

Design based stereological methods for estimating the total number of objects in histological material

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The principle that formed the basis of the most popular “assumption based” stereological methods for counting cells that were available prior to the advent of the more recently developed “design based” methods will be described in general terms. The major weaknesses inherent in the older methods will be described, along with how they have been eliminated by the design based methods.

key words: stereology, unbiased, design based, cell counting, disector

WHAT IS STEREOLOGY?

Stereology is a set of mathematical formulas that describe the interaction between geometric probes and various geometrical features (parameters) of structural entities (Fig. 1). In histological sections they can be used to make estimates of a number of geometrical features of the tissue, such as cell number, surface, length and volume [8,9]. Data of this type can be used to perform cross-sectional studies of structural changes related to development, ageing, evolution, pathology and toxic responses. During the last decades of the last millennium, a number of improved stereological procedures for making estimates of object number, length, surface and volume were described. One salient feature of the new stereological methods was that the probes and the sampling schemes used with the methods were designed so that the investigator did not have to make assumptions about the size shape, orientation or distribution of the objects of interest in the material, a major weakness of previously available methods [3].

DESIGN BASED AND ASSUMPTION BASED STEREOLOGY

Although these procedures were initially often referred to as “unbiased” methods, because the re-

sults were unbiased in the statistical sense, i.e. repetitive estimates made with the same method on average give the true number [17], they are perhaps best described as “design based”. The term “design based” is appropriate because the probes and the cutting and sampling of the histological sections are “designed” in such a way that no assumptions need be made about the object being analysed. Previously available methods, which in large part were based on modelled relationships between the number of objects embedded in a structure and the number of times 2-D sectional probes intercept the objects of known size, shape and orientation, are perhaps best referred to as “assumption based” methods. One often assumed, rather than determined or estimated, the size, shape, orientation and distribution of the objects of interest. This was primarily because it was difficult and time-consuming to actually determine the degree to which the “model” parameters were accurate representations of the true values of the parameters.

METHODS FOR COUNTING OBJECTS

The principle that underlies the most fundamental indirect technique for estimating the number of objects in a unit volume of tissue, N_V , is based on the relationship between the number of objects per unit

