Modern Stereology: A Method for Quantifying the Number of Cells in Histological Sections

Quantifying the number of cells in particular regions of the brain poses certain challenges because cells are numerous, microscopic, and exist in 3-D space. This means that not all cells can be counted, special equipment is required, and cells can only be examined in 2-D planar sections. What is the best way then to determine the total number of cells (or nuclei, synapses, etc.) in a given structure or region of the brain? The field of stereology has developed to address this and similar questions.

Historical background to stereology

The term stereology is derived from the Greek word stereos, meaning solid, and consists of a set of techniques for quantifying the properties (eg. number, length, volume, etc.) of 3-D objects based on their appearance on 2-D sections.[1] Stereology became a formal discipline (complete with its own society—the International Society of Stereology) in the 1960s, and has progressed alongside advances in microscopy and computing. There is a distinction between older stereological methods, which are based on classical geometry, make assumptions about specimens (eg. cells are spherical), and often employ correction factors (eg. the Abercrombie correction[2]), and more modern stereological methods which do not make such assumptions or use correction factors. Since properties of biological tissue rarely conform to perfect geometric shapes, the assumptions of older methods can be unwarranted and can lead to inaccurate estimates (see [1,3-5] for reviews). These older methods have therefore come to be known as biased stereology, while the newer methods are referred to by a variety of names, including unbiased, assumption-free, model-free, design-based, or simply, the new stereology.

Modern stereology

Modern stereological methods typically use a light microscope attached to a motorised stage, a microcator to determine distances in the z-axis, and a digital camera, which are all connected to a computer running commercially available stereological software. The general approach is to sample regions in 3-D space, determine the number of cells in these samples, and then scale this up to estimate the total number of cells in the structure of interest. Estimating population parameters from samples is at the heart of statistical inference and underlies much of the theory of modern stereology. There are three main strengths of this approach. The first is that no assumptions are made regarding the geometry (eg. size, shape, and orientation) of cells, thus eliminating potential biases if these assumptions do not hold. The second advantage is the use of systematic random sampling, which ensures that each cell has an equal probability of being sampled, and thus the sampling procedure is unbiased, in the statistical sense. The third advantage is the use of a 3-D optical probe which ensures that each object is counted once and only once, and that larger cells have the same chance of being counted as smaller cells.

The two-stage method for determining cell number

There are two different methods of determining total cell number, and the most common is the *Nv* x *Vref* or two-stage method first used by Pakkenberg and Gundersen.[6] The logic behind this method is to (1) count the number of cells in a known volume of tissue, then (2) determine the total volume of the structure or region of interest, and (3) multiply these values together to determine the total number of cells in the structure (see equations below). Note that this number is the *total number of cells* in the structure, and is not a density measure (ie. number per unit of volume).

First, brains are sectioned in the standard way (Figure 1A) and equally spaced sections are selected, for example every 6th section or 12th section, with the first section being chosen randomly. After standard histological staining, for example with Cresyl violet (CV), the structure of interest—the striatum in this case (Figure 1B)—is outlined under low power on all the sections on which it appears, and the area of the outlined region is calculated by the software. The software then selects locations within the highlighted region in a systematic random manner (indicated by the black dots). Switching to a higher power objective lens, the software automatically moves the stage to the first location and the user counts the number of cells falling completely within the box (Figure 1C), or cells touching one of the two green lines. Cells falling partially in the box but touching the red lines are not counted, which ensures that the number of cells is not overestimated. The user needs to scan through the depth of the section at each location to ensure that all cells enclosed by the box in x-y-z planes are counted. The stage then moves to the next location (black dot) and another cell count is made. This continues until all areas have been counted, and this procedure is then repeated on the other side of the brain and on all the sections on which the striatum appears. To calculate the total number of CV stained cells (*N*) in the mouse striatum, the number of cells that were counted across all sections is divided by the number of boxes times the area and height of the boxes, and this is referred to as the numerical density (*Nv*; Equation 1). The area and height are constants; the area is determined by the user at the beginning of the experiment while the height is equivalent to the thickness at which the sections were cut. The volume (*Vref*) of the striatum can be calculated by summing the areas of the outlined structures, multiplying by the reciprocal of the sampling fraction (ie. if every 12th section was used, then multiply by 12), and the thickness at which the sections were cut (Equation 2). The final step is to multiply *Nv* by *Vref* to give the total number of cells in the striatum (Equation 3).

