CHAPTER 1

Introduction to Stereology

JUST AS ASTROLOGY BECAME ASTRONOMY and alchemy became chemistry through the application of mathematics, descriptive anatomy can be expected to become more and more quantitative in nature. With precise mathematical descriptions such as those that can be obtained with unbiased stereological techniques, it will be possible to make concise descriptions of the relationships between structure and function, of the dynamics of structure, and to reassert quantitative morphology as an essential part of the evaluation of biological tissues.

1.1 WHAT IS STEREOLOGY?

Measures of structural features such as volume, surface, length, and object number can be used to make quantitative statements regarding function that are useful in comparative and experimental studies of tissues and organs. Because most structures of interest to the biologist are three-dimensional (3D) and opaque, structural features are best visualized on two-dimensional (2D) images or sections through the structure. However, the generation of 2D images results in the loss of information. As a consequence, the relationship between the structural features in the sections and the 3D features in the tissue is not readily apparent. Solids become profiles, surfaces become lines, linear features become points, and objects become an unpredictable number of sectional profiles (Fig. 1.1).

However, it is possible to derive meaningful statements regarding the quantities of these structural parameters in three dimensions by sampling and measuring structural features present in the sections. This is achieved by using mathematical relationship equations that take into account the pertinent structural parameters that are lost during the generation of sections. Stereology is a methodology that does just this. Stereology provides meaningful quantitative descriptions of the geometry of 3D structures from measurements that are made on 2D images (Weibel 1979; Cruz-Orive 1997; DeHoff 2000).

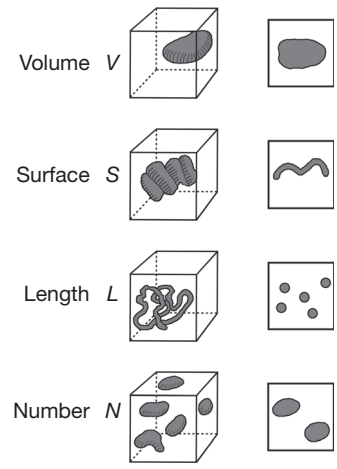

FIGURE 1.1 Structural information is lost when embedded structures are sectioned. Volumes appear as areas, surfaces appear as lines, and linear features appear as intersections. Object number becomes obscure. *Stereology* is a set of methods that allows the application of mathematical rules to information present in 2D sections. The application of these rules makes it possible to work back to a quantitative measure of a structural parameter in 3D space.

Seldom, if ever, is it necessary to use these methods to make an actual determination of the total amount of any structural parameter. Doing so is both time-consuming and unnecessary. In the vast majority of cases, estimates—that is, approximations with statistically defined margins of error—are sufficient to achieve the goals of a study. As discussed in more detail below, the margins of error of stereological estimates can be controlled by the amount of sampling that is performed. Increasing the amount of sampling will decrease the margin of error of an estimate. This is completely analogous to the results of public opinion polls, with which one refers to an "estimate" and a "margin of error."

There is a hierarchy of levels of sampling that ranges from groups, to individuals, to sections, to the actual stereological probes that are used to make the measurements from which the estimates are derived. In Chapters 7 and 10, it is shown how it is possible to analyze this hierarchy in order to determine how much sampling is necessary for optimal stereological estimates, that is, how many animals, how many sections, and how many measures are required to obtain an estimate that has enough precision to realize the goal of a study, but not more.

1.2 DO NOT SECTION YOUR MATERIAL BEFORE YOU HAVE READ THIS BOOK

The first critical step in the sampling of the structure of interest at the level of individuals is the sectioning of the tissue. For the estimates of any particular parameter to be meaningful, they have to be representative of the entire structure. This means that the structure of interest has to be sectioned in such a manner that all parts of

BOX 1.1 Isotrophy

Surfaces and lines that are equally oriented in all directions in 3D space have isotropic orientations. Spaghetti in a box at the store has a preferred orientation and an anisotropic orientation. Spaghetti that has been boiled and thoroughly mixed has no preferred orientation and an isotropic orientation.

that structure have equal probabilities of being present in the sections that are to be used in the analysis. That is, one needs a representative sample of the sections from the entire structure. One does not need to collect or prepare all of the sections from a structure in order to perform a stereological analysis. However, for all parts of a structure to have equal probabilities of being present in the sections, one does need to have access to the entire structure of interest at the start of the analysis to collect and prepare a representative sample of sections.

