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The Optical Fractionator

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Stereological methods are precise tools which gives us information about three-dimensional (3D) objects from essentially flat two-dimensional (2D) images, which may be physical or optical slices (Gundersen et al. 1988b, Mayhew 1992). To use these techniques, a rigorously defined and uniform random sampling must be executed, giving every element of the object under investigation the same chance of being selected. This is the only way of ensuring an unbiased sample (Mayhew 1992). Systematic sampling is frequently used in biology and materials science, and it has been found to be much more efficient than simple random sampling (Gundersen and Jensen 1987, Thioulouse et al. 1993). It provides an "unbiased" and "efficient" estimator, which mean respectively "without systematic deviation from the true value" and "with low variability after spending a moderate amount of time" (Gundersen et al. 1988b). "New stereology" does not rely on the use of simplistic models of real objects and makes no assumptions about object shape, size and orientation (Mayhew 1992).

Stereological methods can be applied to all levels of organization, estimating numbers of particles, lenghts, surface areas and volumes. The stereological estimators can be divided into two groups, according to the necessity of randomizing only the location of the section planes (volume, number) and also the orientation (surface, area, lenght, particle spacing, layer thickness) (Cruz-Orive and Weibel 1990, Gundersen et al. 1988a and b, Mayhew 1992).

PARTICLE NUMBER

Number is a dimensionless quantity, so counting particles is essentially equivalent to counting points. Particle number in 3D cannot be estimated unless a 3D probe is used (Cruz-Orive and Weibel 1990, Gundersen et al. 1988a).

THE DISECTOR

The disector (Sterio 1984) is a probe which samples isolated objects or particles with a uniform probability in 3D space, irrespective of their size and shape. This device counts particles between randomly positioned pairs of parallel slice planes separated by a known distance. Complete transects (one or more profiles in the same particle) are sampled if they appear in an unbiased counting frame on one slice plane (the "reference plane") but not on its partner (the "look-up plane") (Fig. 1). The number (**N**) of particles so counted ($\mathbf{Q^-}$) is contained within a volume (**V**) equal to the area of the counting frame (A), multiplied by the distance between the upper (or lower) faces of the slices **(d): est. N/V = Q^-/(A.d)**

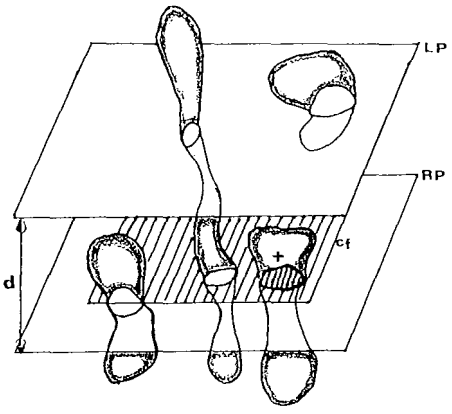

Fig. 1. The disector counts particles between randomly positioned pairs of parallel slices separated by a known distance (d). Particle labeled + is counted because it is hit by reference plane (RP), its transects are captured by the unbiased counting frame (cf), and it is not hit by look-up plane (LP). The other particles are not counted because they do not fulfil these requirements. Direct estimate of N/V is obtained by dividing the number of particles counted (Q-) by the volume (V) , which is equal to the area of the counting frame (A) multiplied by the distance between the planes: est. N/V = Q^- / (A.d).

THE OPTICAL

Disector the ordinary or "physical" disector requires cutting at least two sections, measuring their thickness accurately and using two microscopes to observe the reference and the look-up sections simultaneously. By taking a reletively thick section (25-50 mm) and using optical sections planes by moving the focal plane up or down these problems can be avoided, and much labour can be saved (Gundersen et al. 1988a).

Optical sectioning will be at its best when used on a confocal microscope; if the particles exhibit fluorescence or reflectance they can be counted using un unbiased counting brick (Howard et al. 1985, Petran et al. 1968). However a conventional light microscope, modified in various ways, can be used when working with thick plastic sections.

The two main modifications are an electronic microcator which has been fitted to the microscope to measure the movements of the stage in the z-axis with high precision and that the microscope is fitted with a projection arm (or a television monitor). It is essential to use high numerical aperture, oil-immersion objectives, so that movements in the z-direction reported by the microcator represent the true distance moved by the focal plane through the thick section (Gundersen et al. 1988a) and also that the optical section thickness is tolerably thin (about 0.5 μm for a 1.4 NA lens). The use of glycol methacrylate as an embedding medium permits optical sectioning in thick sections (25 mm) to be performed with minimal shrinkage effects (Bjugn 1993, Braendgaard et al. 1990, Jensen and Pakkenberg 1993, Korbo et al. 1993, West and Gundersen 1990, West et al. 1991). The importance of preparation artefacts such as fixation shrinkage is due to the fact that the disector yelds numerical density, N/V, rather than N itself. However, when combined with Cavalieri estimates of volume, these difficulties can be circumvented.