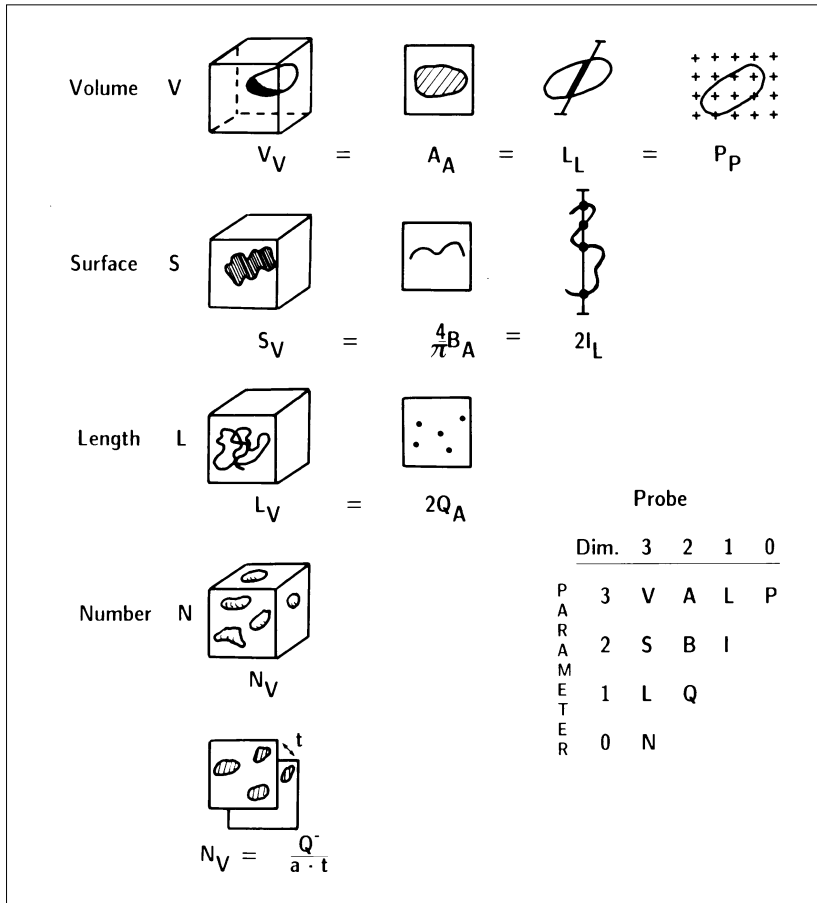


Figure 1. Diagram showing the relationship between the dimensions of stereological probes required to obtain assumption free estimates of structural parameters of various dimensions. Estimates of surface and length require uniform random position and isotropy of the probes relative to the structure, whereas those for volume and number require uniform random position only. Total Volume, V ; Surface, S ; Length, L ; and Number, N , can be estimated by multiplying the density parameters V_V , S_V , L_V and N_V by the volume of the region in which the measures were made. Volume: The top line shows the direct (assumption free) relationship between point ratio, P_P ; line intercept length, L_L ; area ratio, A_A , and the volume density, V_V , shown on the left. Surface: The second line shows the mathematical relationship between the number intercepts per unit length of test probe, $2I_L$; and between boundary per unit area of test probe, $\frac{4}{\pi} \cdot B_A$, and surface density, S_V . Length: The third line shows the relationship between the number of profiles per unit area of 2 dimensional probes (sections) and the length density, L_V . A tabular representation of the relationships between the dimensions of probes that produce assumption free estimates and structural parameters of various dimensions is shown on the lower right. Note that the sum of the dimensions of the parameters estimated, V , A , and L , and the dimensions of the probes that result in unbiased estimates is 3 or greater in all cases. It would therefore be expected that estimates of number, N , a zero dimensional parameter, can only be estimated by a 3 dimensional probe. The simplest three dimensional probe is a disector, so named because it is composed of two sections. Accordingly, N_V can be obtained without making any assumptions about the size shape or orientation of the objects being counted if one can define a unique point associated with each object and determine whether or not these unique points (e.g. the leading edge, Q^- , of an object that is present on one section and not the next) appear in the disector volume which is defined by the area of the sections, a , and the distance between them, t . With permission, Pergamon Press [16].

area of the sections, Q_A ; the mean height of the objects measured orthogonal to the sectioning plane, H ; and the thickness of the sections, h : $N_V = Q_A / (H+h)$ [2]. Note that this method requires either 1) a determination of H from serial constructions, in that it is not possible to measure H with a single section, or 2) an assumption about H , which historically has most often been the case and hence the name "assumption based" methods. $H+h$ is the average number of times that a profile of an object can be identified in a section series. In Figure 2, the mean

height, H , of the objects, orthogonal to the plane of sectioning, is approximately four section thicknesses (actually 3.94) and there are 326 sectional profiles. According to the formula presented above, $N_V = Q_A / (H+h) = 326/5$ (which is approximately 66). In this case the $V_{(REF)}$ would be the same as the volume sampled, i.e. the sum of the areas of all of the sections multiplied by the thickness of the sections, and **est** $N = N_V \cdot V_{(REF)}$, that is $66 = 66 \cdot 1$.

With this approach, the accuracy of the determination is dependent upon the accuracy of the geo-

