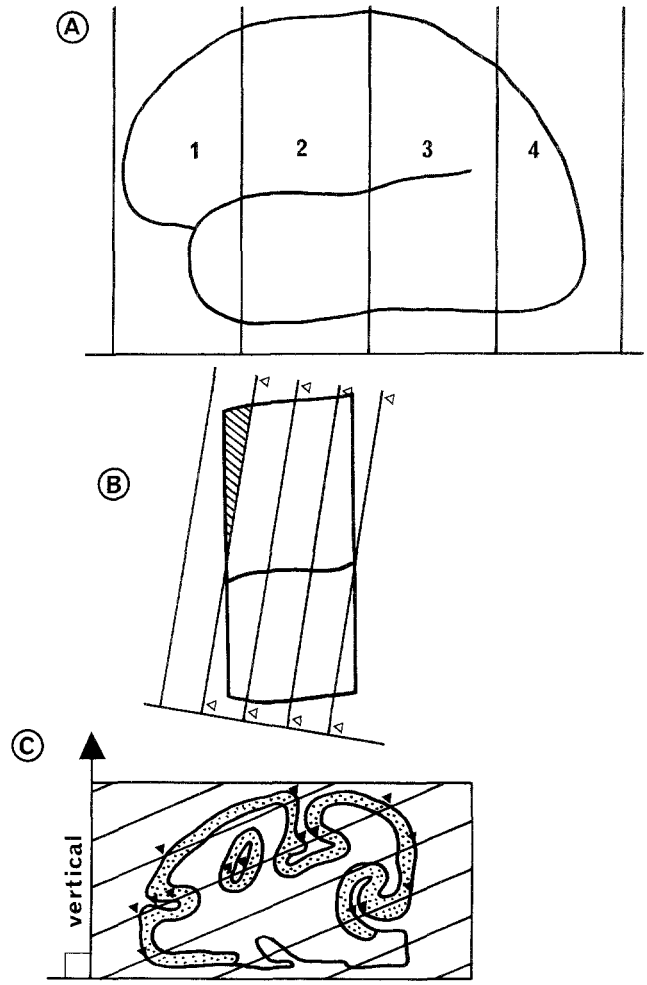

Fig. 5. Vertical sectioning. (A) A single cerebral hemisphere is placed on its medial aspect on a 'horizontal' worksurface. Any section orthogonal to this reference surface is, by definition, a 'vertical' section. The hemisphere is divided into four slabs (labelled 1–4). (B) Each slab (here slab 2) is cut at a random angle into parallel vertical sections. Only one face of each section (say, the more frontal, open arrowheads) is analyzed and one end-fragment (hatched) must therefore be discarded. (C) Each vertical section, with its defined vertical axis, has an overlay of test lines superimposed at a random angle which must be sine-weighted with respect to the vertical axis. The test lines form intersections (solid arrowheads) with the outer aspect of the cortex and summing them over all slabs and all sections provides a basis for unbiasedly estimating cortical surface area. An overlay of cycloid test lines would substitute equally well for the lines shown here (see text).

or a creation of the experimenter. A detailed description of the method, with some examples, is given by Baddeley and colleagues (1986). It has been used to study surfaces on and within different cells and organs (Michel & Cruz Orive, 1988; Griffiths *et al.*, 1989; Henery & Mayhew, 1989; Mayhew, 1990b; Mayhew *et al.*, 1990a).

Any straight line in 3-D space can be contained within a unique vertical plane. The practical implication of this is that S/V can be estimated by cutting objects vertically and applying test lines to the vertical planes. If the vertical sections are positioned uniform randomly within the reference space, the test lines will be IUR in 3-D. In fact, the lines must have lengths which are sine-weighted with respect to the vertical direction. A particular geometric curve, the cycloid arc (see Baddeley *et al.*, 1986) possesses this characteristic but sets of sine-weighted straight lines are also appropriate (see Fig. 5 and Cruz-Orive & Hunziker, 1986; Gundersen *et al.*, 1988a).