Another important consideration when sectioning the structure of interest is the orientation of the sections in the tissue being analyzed. As discussed in Chapter 4, unbiased estimates of length and surface are orientation sensitive. Both length and surface features may have preferred orientations in 3D space, that is, they may not be isotropic (see Box 1.1). In that biological structures are seldom if ever isotropic, it is necessary to ensure that the probes interact isotropically with length and surface features in order to produce unbiased estimates of length and surface. In Chapters 5 and 6, methods for sectioning and analysis that ensure the isotropic interaction of length and area features and probes are described.

1.3 GEOMETRICAL PROBES INTERACT WITH FEATURES TO PRODUCE EVENTS

At the level of sections, stereology involves sampling structural features of interest with geometrical probes that are sensitive to the structural features of interest. A geometrical probe is a geometrical construct characterized by its dimensions. There are four basic types of probes that can be used to sample structural features in sections (Fig. 1.2):

- A point is a zero-dimensional probe. A point is characterized solely by its position and has neither direction nor orientation.
- A line is a one-dimensional probe and is characterized by both its position and its orientation in 3D space.

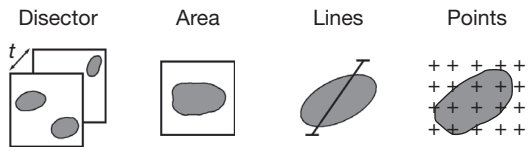

FIGURE 1.2 Examples of geometrical probes. (*Right to left*) Point probes on a section, line probes on a section, area probes or sections themselves, and a pair of sections that defines a volume probe.

- A section is a 2D probe (area) that is characterized by its position and its orientation in 3D space.
- A 3D probe can be constructed by using two sections, hence the name *disector*. The distance between the sections and the area of the two sections define the volume of a disector probe. Disectors are characterized by their position in 3D space and are not orientation sensitive.

Because point probes and disector probes are not direction sensitive, the tissue to be analyzed with these probes can be cut in any direction. This is not necessarily the case for estimators of length and surface area, as described in Chapter 4.

1.4 PROBES AND MATHEMATICAL RELATIONSHIP EQUATIONS

The interaction between probes and particular structural features results in *events* that can be related to the quantity of a particular feature through *mathematical relationship equations*. These equations relate probe/feature interactions on sections to corresponding geometric properties in 3D space. Although the derivation of the mathematical relationships often can be complex and not intuitively apparent, the equations themselves are relatively simple and the calculations involved in quantifying the most complex structural parameters are straightforward (Jensen and Gundersen 1989; DeHoff 2000).

Examples of probe interactions and relationship equations for volume estimates can illustrate this point (Fig. 1.3). When randomly chosen sections that pass through an object contained within a structure are probed with a series of points, the ratio of points that interact with (hit) the sectional profiles of the object, to the total number of points probed on the sections P_P will, on average, be the same as the ratio of the volume of the object to the volume of the entire structure V_V.

Similarly, the ratio of the length of linear probes that interact with (i.e., lie over) the sectional profiles of an embedded object, to the total length of the linear probes L_L, on average, will be equal to the ratio of the volume of an embedded object to the volume of the structure that contains the object V_V. One stipulates that the relationships hold

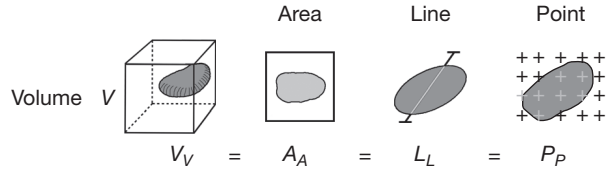

FIGURE 1.3 Mathematical relationship equations for interactions between probes of various dimensions and a sectional profile of an object that allow the estimation of the volume of the object. (*Right* to *left*) The ratio of the points hitting the profile of the object on a section to the total number of points hitting the region of interest P_P is, on average, equal to the ratio of the volume of the object to the volume of the structure in which it is embedded, V_V. The ratio of the length of randomly oriented lines that lie over sectional profiles of objects to the total length of the line probes L_L is, on average, equal to V_V. The ratio of the area of a sectional profile of an object to the area of the section A_A is, on average, equal to V_V.