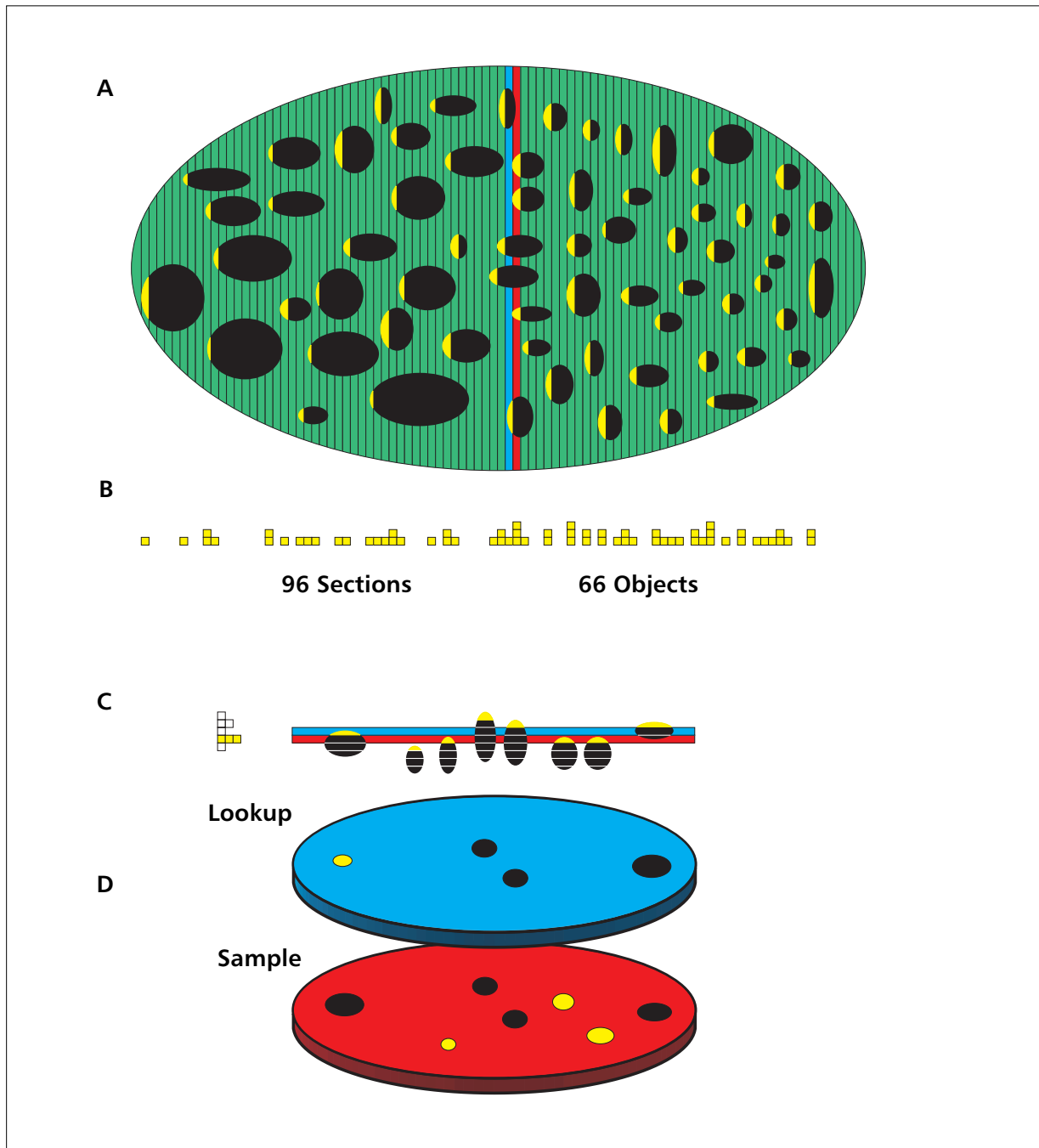


Figure 2. Assumption based and disector counting.

A) Representation of a green structure that contains 66 objects of different size, shape and orientation and that are unevenly distributed throughout the structure. The structure has been serially sectioned into 96 sections and is viewed orthogonal to the plane of sectioning. The number of objects can be determined by 1) counting the total number of sectional profiles that appear in the sections, 326, and dividing this number by the mean number of sectional profiles per object, 5, which gives approximately 66 objects (indirect or assumption based counting) or 2) counting the first profiles of the objects as they are encountered, that is the leading edges shown in yellow, as one proceeds sequentially through the series (design based or disector counting). B) A histogram in which the number of leading edges, small yellow squares, is plotted as a function of the position in the series where the first profile of an object appears when proceeding from left to right. Note that the distribution of objects is not even along the sectioning axis. C) A disector composed of two sections, the red and blue sections shown in (A) after being rotated 90 degrees. The objects that have sectional profiles within these sections are shown in their entirety. Using the leading edge counting rule, three objects are counted in the red section. D) An expanded view of the disector seen in (C) which shows the spatial positions of the sectional profiles. The red section is referred to as the "sample section" and the blue as the "lookup section". There are sectional profiles of objects (yellow) in the sample section that do not have sectional profiles in the "lookup" section. With permission, Elsevier [17].

metrical description of the objects. If an assumption about the H of the object is not accurate, the resulting determination of N will systematically deviate from the true number and, in a statistical sense, be biased. An error in measurement or false assumption about object H , comparable to one section thickness, will in this case result in a 20% bias, that is $est N$ will be $326/4$ or 82 rather than 66.

The new stereological methods eliminate the need for assumptions about the size, shape and orientation of the objects being counted. They do this through the use of a 3-D counting probe, the disector [15]. Unlike the assumption based approach described above, in which the numerical density, N_v , is derived from a model relationship between the number of object profiles counted on 2-D probes (i.e. sections), disector counting involves the direct counting of objects in a known volume of tissue (Fig. 2). In its simplest form, a disector is composed of two sections: a "sample section" and a "lookup section". The volume being probed by the disector is the product of the area of the "sample" section and the distance between the sections. The only requirements for proper use of the disector probe is that any object placed within the region of interest can be identified on at least one of the sections that pass through

the region and that one can identify sectional profiles that belong to the same object. The disector counting rule is then: An object is considered to be in a disector probe when a sectional profile of the object is apparent in the second section, the "sample section", and not in the first, the "lookup section", as one proceeds through the section series. Essentially what one is doing is directly counting the number of leading edges — "tops" — present in the volume defined by the disector. For any direction of sectioning, there will be only one leading edge for each object, regardless of the latter's size, shape or orientation. In order to determine which sectional profiles belong to the same object it may, in the case of branching objects, be necessary to have access to additional sections that are between and adjacent to the disector pair (pair).