The basic sampling protocol (Fig. 5) is as follows:

(a) The object must possess a 'horizontal' or one must be invented for it.

(b) Vertical sections must be cut perpendicular to the horizontal and the vertical direction must be recognizable on each of those sections.

(c) Vertical sections must be randomly positioned and IUR on the horizontal.

(d) Intersections between the surface boundary traces and the test lines are counted using a systematic array of cycloid arcs whose minor axes are aligned with respect to the vertical direction on each section.

Note well that whilst the lines are IUR in 3-D, the vertical section planes are isotropic only in 2-D. In consequence, vertical sections cannot be used to estimate the length of a curved filament from counts of its profiles or the thickness of a sheet from measurements of orthogonal intercept lengths. For these purposes, IUR sections (produced by the orientator or the isector) must be cut.

A bias associated with estimating the surface area of cellular membranes by transmission electron microscopy is membrane image loss. This occurs when membranes are tilted beyond about 20–25 degrees from the electron beam axis. An impression of the magnitude of this bias is given in Mayhew and Reith (1988).

ESTIMATING LENGTH, *L*

Length here refers to connected or separate filaments, fibres, or tubules. These may be capillaries, axons, dendrites, neurofilaments, neurotubules, etc. Let these filaments (overall length, L) be embedded in a reference volume. Sections through the latter will transect the filaments to produce a number of profiles, Q. In fact, the areal packing density of profiles on the section, Q/A, is directly proportional to the length density, L/V. The relationship is

$$est\ L/V = 2 \times \Sigma Q/\Sigma A$$

where ΣQ and ΣA are summed over the section set (e.g. Weibel, 1979). The factor 2 is valid given IUR encounters between the linear structures and the planes and the relation yields practically unbiased estimates provided that the filaments are negligibly thick in comparison to their length (Gundersen, 1979). In practice, of course, total sectional area would be estimated most efficiently by point counting, as described under the Cavalieri principle. Estimating L/V then reduces to counting chance events (points and profiles). Absolute L (in cm) can be calculated from L/V (in cm/cm^3) if V (in cm^3) is known.

To obtain slice planes which are IUR in 3-D, the isector and the orientator (but *not* vertical sectioning) can be used. A practical problem arises in counting profiles for which purpose an unbiased counting frame and counting rule should be adopted (see Fig. 2 and Gundersen (1977), Jensen and Sundberg (1986) and Mayhew (1990a) for examples). If these are not used, profiles may be counted incorrectly, especially those which obtrude into the slice area at its edges and corners.

Although it cannot be estimated from vertical sections, it has been shown (Gokhale, 1990) that L/V can be calculated from the orthogonal projections of filaments within uniform vertical slices of known thickness, t. The rationale behind this approach is that a cycloid test arc on a vertical plane can be treated as the 2-D projection of a cycloid test surface which is perpendicular to that plane. Therefore, the projection of a curved filament on to the vertical plane can be used to generate and count test intersections between the projected filaments and the cycloid arc image of the cycloid surface. In contrast to the situation for estimating S with cycloid arcs, the minor axis of the cycloid arcs must be aligned with respect to the *horizontal* direction on the vertical slices.

The sampling rules are:

(a) Slices through the specimen must be vertical and uniform random in location.

(b) All vertical slices must have the same thickness.

(c) The projected images of the filaments are observed through the section thickness (e.g. by transmitted light microscopy where the beam is orthogonal to the vertical slice).

(d) Count intersections with cycloid arc test arrays. length density, L/V, is then estimated by the relation

$$est\ L/V = 2 \times (\overline{I/l})/t$$

(Gokhale, 1990) where t is slice thickness and $(\overline{I/l})$ is the number of intersections between the projected images

of the filaments and cycloid arcs expressed per unit test arc length. An inconsequential degree of bias is introduced because l is random in general.