THE OPTICAL FRACTIONATOR

A recent development involving the combination of the optical disector with a fractionator sampling scheme, referred as the Optical Fractionator (West et al. 1991), involves counting particles with optical disectors in a uniform and systematic sample, that constitutes a known fraction of the region to be analysed. It is unnafected by tissue shrinkage or expansion, and makes possible the analysis of frozen, vibratome, celloidin and paraffin sections.

The **fractionator** (Gundersen 1986) is an application of systematic sampling. To estimate some quantity within an object we may cut it into a few fragments. If we pick every f^{th} fragment, with random start between **1** and **f**, and measure for all these fragments the quantity of interest, then we only need to multiply this measured quantity by **f** to obtain an unbiased estimate of the total quantity in the whole object. This can be extended to several subsampling stages; every $\mathbf{f_1}^{th}$ fragment of the first fractionation step is cut into subfragments, of which every $\mathbf{f_2}^{th}$ fragment is picked, and so on. To obtain the total quantitiy we seek, the last $(\mathbf{k}^{th})$ subsample is multiplied with $\mathbf{f_1 . f_2 . (...) . f_k}$ (Cruz-Orive and Weibel 1990, Gundersen et al. 1988a, Mayhew 1992).

West et al., in 1991, estimated the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. After an exhaustive sectioning of the hippocampus, a random systematic sampling of the sections was executed. In each sampled section, counts of neurons were made with optical disectors at regular predetermined x,y positions within the subdivisions, again after random placement of the first counting frame within the first x,y interval, moving each section in a raster pattern with stepping motors. The area of the unbiased counting frame of the disector, a(frame), was known relative to the area associated with each x,y movement, a(x,y step). The latter was calculated from the size of the step movements made on x and y axes (dx.dy = a(x,y step)). The area sampling fraction (asf) was then a(frame) / a(x,y step). The height (h) of the disector was known relative to the thickness of the sections (t) - mean thickness estimated with a microcator - as was the section sampling fraction (ssf). The number of neurons in the subdivisions (N) was estimated as:

$$\text{est } N = SQ^{-} \cdot t/h \cdot 1/asf \cdot 1/ssf$$

SQ^{-} refers to the total number of neurons actually counted in the disectors that fell within the sectional profiles of the subdivisions seen on the sampled sections (Fig. 2).

OPTIMIZING THE SAMPLING SCHEME

One of the most valuable features of this metod is that the evaluation of the precision of the individual estimates, the coefficient of error

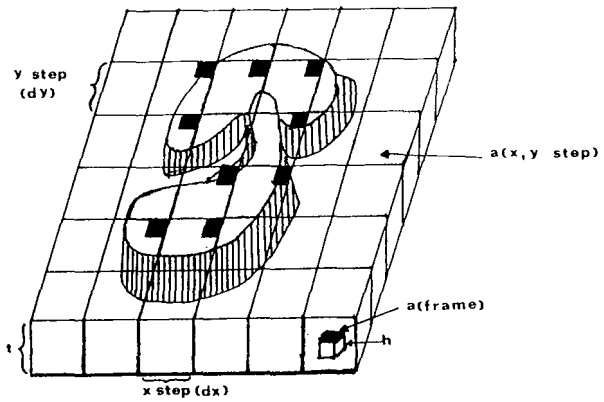

Fig. 2. Hippocampal section, sampled by the fractionator technique. Each section is moved in a raster pattern with stepping motors, so that in the center of the field viewed on a video monitor we see those positions indicated by the intersections of the lines of the sampling lattice. An unbiased counting frame (black square) is superimposed on he video image, and at positions where it falls over the neurons of a subdivision, samples of neuron number are made with an optical disector (Q^-). Area of the sampling fraction is the area of the counting frame, a (frame), relative to the area assoicated with each step of the stepping motors, a (x, y step): asf=(a (frame)/a (x,y step)). The thickness sampling fraction is the height, h / t. Adapted from West et al. 1991.

(CE), provides the information necessary for determining whether or not a particular sampling scheme is appropriate for a particular study. In this case, in order to do a complete study, the sampling must be organized into a hierarchy of levels such as individuals, sections, and disector samples. The variance of the estimates at each level contributes to the overall variance of the estimate at the top level. For biological material, the level that usually contributes most to the total variance is exactly the biological variance between individuals at the top level. This can be easily solved by using larger experimental groups. The strategy for effecting optimal sampling is to ensure that the variance of the estimate at a particular level (j), contributed by the variance of the estimate at the next lower level (j + 1), is a minor part of the variance observed at level j.

It should also be pointed out that although the sampling and the estimates described for the optical fractionator are unbiased, the CEs used in the evaluation of the efficiency of the sampling scheme are aproximations. Exact CEs can only be obtained with simple random sampling schemes (the expected CE for n independent observations made from a Poisson distribution is 1/ n), a procedure impossible to realize in 3D space. The method currently used to to estimate the CE (or the precision of the estimate of **N**) uses the quadratic approximation formula described in Gundersen & Jensen (1987). This approach to estimating CE has been improved and provided with a more thorough theoretical background by Cruz-Orive (1990) and Thioulouse et al.(1993).

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