$$N_v = \frac{\sum (\text{Number of cells counted})}{\text{Number of boxes} \times \text{Area of box} \times \text{Height of box}} \qquad (Eq.\,1)$$

$$V_{ref} = \sum \text{Structure areas} \times \frac{1}{Freq} \times \text{Section thickness} \qquad (Eq.\,2)$$

$$N = N_v \times V_{ref} \qquad (Eq.\,3)$$

Drawbacks and controversies

Despite the advantages of modern stereological methods they have some disadvantages. They can still lead to biased estimates,[7] and vastly different estimates of neuron numbers have been obtained for the same structure in the same species, using the same ostensibly unbiased method (eg. 80,000 vs. 205,000 hippocampal CA1 neurons per half mouse brain).[8,9] In addition, modern methods have some assumptions as well which are occasionally not met in practice, such as 100% of the tissue being available for analysis (occasionally sections are torn or lost during pro-

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cessing). Furthermore, these methods can be time consuming and require special equipment and a knowledge of stereological theory. Finally, Schmitz and Korr suggest that modern methods may have reduced power to detect significant differences and thus researchers may be disinclined to trade increased accuracy for decreased power.[10]

Summary

Modern stereological methods offer a theoretically unbiased way of determining the total number of objects in a given structure or region of interest. They are not without their limitations however, and researchers will continue to use both older and new methods depending on their appropriateness for addressing the objectives of the experiment.

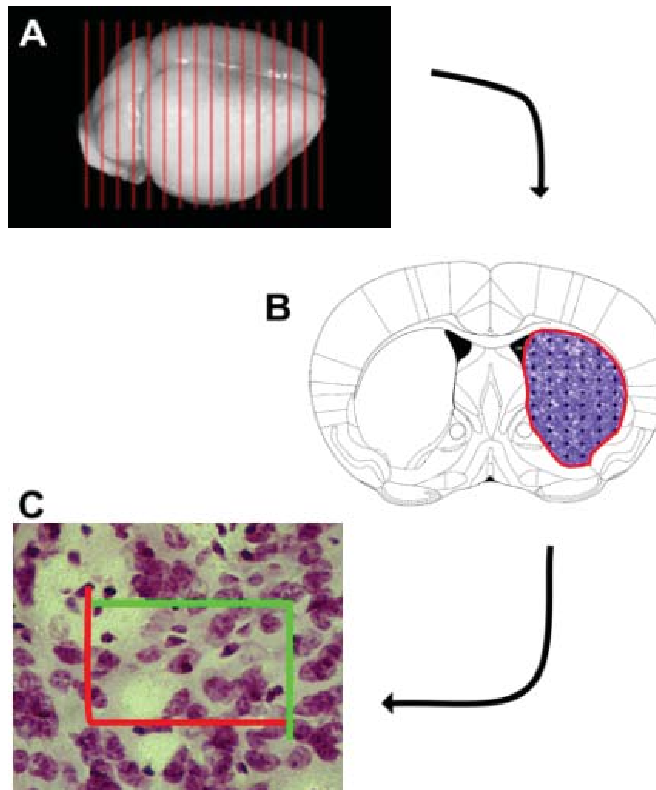

Figure 1: Sampling procedure for stereology. Sections through the brain are sampled at a certain frequency (eg. every 12th section; A). The structure or region of interest is outlined and locations (black dots) selected by the software in a systematic random manner (B). Under high magnification the number of cells that fall within the box or partially within but touching a green line are counted, while cells touching a red line are excluded. Schematic diagram adapted from Paxinos & Franklin.[11]

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