A practical nuisance is the need to measure t. To circumvent this, Cruz-Orive and Howard (1991) have proposed a simple solution which avoids having to physically slice the specimen. The trick is to rotate the curved filament about a fixed vertical axis and project it on to a fixed vertical plane. By using these total vertical projections and counting intersections at different angles of rotation, the Gokhale formula can be re-arranged to

$$est\ L = 2 \times \Sigma I/n \times (a/l)$$

where a/l represents the area per test length for the cycloid arc test system, $\Sigma I/n$ is the mean number of intersections averaged over the n different rotation angles. This modification is illustrated with a synthetic filament in Cruz-Orive and Howard (1991). It has great potential for estimating the lengths of neurites, neurofilaments and neurotubules in situations where the containing matrix is sufficiently transparent and where filament length density is not so high that overlap effects become problematic. The use of confocal microscopy is an obvious possibility.

ESTIMATING PARTICLE VOLUME, $\bar{v}_V$ AND $\bar{v}_N$

Several direct estimators of mean particle volume are available and they are more efficient than the indirect ones (e.g. dividing total V by total N or dividing V/V by N/V).

Volume-weighted mean volume

This volume, $\bar{v}_V$ (in, say, cm^3) is the mean value of particle size obtained from the volume distribution of particle volumes. It is different to the more familiar number-weighted mean volume, $\bar{v}_N$ (also in cm^3), which is determined from the numerical frequency distribution of particle volumes. However, the two quantities are related as follows:

$$\bar{v}_V = \bar{v}_N\,(1 + CV_N^2)$$

where CV_N is the coefficient of variation of the *number* distribution of particle volumes (Gundersen & Jensen, 1985; Cruz-Orive & Hunziker, 1986).

Consider a population of arbitrary particles (e.g., neuronal perikarya). Individual perikarya can be selected for volume estimation in a volume-weighted manner by allowing them to be hit by uniform random test points (see Fig. 6). For each point hitting a perikaryal profile, an independent isotropic direction in 3-D is chosen. This direction is used to draw a line which passes through the point. The part of the line which intercepts the perikaryon constitutes an isotropic point-sampled intercept and its length provides the basis of the method for estimating volume-weighted mean volume (Fig. 6).

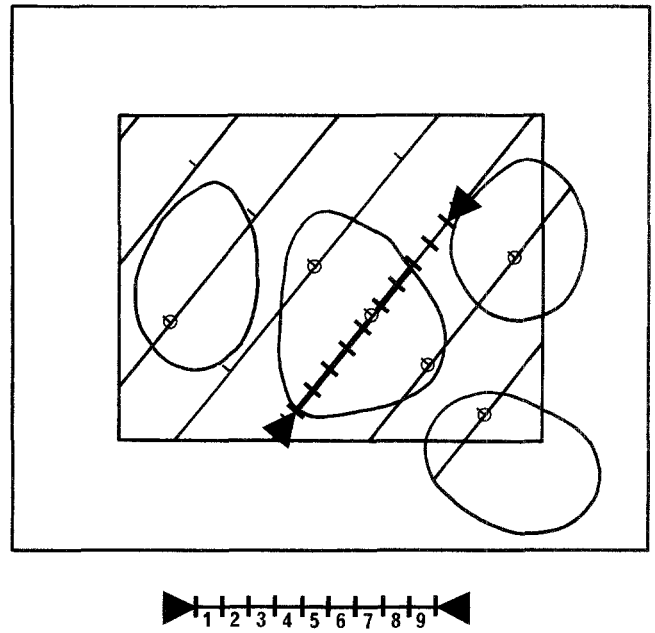

Fig. 6. Volume estimation from point-sampled intercept lengths. A group of nerve cell profiles appears on a single section on which is represented a frame with a surrounding guard area. An overlay of randomly oriented lines bearing a systematic pattern of marked test points is superimposed. Some of these points (the six open circles) fall on cell profiles. The lengths of intercepts which cross cell profiles through these points are measured using a graduated scale as a classifier. The point-sampled intercept shown falls in class 7 of the scale. The guard area allows profiles hit by points but outside the frame to be classified. If the directions of lines are IUR in 3-D space, the random intercept lengths through all six points can be used to provide an unbiased estimate of the volume-weighted mean volume of the neurons. If neurons are selected using disector pairs, the same procedure can be used to determine the number-weighted mean volume (see also text and Fig. 7).