While this method of counting has been discovered and rediscovered over the centuries [1], a relatively recent development has made disector counting feasible in histological tissue, in which the numbers of objects reaches thousand and millions. This is the unbiased areal counting frame (Fig. 3), which enables one to perform unbiased sub-sampling of sections that have large numbers of sectional profiles of objects of interest. While there are a number

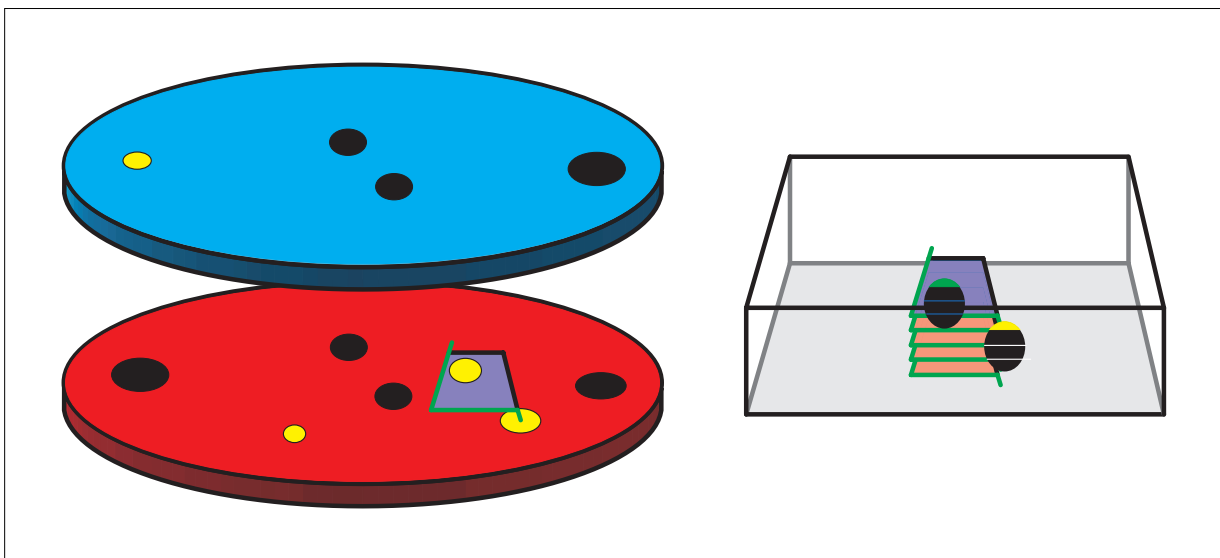


Figure 3. Subsampling sections.

A) A physical disector consisting of two separate sections. The small blue square in the sample section (red) represents an unbiased 2D counting frame that can be used to sample a limited part of the section. Profiles of objects that are either entirely within the frame or partially within the frame, but do not touch the green "forbidden" line, are sampled. (Not shown is the infinite extension of the forbidden line in both directions.) When disector counting rules are used, only one object is counted (yellow profile in upper left of frame). The volume of the disector is defined by the area of the counting frame and the thickness of the sample section. B) A diagrammatic representation of an optical disector. In this case, the counting grid is superimposed on an image of a thin focal plane that is moved a known distance through a thick section. An object is counted if its leading edge comes into focus within the counting frame, as the latter is moved through the section. The volume of the disector is defined by the area of the counting frame and the extent of the movement of the frame through the thick section. In this example, only one object is counted. With permission, Elsevier [17].

of ways to obtain unbiased counts of the number of profiles per unit area of a section, the most practical one is that described by Gundersen [6]. Accordingly, one samples, at random, an area of the test section with an unbiased areal counting frame. The profiles that lie partially or entirely within the frame and do not intercept the forbidden line (i.e. hyper plane that divides the sampling field) are defined as the objects that are to be "tested". One then applies disector counting rules to the profiles sampled by the frame and the corresponding part of the "lookup" sector. If the sectional profiles of an object that is "sampled" by the areal counting frame placed on the "sample" section does not have a profile in the "lookup" section, it is defined as an object that should be counted in the volume defined by the disector. In this case, the latter is a product of the area of the counting frame and the distance between the corresponding surfaces of the two sections.

A short time after the first descriptions of the application of disector counting rules to unbiased sub-samples of large sections, it became apparent that the sections used to define a disector need not be physically separate sections [7]. By adjusting the optics of the light microscope so that the depth of focus was minimised (i.e. opening the diaphragm of the substage condenser lens), it was possible to apply disector counting rules to optical sections positioned within thick sections. It was also possible to increase the volume of the sample by increasing the number of consecutive optical sections, so that a virtual "stack" of optical sections then defined the optical disector [18]. This probe was subsequently referred to as an optical disector and the original disector referred to as a physical disector, to distinguish between the two. The volume of an optical disector is then the product of the area of the unbiased areal counting frame and the distance between the corresponding surfaces of the upper and lower optical sections in the stack. Optical disector counting is performed by superimposing an unbiased areal counting frame on an image of an optical section and "moving" the counting frame a known distance, through the thickness of the section, with the focus control of the microscope (Fig. 4). An object is considered to be in an optical disector if it first comes into focus within the unbiased counting frame, as one focuses through the section. Objects in focus at the top of the optical disector are not counted because this represents the "look up section" of the first disector pair in the stack. They are counted at the bottom level, since this is the "test section" of the last disector in the stack.