"on average," because this may not be so for any one sample, but will approach the true value as the amount of sampling is increased.

These relationship equations are not recent developments. The proof of the relationship $V_V = A_A$ was described more than 150 years ago (Delesse 1847). The $V_V = L_L$ relationship was first described more than 100 years ago (Rosiwal 1898), and the $V_V = P_P$ relationship is more than 80 years old (Thomson 1930).

Mathematical relationships also exist between specific probes and the other structural parameters (Fig. 1.4). As indicated in the second line of Figure 1.4, surfaces appear as lines in sections. The interaction between line probes and the linear representation of the surface in the section results in *intercepts*, *I*. There is a well-defined relationship between the number of intercepts, per length of probe, and the amount of surface per volume sampled. Two times the number of intercepts, per length of probe, $2I_L$, is, on average, the surface area per unit volume, S_V (Smith and Guttman 1953; Baddeley et al. 1986). It is also possible to use the ratio of the length of the border of the sectional profile of a 3D surface feature *B* (border) to the area of the section sampled *A* to estimate the surface per unit volume, S_V, that is, $S_V = 4/\pi \times B_A$. Note that it is not possible to use point probes to measure linear features (Fig. 1.4, line 3). Points do not interact with lines, because neither mathematical lines nor points have width. The chance that a point falls onto a line is therefore infinitely small.

As pointed out above, length and surface area are direction-sensitive parameters. As a result, this relationship is only valid if one ensures an isotropic interaction between probe and feature. Because one wants to avoid making any assumptions regarding the orientation of linear features (to avoid biases in the estimate), one must either cut the tissue or orient the probes in a manner that ensures that this happens. Different techniques for achieving this are described in Chapter 4.

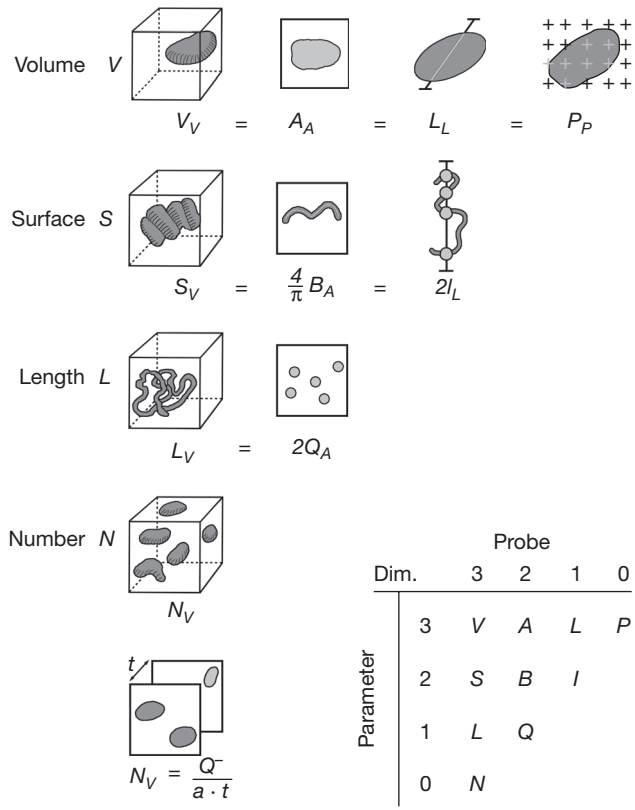

FIGURE 1.4 Diagram showing the mathematical relationships between 3D structural features of objects (left column) and the interaction of probes of various dimensions, with features of the object apparent in sections. (*Lower right*) A table showing the dimensions of the probes that can be related to structural parameters of varying dimensions: (*V*) Volume, (*A*) area, (*L*) length, (*P*) points, (*S*) surface, (*B*) boundary, (*I*) intercepts, (*Q*) cross sections, (*N*) number. (Redrawn from West 1993.)

The third cardinal structural feature, length, can only be analyzed with area probes (Fig. 1.4, line 3). Linear features appear on sections as cross sections or points that have no dimensions. As a consequence, linear features interact with neither point nor line probes. Although the derivation of the relationship between intercepts per unit area of sections Q_A and length per volume L_V is somewhat involved (Smith and Guttman 1953), the mathematical relationship between the number of times linear features intercept the planar probe Q (see Box 1.2) is simple and calculations are straightforward, $L_V = 2Q_A$.