In fact, the third power of the point-sampled intercept lengths is averaged over all intercepts and multiplied by $\pi/3$. For convex particles (which are hit only once by a section plane), point-sampled intercepts are unbroken straight lines. In contrast, non-convex particles (which may be hit more than once by the section plane), can produce intercepts which are split into two or more separate elements of length. Despite this, an unbiased volume estimate can still be computed if every particle profile can be ascribed unequivocally to the same parent particle (Gundersen & Jensen, 1985; Gundersen, 1986).

To apply the method, a test lattice of points is placed on an IUR section. Wherever a test point hits a perikaryal profile, a line is drawn through this point across the entire profile and at a randomly selected angle. This procedure is repeated for all those test points which hit perikaryal profiles (Fig. 6). If the tissue is sampled by vertical sectioning, the angles are

defined with reference to the vertical direction. In this case, the test lattice should bear sine-weighted lines.

For applications to the sizing of neurons, see Braendgaard and Gundersen (1986) and Mayhew (1989). A consequence of the sensitivity to both number-weighted volume and CV_N is that estimates of volume-weighted mean volume are useful for analysing certain structural manifestations of pathological processes, viz. alterations in the size and/or size distributions of cells and their nuclei (Nielsen *et al.*, 1986).

Recently, Gittes (1990) has modified the point-sampled intercept approach to obtain a boundary-sampled intercept volume. In this method, random intersections between test lines and profile boundaries are used as a basis for measuring intercept lengths. This procedure samples particles with probabilities proportional to their surface and the resulting volume is a *surface*-weighted mean volume. The method may be used to size (and count) particles that show little variation in surface area.

The selector

Whilst the selector arose out of the disector, it does not provide an estimator of particle number. Instead, it is a device for selecting particles in an unbiased way for estimating directly number-weighted mean volume (Cruz-Orive, 1987).

Stacks of serial sections (which must be IUR in 3-D or vertical sections) are cut and a disector pair from each set is drawn to select particles according to their presence and *not* their size. The number-weighted mean volume of the chosen particles is obtained from their point-sampled intercept lengths. Note that this is achieved *without* needing to know the distance between the disector planes. Again, the lines must be IUR in 3-D space, hence the necessity of cutting IUR sections in 3-D or cutting vertical sections and applying sine-weighted test lines (Baddeley *et al.*, 1986; Cruz-Orive & Hunziker, 1986; Gundersen *et al.*, 1988a,b). For an application to ependymal cells in spinal medulla, see Bjugn and colleagues (1989).

The nucleator

The nucleator (Gundersen, 1988) is similar to the selector but is a more efficient way of estimating the number-weighted mean volume for particles which possess a single identifiable point-like inclusion (for convenience referred to as the 'nucleus', see Fig. 7). An ideal candidate in many types of neuron is the nucleolus.

The nucleator requires a stack of sections which is as high as the largest diameter 'nucleus'. Having sampled the 'nucleus' with a disector pair from the stack, test points are applied and, for each point, an isotropic direction is chosen. Then a line is drawn in the chosen direction from the point to the boundary of the particle. An unbiased estimate of number-weighted mean volume is derived by averaging, over all intercepts, the third powers of intercept lengths and multiplying by $4\pi/3$ (Gundersen, 1988). If lines are drawn across the particle profiles passing through the chosen points then two possible intercepts may be measured, each passing from the point to the particle boundary, as illustrated in Fig. 7. Estimates of volume can also be made using both of these intercepts (for a practical example, see Jacks *et al.*, 1990).