The optical disector has a number of advantages over the physical disector when used at the light microscopic level. First and foremost, one's ability to find the corresponding parts of the sections that have to be compared, when applying the counting rules, is greatly simplified in that one only has to focus up and down to find the corresponding areas of the "sample" and "lookup" sections. This is a major problem when using physically separate sections that contain large numbers of profiles of the objects of interest. When using the optical disector it is also considerably easier to look at other "sections" when attempting to determine whether or not profiles of objects at one level belong to the same object. Unfortunately, the optical disector concept cannot be used at the electron microscopic (EM) level. This is because of the depth of focus of an electron image is very large (in the order of meters) and cannot be positioned or moved as a section within an EM section.

UNBIASED COUNTING METHODS ARE NOT ENOUGH

In order to obtain a truly unbiased estimate of total object number, it is not enough to just count the objects with disectors, it is also important that the sections used in the analysis and the positions on those sections to be sampled with disectors be chosen in a statistically unbiased manner (Fig. 5). In order to make this point clear, one should recall that there are two, basic, methods for making unbiased estimates of total object number, N , using optical disectors [15]. One is the "two step" method, which involves: a) estimates of the numerical density of objects, N_v , obtained from multiple optical disector samples and b) estimates of the volume of the tissue in which they are found, $V_{(REF)}$, which can be readily and efficiently obtained by point counting. According to the first method, $N_v \cdot V_{(REF)} = N$. The other method is the "optical fractionator" method [19], with which one counts with optical disectors the number of objects, ΣQ^- , in a known fraction, f , of the volume of the structure of interest. In this case, $\Sigma Q^- \cdot 1/f = N$. The proper implementation of both of these methods involves unbiased sampling at two additional levels of the sampling scheme. In order to make an unbiased estimate of cell number (i.e. an estimate obtained with a method that on average gives the true number) with either method, there must be a random selection of 1) the sections used in the analysis and 2) the positions on those sections that are sampled with the optical di-