BOX 1.2 *Q* is for cross section

The use of the letter *Q* to refer to the intercepts of linear features with an area probe originates from the German term *Querschnitt* originally used by Weibel in his pioneering work involving the application of stereological methods to biological tissue (Weibel 1979). This term is widely used in the stereological literature, which we have made an effort to respect in order to support a standardized terminology.

Again, this relationship is only valid if one ensures that the interaction between the area probe and the linear feature is isotropic.

1.5 THE DISECTOR: A 3D PROBE

Object number *N*, a zero-dimensional structural parameter, is unique in that it cannot be derived from the information on a single, 2D section without additional information regarding the size, shape, and orientation of the objects. It can, however, be derived from information present in two sections. Two sections constitute a 3D volumetric probe referred to as a *disector*. The volume of a disector is defined by the area of the sections and the distance between the sections. By counting unique points associated with objects that lie within the volume defined by the disector, it is possible to directly count objects in a known volume of tissue.

Estimating object number with disector probes requires no information regarding the size, shape, or orientation of the objects. In the simplest case, this involves determining whether there is a profile of a specific object in one section of an adjacent pair and not in the other. If so, the leading edge of the object must be in that sample. The leading edge is a unique point on every object, regardless of its size, shape, and orientation. This method of counting was popularized in the 1980s by Gundersen and coworkers (Sterio 1984; Gundersen 1986), although its origins can be traced back to the earlier 20th and late 19th centuries (Bentsen and Nyengaard 1998). Chapter 3 introduces the counting of objects in sectioned tissue and the use of the disector probe.

As described above and depicted in Figure 1.4, the number of different probes that can be used on sections to analyze different structural features decreases as the dimensions of the features decrease. Volume, a 3D parameter, can be probed with points, lines, and areas. Surface, a 2D feature, can be probed with lines and areas. Linear features can only be probed with area probes. From this relationship between probe dimension and parameter dimension, one can conclude, on an empirical basis, that the sum of the dimensions of the probe and the dimensions of the structural

feature must sum to three or more in order to be able to make an analysis that does not involve assumptions regarding the size, shape, or orientation of the structures. It can therefore be expected that assumption-free estimates of object number, a zero-dimensional parameter, require the use of a 3D probe (Cruz-Orive and Weibel 1990).

1.6 WORKING ON THE EDGE: CHOOSING A PROBE

From Figure 1.4, which depicts the relationships between probes and structural parameters, it becomes apparent that all structural parameters can be analyzed by counting events. For volumes, one can count points that lie over profiles. For areas, one can count the intercepts between line probes and linear profiles of surfaces. For lengths, one can count the number of cross sections of linear features on the surface of an areal probe. For object number, one can count the number of unique points associated with objects within a volume of known size. All of the probes depicted in Figure 1.4 can be used to obtain unbiased, assumption-free stereological estimates. However, choosing probes that permit one to count events is generally a more efficient way to collect data, in that they involve all-or-none decisions. As described later in this book (Chapter 10), sampling rarely involves recording more than 150 events in an individual. Once the material is prepared, this can be accomplished in very little time by working on the edge of the scheme presented in Figure 1.4, that is, by counting events.

The choice of the probe and the design of the sampling scheme are critical aspects of designing stereological studies. Because stereology involves sampling, the application of this methodology generally results in estimates of particular parameters, that is, approximations that have a margin of error. If the probe is appropriate for the parameter of interest and the sampling is performed in a representative manner, the estimates can be considered to be unbiased, that is, they will arbitrarily approach the true value of the parameter as the amount of sampling is increased.