It is useful to note that if a single, small nucleolus of approximately constant size is adopted as the 'nucleus', the number-weighted mean volume can be estimated from just one section or from several

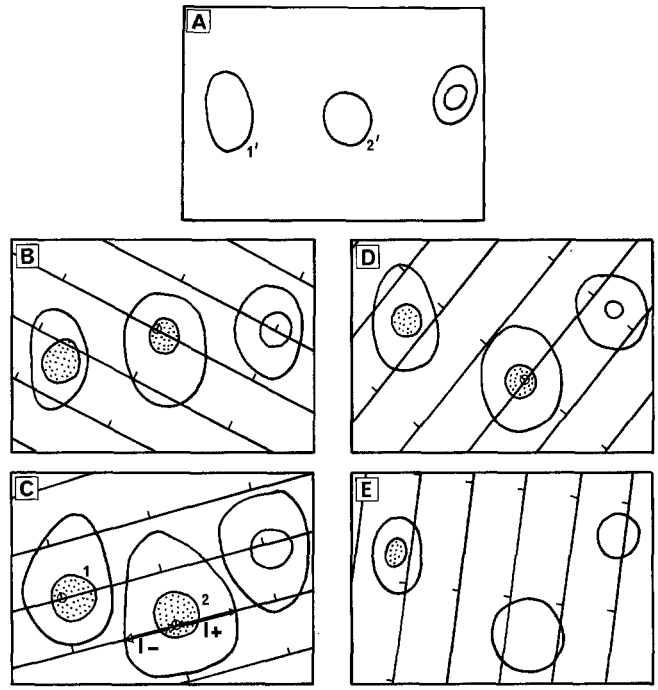

Fig. 7. The nucleator and cell volume estimation. The sequence A–E represents a set of serial sections through three neurons, each of which contains a single nucleus. The pair C and A is used as a nucleator, i.e. as a disector pair (C is the reference and A is the look-up section) for selecting nuclei within cells according to their number alone. On this basis, two nuclei (1,2) are cut on section C and disappear by section A (see 1',2'). The profiles of these two nuclei (stippled) are sampled on all sections to estimate number-weighted cell volume. An overlay of lines (which must be IUR in 3-D) bearing test points is rotated systematically on each section in turn and some test points hit the identified nuclei (see 4 open circles). Distances from these points to the cell boundary form the basis for unbiasedly estimating the number-weighted mean volume of each cell and, hence, the mean for the two cells. The intercepts in just one direction may be used (see text) or volumes can be estimated using intercepts passing in both directions from the point (see intercepts. 1+, solid arrowheads, and 1−, open arrowheads, on section C).

independent sections (Gundersen, 1988). Several different illustrations of the use of the nucleator are available (Gundersen *et al.*, 1988a; Møller *et al.*, 1990; Tandrup, unpublished). Møller and colleagues (1990) have justified some modifications which improve efficiency and are aimed specifically at neurons.

PARTICLE SPACING

The 3-D spatial relationships between different particles may be of experimental interest. For instance, the clustering of glial cells around neurons seems to be particularly prominent in aged human brains. However, the apparent distances between particles visible on 2-D sections can be very misleading (see, for example, Braendgaard & Gundersen, 1986). Fortunately, the nucleator method, using just one thick section, will yield information about the way in which particle number (and size) varies with 3-D location.

The method (Evans & Gundersen, 1989) requires the use of vertical sections or of sections which are IUR in 3-D. Recently, it has been applied to quantify peri-neuronal clustering of glial cells in human neocortex. Using vertical sections combined with optical disectors, neuronal nucleoli were selected. Estimates of distances were then made with reference to the centre of each selected nucleolus and to the glial cells whose clearest nuclear profiles occurred within the *same* disector pair of optical sections. On this sampling scheme, estimates of the numerical density, N/V, of glial cells occurring at specific distances from the neuronal nucleolus were obtained by measuring two distances from the centre of each glial cell nuclear profile: (a) to the centre of the neuronal nucleolus and (b) to the vertical axis which passes through the nucleolus. Further details and results will be found in Evans (1990) and Evans and Gundersen (1989).