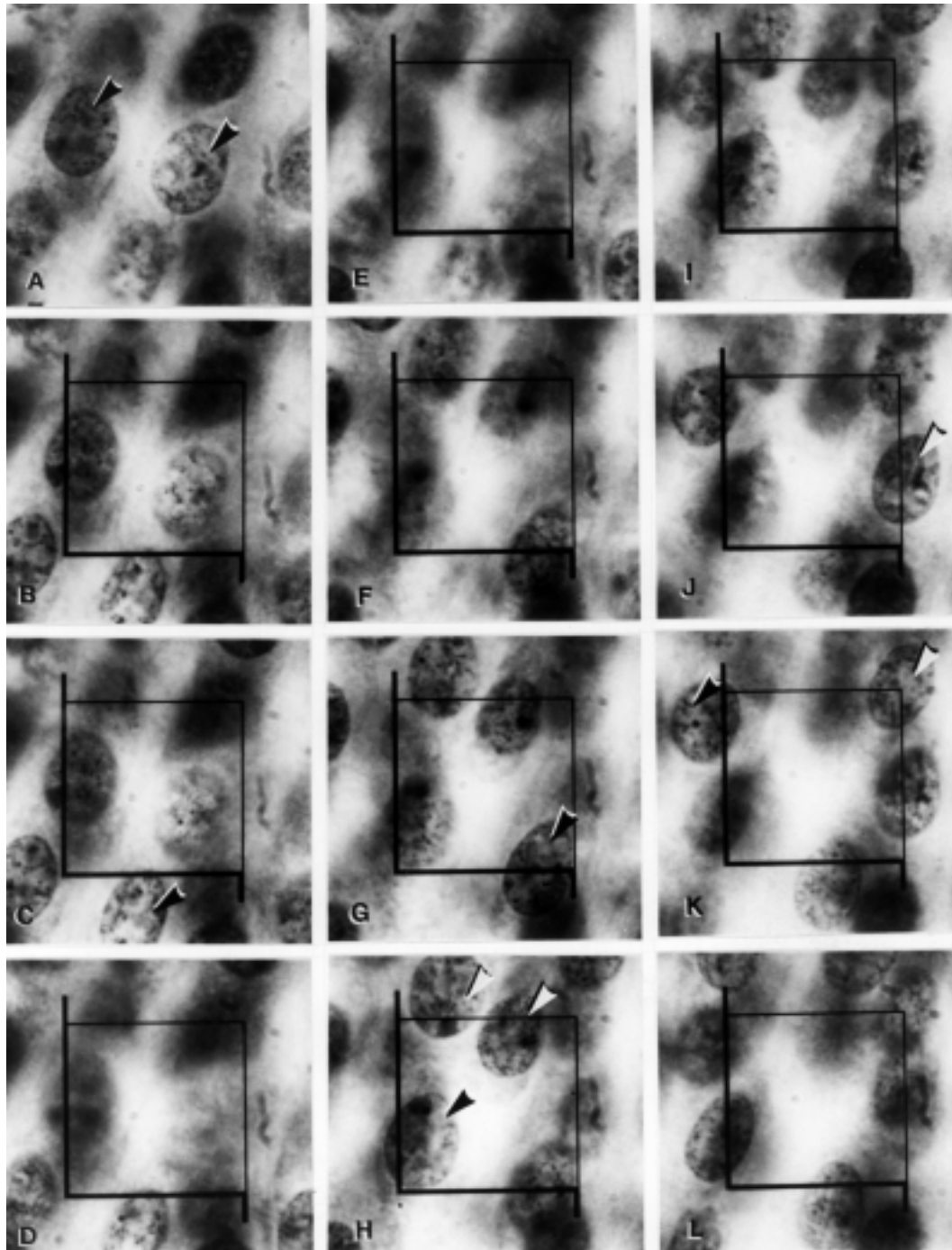


Figure 4. An optical disector. A stack of optical sections through the granule cell layer of the dentate gyrus of the human hippocampus, used to make an estimate of the numerical density N_V of granule cells with the optical disector technique. An unbiased counting frame of known area ($0.02 \text{ mm} \times 0.02 \text{ mm}$) is superimposed on an optical section obtained with a high numerical aperture oil immersion objective. Each optical section (A–L) is separated by 0.002 mm . Starting with the first lookup section, **A**, the nuclei sampled by the frame are counted as one proceeds to focus through a known distance of the section thickness. (In this example nuclei, rather than cell bodies, are counted because it is easier and because there is only one nucleus per granule cell.) The profiles of nuclei within the frame or in contact with the thin lines of the frame are considered to be inside the counting frame. Those touching the thick forbidden line are defined as being outside the frame. The two nuclei in focus at the top level of the optical disector, level **A** (black arrows are not counted because they do not come into focus as one proceeds to focus through the section). That is, the top of the disector is also a forbidden line. This point is emphasised by omitting the counting frame from this level. In this optical disector, 4 nuclei are counted (white arrow heads). Other nuclei that come into focus within the field but are not sampled by the optical disector are shown in black. Note that the bottom level of the optical disector is **K**. Profiles sampled at this level are counted, unlike those at level **A**. Level **L** has only been included to resolve ambiguities that may arise with branched objects (seldom the case with convex structures such as nuclei) and is not used for counting. With permission, Wiley-Liss [18].