1.7 ONLY TOTAL QUANTITIES WILL DO: BEWARE THE REFERENCE TRAP

From the preceding, it can be appreciated that the volume density V_V, surface density S_V, length density L_V, and numerical density N_V can be estimated in an unbiased fashion with data collected from relatively simple geometric probes. Although density measures may be used for intraorgan analyses, they are of limited value in comparative and experimental studies. This is because they are ratios: volume per unit volume, surface per unit volume, length per unit volume, and number per unit volume. Each has a numerator (number, length, surface, volume) and a denominator (volume). As such, they cannot be used to evaluate differences in total amounts

of the structural parameters, without making assumptions regarding the volume of the structure in which the densities were estimated. For example, there can be a decrease in the number of cells and a proportionate decrease in the volume of the region of interest without a change in the number of cells per unit volume. A nice example of the impact of changes in volume on density measures has been reported by Yamamura et al. (2011), who observed increases in the number of cells yet decreases in cell density in some of their experiments. Drawing conclusions regarding total amounts of specific parameters from density measures without taking into consideration the reference space can lead one into the "reference trap."

There are two methods by which the density measures can be used to obtain estimates of the total amounts of the volume, surface, length, and number of particular features. Total values can be obtained by multiplying the volume density of a particular parameter by the volume of the structure in which the densities were estimated, V_{REF} (Fig. 1.5). The volume of the structure in which the estimates are made, referred to as the reference volume V_{REF}, can itself also be estimated with unbiased stereological techniques. This is most readily achieved with point-counting techniques.

The other way in which the volume densities can be used to estimate the total amount of a particular parameter is to use the fractionator principle (Gundersen et al. 1988a; West et al. 1991). One first determines the fraction $1/k$ of the reference volume sampled and then multiplies the amount of a particular parameter measured in that fraction by the reciprocal of the fraction (Eq. 1.1). For example, if one counted all the cells in one-tenth of all the sections, an estimate of the total number N_{TOTAL} would be 10 times the number of cells counted, provided disector counting was used and the sections were chosen at random:

$$N_{TOTAL} = \text{sum counts} \times k. \tag{1.1}$$

In addition to providing unambiguous data that are easy to understand and can be readily related to function, total amounts are also amenable to straightforward statistical analyses.

The main focus of this book is on estimators of total number, length, surface, and volume, which can be referred to as "global" estimators. There are other categories of stereological estimators, however. These include "local" estimators (Chapter 12) and "second-order estimators" (Chapters 7 and 10).

$V_{TOTAL} = V_v \times V_{REF}$
$S_{TOTAL} = S_v \times V_{REF}$
$L_{TOTAL} = L_v \times V_{REF}$
$N_{TOTAL} = N_v \times V_{REF}$

FIGURE 1.5 Total quantities of a specific structural parameter can be obtained by multiplying the volume density by the volume of the structure of interest or reference volume V_{REF}.

1.8 STEREOLOGY IS SAMPLING

Stereology involves sampling sections with probes. When designing a stereological study, it is essential that the selection of the sections and the selection of the positions in the sections to be probed be performed in a statistically representative manner, that is, all parts of the structure of interest have an equal probability of being sampled. This is much like conducting a public opinion poll in which everyone in the population of interest has to have an equal probability of being polled even though not everyone will be polled. One of the ways in which this can be achieved in histological material is to make systematic random samples of sections and positions on the sections.

On first encounter, the concept of a systematic random sample may appear to be oxymoronic and counterintuitive. What it means, however, is that the set of sections to be collected for the analysis is spaced at equal intervals throughout the entire region of interest, and, most importantly, the first section in the set is randomly positioned within the first interval. This is analogous to slicing an egg with an egg slicer after positioning the egg in a random position in the slicer. This method of sampling ensures that all positions along the sectioning axis have an equal probability of being sliced. The same holds true for the systematic sampling of the 2D sections with probes. In this case, a coordinate system of a grid can be used to specify the positions of the probes after the grid is randomly positioned on the section (Fig. 1.6).

Systematic random sampling is well suited for stereological analyses of histological material, because the material is most often sectioned along one axis of the tissue. It also has the advantage of being more efficient than independent random sampling, that is, when each section is selected independently of the other sections (Chapter 8). Systematic sampling is presented as an integral feature of most of the estimation procedures described in this book. The efficiency and precision of systematic random sampling are discussed in detail in Chapter 7. The amount of sections and probes that should be used in an analysis is discussed in detail in Chapter 10.

1.9 LOCAL ESTIMATORS

Local estimators involve the estimation of features such as the volume or surface of individual objects such as cells (Gundersen et al. 1988a; Jensen 1998). They involve both linear and surface probes and are subject to the same constraints as global estimators that are orientation specific. They also require the use of representative sampling. In Chapter 12, methods for estimating the mean and distributions of these parameters for populations of small objects are presented.