ESTIMATING THICKNESS AND SHAPE

These terms refer to cortical thickness or depth and to a useful descriptor of brain 'shape'. The former is of interest because it is known that the depth of cerebral cortex within a brain varies widely with location. If a global quantity (e.g. arithmetic mean cortical thickness) is required, then all these locations must be sampled with equal probability. Shape provides a useful basis for drawing comparisons within brains (e.g. for studying lateral asymmetries) and between brains (e.g. changes due to ontogenetic, phylogenetic and gender differences).

Cortical thickness

On random sections, apparent cortical thicknesses tend to over-estimate true thicknesses because the cortex will be cut obliquely as well as perpendicularly. Indirect and direct solutions to this problem are possible.

An indirect approach for computing the average of all local thicknesses through the cortex (known as the arithmetic mean thickness, T_a) was used by Henery and Mayhew (1989). The method exploits information obtained already for cortical volume (estimated by the Cavalieri principle) and cortical surface area (estimated by vertical sectioning). In fact, two surfaces are determined, viz. those of the outer (pial) and inner (white matter) aspects of the cortex. A sampling scheme is devised which satisfies the sampling requirements for valid estimates of volume and surface. An estimate of T_a is calculated by dividing cortical volume by the mean of the two surface areas.

The direct approach uses test lines which are IUR in 3-D space. These test lines provide the basis for randomly sampling sites on the cortical surface (e.g. its outer aspect) from which intercepts can be drawn across the cortex (i.e. to its inner aspect) and their lengths estimated. Two sorts of intercept may be drawn. If the intercept follows the direction of the test lines, it is referred to as a *random* intercept. If each intercept is drawn to cross the cortex in a direction which is perpendicular to the outer aspect, these are referred to as *orthogonal* intercepts. Vertical sectioning with sine-weighted test lines provides a convenient way of generating random intercepts but is *not* permissible for producing orthogonal intercepts. For this, IUR planes generated by the orientator or by the isector will be necessary.

The arithmetic mean of random intercept lengths, divided by 2, yields an estimate of T_a (Gundersen *et al.*, 1978). For orthogonal intercepts, the arithmetic mean length, multiplied by $\pi/4$, provides a minimally biased and efficient estimate of T_a (Jensen *et al.*, 1979).

A 'shape' descriptor

A shape descriptor must be size-independent. A convenient way of achieving a dimensionless coefficient for monitoring brain shape (for cerebrum or cerebellum) is to exploit volume and surface area estimates, e.g. to raise the cortical (pial) surface area of the cerebellum to the power 3/2 and divide by cerebellar volume. This coefficient is constant for objects of the same shape and, provided that fixation distortions are uniform and concentric, is identical for fixed and fresh specimens. Again, these estimates can be made by designing a sampling scheme which combines Cavalieri sectioning with vertical sectioning (Henery & Mayhew, 1989; Mayhew *et al.*, 1990c).

This type of dimensionless coefficient is really a device for testing for isomorphic change rather than being a true shape descriptor. Objects of different shapes may share the same coefficient. However, mammalian brains are sufficiently similar in shape for the descriptor to be of real comparative worth.

Conclusions

Recent advances in stereology have made the efficient and unbiased estimation of structural quantities a practical reality. The methods are based on rigorous sampling which frees them from the constraints and dangers of making assumptions about object shape, size and spatial orientation. Their ability to provide 3-D spatial information makes them the only sensible option for analysing 2-D flat images, however these are generated. Their applicability to arbitrary objects makes them especially attractive for use in neuromorphometry. Indeed, in future studies on neurons, synapses and other neural elements there can be no excuses for adopting the assumption-dependent, inefficient and biased methods which our predecessors were obliged to employ.

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