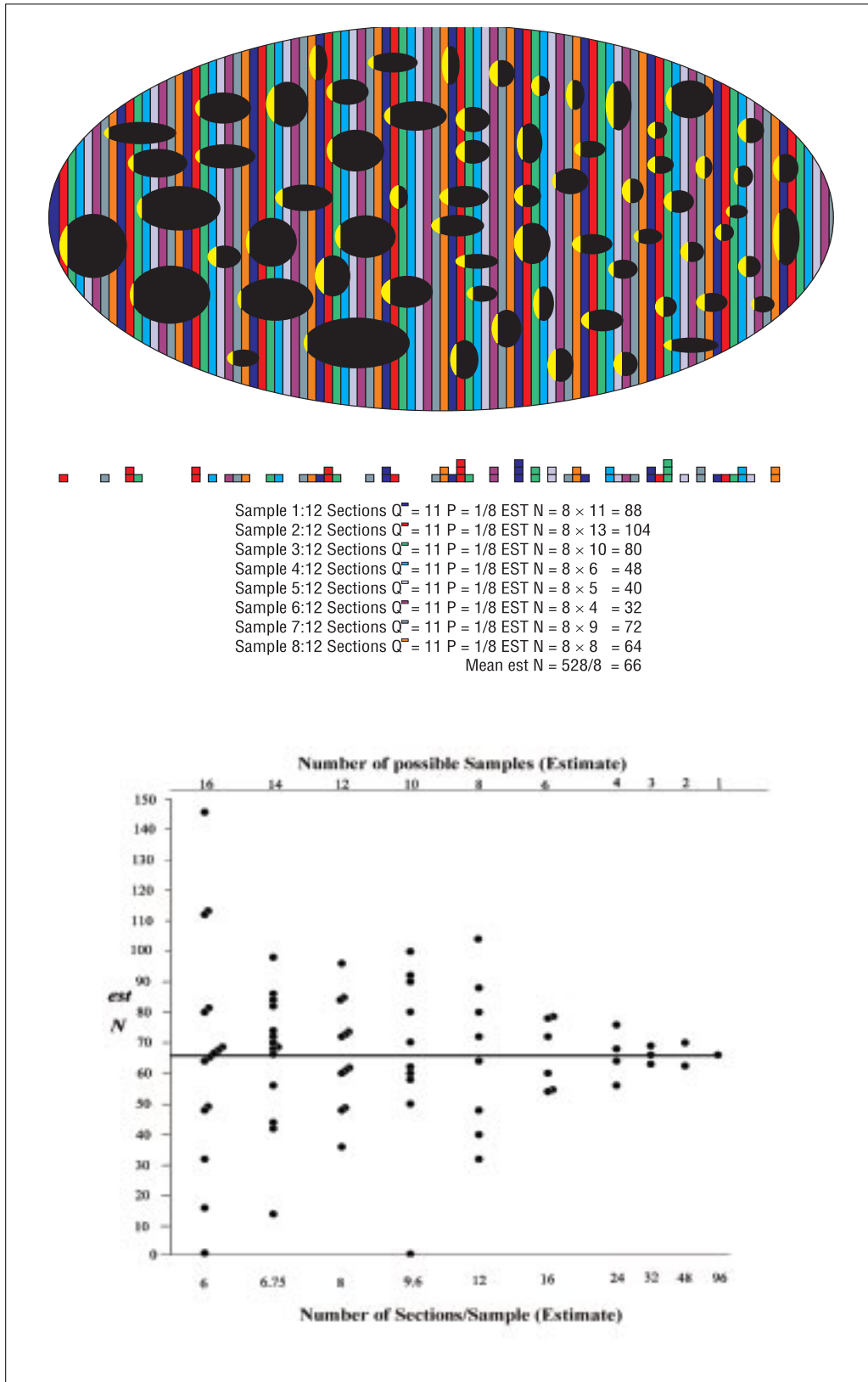


Figure 5. Unbiased Sampling. The 98 sections shown in Figure 1 are divided into eight systematic, uniform random, samples of sections. The sections in each sample have the same color. There are eight possible estimates of the total number of objects from this sampling scheme. If one further designs the sampling scheme so that all samples have the same probability of being chosen, i.e. $p = 1/8$, the estimation procedure can be considered to be unbiased, i.e. the mean of repeated estimates will be the true number. With permission, Elsevier [17].

sectors (Fig. 6). If this is not done at both levels, there are constraints with regard to the conclusions that can be drawn from the resulting estimate. For example if one uses a "standardised" section [10] or a set of sections taken from one end of the region of interest to make counts, the estimate can only be

considered to be representative of the entire region when, and only when, 1) the N_V estimated in that section is the same as the ratio of the total number of neurones to the reference volume, i.e. $N_{(TOT)}/V_{(REF)}$ and 2) the reference volumes, $V_{(REF)}$, are the same in all individuals. The same will be the case if one only sam-