Another group of estimators is often referred to as second-order estimators. Although they are based on many of the same principles when probing and sampling to obtain global estimates, they do not deal with the cardinal structural parameters.

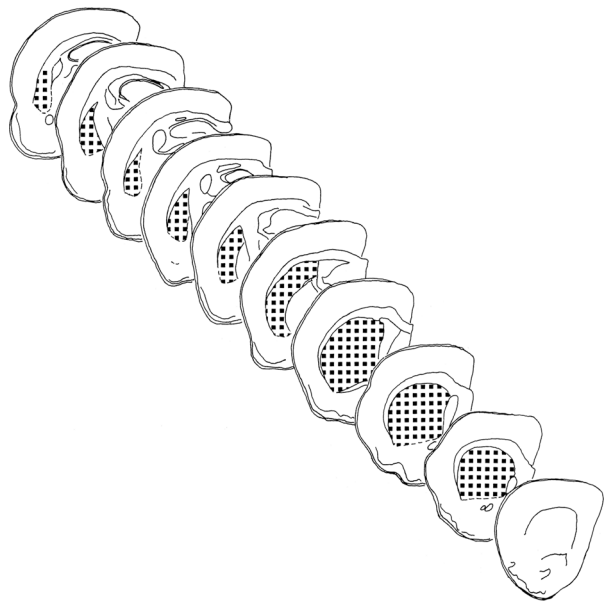

FIGURE 1.6 A diagram of a systematic random sample of 10 sections through one side of a rat brain showing a systematic random set of sampling positions within the striatum (dots). (Redrawn from West et al. 1996.)

Methods for measuring the precision of estimates, that is, the size of the margin of error, are often considered to be second-order estimators. Considerable attention is paid to these methods in Chapter 7, because they are essential when designing and evaluating sampling schemes. Other examples of second-order estimators include measures of spatial distributions or nearest-neighbor analysis (Evans and Gundersen 1989) and connectivity (Gundersen et al. 1993).

1.10 UNBIASED STEREOLOGY: DESIGN-BASED VERSUS MODEL-BASED METHODS

In the preceding, the term *unbiased* has been used to refer to the estimates obtained with the stereological methods on which this book focuses. It is important to point out here that this term is used in the statistical sense and not the pejorative sense. It is used to refer to procedures that result in estimates that approach the true value as the amount of sampling increases. Returning to the analogy with public opinion polls, the margin of error of the estimate will decrease as increasing numbers of people are polled, until all persons are polled and no margin of error exists, that is, when one has

actually made a determination of the true value. The unbiased nature of the estimation procedures described in this book is their most salient feature. Unbiased estimates require the use of unbiased probes and unbiased sampling. This, in turn, means that probes and the sampling have to be designed so that their application will result in values that will approach the true value of a particular parameter as sampling is increased.

As a consequence, it is desirable to use procedures that do not require additional information regarding the size, shape, or orientation of the structures being quantified. One wants to use probes and sampling procedures that are designed to be independent of variations in the size, shape, orientation, and distribution of structural features. These methods are therefore often referred to as design-based methods and are the main focus of this book (Gundersen et al. 1988a,b).

There is an alternative group of stereological methods that is based on the modeling of structural features. Modeling requires a priori knowledge of some aspect of the structural organization. Historically, one of the most popular stereological techniques has been the Abercrombie method for counting cells (Abercrombie 1946). This is a model-based method. Accordingly, one converts the number of sectional profiles of objects to the number of cells by knowing the relationship between section thickness and the average height of the objects. When one knows how many profiles correspond to one object, and therefore has a correct model, this method can be used to make accurate estimates of object number from sectioned material. The difficulty with this method lies in obtaining information regarding object height. To be used properly, this parameter must also be modeled or validated for each sample, which is difficult and time-consuming (West 2002). As a result, object diameter, which is easier to measure, has often been used to model object height (or number of sectional profiles per object). Deviations from the underlying assumption of the model—that objects are on average as high as they are wide—has the potential to lead to biases, that is, systematic deviations from the true values that do not decrease as the sampling decreases.

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