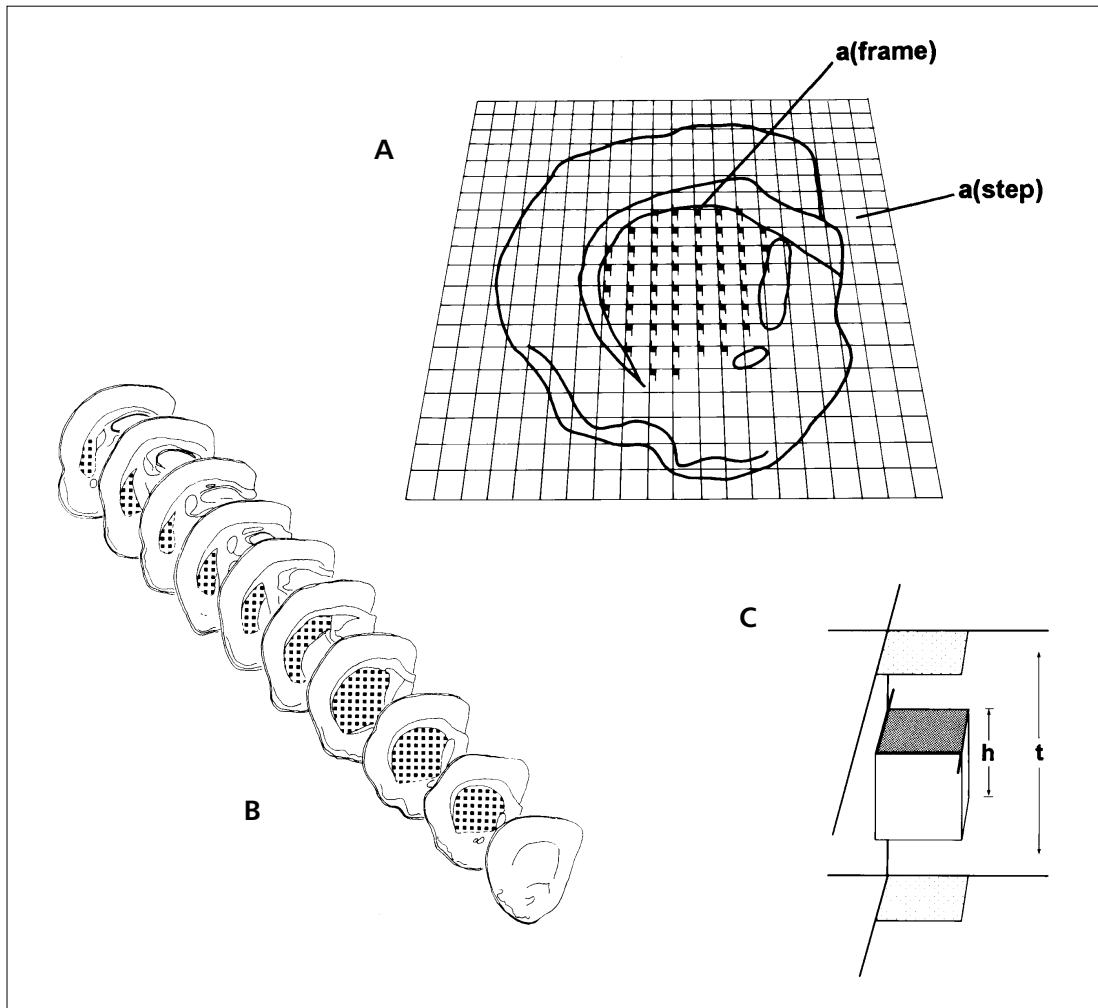


Figure 6. A diagrammatic representation of the optical fractionator sampling scheme for estimating total number of neurones expressing Somato-statin mRNA in the striatum of the rat (ref).

A) A systematic random sample of 10–13 sections that span the entire length of the striatum are selected for analysis. The sections are selected at equal intervals, i.e. every n th section after a random start within the first interval, to ensure that all parts of the striatum have equal probabilities of being in the sample. The selected sections therefore constitute a known fraction of the sections in the series, the *section sampling fraction (ssf)*.

B) The labelled neurones are counted under a known fraction of the section area, the *area sampling fraction (asf)*. This fraction corresponds to the ratio of the area of the disector counting frame, $a(frame)$ (shown here as small black rectangles), to the area associated with each step movement of the slide, $a(step)$ (shown here as large white rectangles); $asf = a(step)/a(frame)$.

C) The neurones are counted in optical disectors positioned in the central part of the section thickness. The *height of the optical disector, h*, constitutes a known fraction of the *section thickness (t)*. The ratio h/t is the *thickness sampling fraction, (tsf)*. The area of the counting frame, $a(frame)$, is shaded.

After systematically sampling at all levels, one has directly counted the number of neurones ΣQ^- in a known fraction of the region of interest without having to make assumptions about the size, shape and orientation of the objects. The sum of the number of neurones in the disectors, ΣQ^- , times the product of the inverse of the fractions, constitutes an unbiased estimate of the total number of labelled neurones in the striatum.

$$est N = \Sigma Q^- \cdot 1/ssf \cdot 1/asf \cdot t/h.$$

Note that the volume of the structure and the numerical density are never estimated. With permission, Wiley-Liss [20].

ples on one edge or side of the region of interest. Without *a priori* knowledge about $N_{(TOT)}/V_{(REF)}$, this approach would fall into the category of “assumption based” methods because the validity of the resulting data is dependent upon the validity of the assumption about N_V , stated above and the assumption about the reference volumes being the same in all individuals in the study. In the biological world, these assumptions are generally weak and must be discussed openly in the discussion of any data of this type.

The potential biases inherent in the assumption based approach described above can be eliminated by designing the selection and sampling of sections in such a manner that one does not have to make assumptions about the distribution of the objects of interest. As already alluded to above, the assumption about N_V being the same as the ratio $N_{(TOT)}/V_{(REF)}$ can be eliminated if the sections and the positions within the sections sampled by disectors are randomly sampled. That is, one uses a method of selecting: 1) the sections from all of the sections that pass through the region of interest, and 2) the positions within the sections so that all parts of the region of interest (i.e. along all three spatial axes) have equal probabilities of being sampled. The random selection and sampling procedures can be either independent random or systematic random. Systematic random is preferred because in general it is more efficient and more readily applied to histological preparations because they are cut along one axis. Figure 6 depicts the application of such a sampling scheme. A scheme for the unbiased sampling of structures that can only be identified at the electron microscopic level (e.g. synapses) can be found in Guinisman et al. [4].

An appreciation of the difference between the recently developed “design based” methods and previously available “assumption based” methods can help the investigator of structural dynamics to understand why the newer “designed based” stereological methods provide more robust useful data. This is true for methods for counting as well as those for making global and local estimates of length [13,14], surface [5] and volume [11].

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