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A BRIEF INTRODUCTION TO PARTICLE NUMBER ESTIMATION

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ABSTRACT

The principle of particle number estimation using the disector is described emphasising the practical similarities and differences in the application of the principle in biomedicine and non-biological sciences.

Key words: disector, number density, optical sectioning, particle sampling.

INTRODUCTION

One of the most important principles in modern design-based stereology is unbiased counting, i.e. unbiased number-weighted sampling in 3D space. Without the uniform sampling of the objects of interest, all number-weighted estimates are unreachable – i.e. no simple (number-weighted) mean of any kind relating to the objects as individuals can be estimated without significant assumptions of shape, size and orientation. The older, model-based stereological methods are genuine dependent on such assumptions and are therefore always facing the (often) almost impossible task of verifying the model.

DESIGN-BASED SAMPLING OF NUMBER

It is well known that a safe way of unbiased sampling of a zero-dimensional quality (i.e. number) in 3D space is by the use of a 3D probe - the disector. The disector (Sterio, 1984) consists of two parallel section planes positioned uniformly random in the sampling space. The distance h separating the planes must be less than the height of the smallest particle (in the direction perpendicular to the section planes) and all profiles in the sections must be identifiable. That is, it must be possible to determine if profiles in both sections are from the same particle. The counting rule is as follows: count the number Q^- of particles which are *not* transected by the first section plane (the look-up plane) but are hit by the second (the reference plane). If the sections are not complete sections spanning the whole structure, i.e. if the sections contain artificial borders, sub-sampling in the plane using an integral test system, based on the unbiased counting frame (Gundersen, 1977; Gundersen et al., 1988a), is needed – see Figure 1. The estimated number density N_V is Q^- divided by the product of the sampled area a and the disector height h. Both Q^- and a are summed over the observed disectors:

$$N_V = \frac{\sum Q^-}{h \cdot \sum a} \qquad (1)$$

The disector estimate is independent of shape, size and orientation of the particles. In practice, the observer can use two thin sections where the distance between the sections (and the section thickness) is smaller than the smallest particle height. This is called a *physical disector* as physical sections substitute the planes. If the region of interest is transparent, as most biological specimens, one may instead cut thick sections (25 to 200 μm or more) and optically scan through the height of the section using a thin (0.5 to 1 μm) focal plane. In this way, the observer actually places a small stack of thin (optical) sections inside the thick section. This quite efficient method is called an *optical disector* (Gundersen, 1986; West et al., 1991). A microscope is needed with high numerical aperture oil immersion lenses and a microcator for the correct definition of the position of the focal plane.

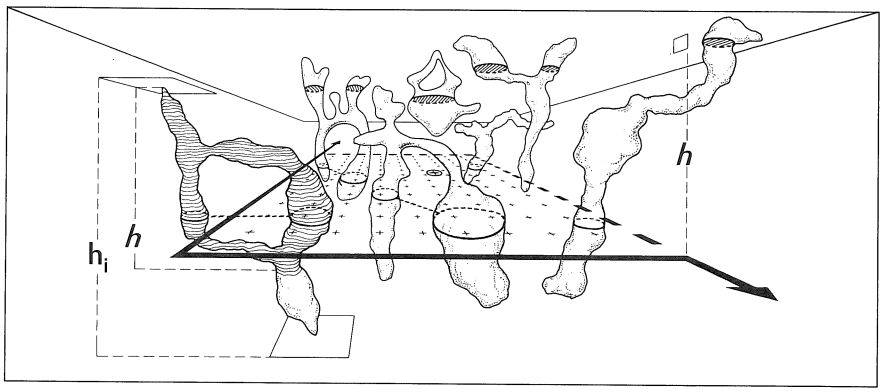

Figure 1. The disector. Two parallel section planes a known distance h apart with an unbiased counting frame of area a in the reference plane. Notice that h is smaller than any particle height h_i. Complete transects (one or more profiles in the same particle) are sampled if they are partly or totally inside the frame provided they do *not in any way* intersect the fully drawn exclusion edges or their extension. There are $Q = 4$ such transects sampled in the Figure. Of these four, two are intersected by the upper look-up plane and are *not* counted. The number of particles in the probe is the remaining $Q^- = 2$. (Slightly modified from Gundersen et al., 1988b).

Four main sampling schemes for estimation of total number of particles exist using the disector as a sampling probe. First, *the disector design*, where the volume of interest, i.e. the reference volume, must be defined and estimated e.g. by the Cavalieri method (Gundersen and Jensen, 1987). The estimate of the number density is then multiplied by the estimate of the reference volume to obtain an estimate of the total number of particles. Secondly, in *the fractionator design* (Gundersen, 1986), the particles are contained in a space with no need for exact delineation but at least large enough to contain all particles of interest. The number of Q^- is counted in a known fraction of the containing space made by one or several levels of sub-sampling. The multiple of Q^- and the inverse fractions is an unbiased estimate of the total number. Using a physical disector as a sampling probe it is unnecessary to know the height h of the disector, because the fraction of the sections used are known from sectioning the containing space exhaustively. Thirdly, *the selector design* (Cruz-Orive, 1987) estimates the number of the particles without knowledge of the section thickness. The number density N_V is estimated indirectly: using disectors of unknown thickness particles are sampled with an unknown but uniform probability; the volume of every sampled particle is estimated using point-sampled intercepts providing an unbiased estimate of the number-weighted mean particle volume $\overline{v}_N$; the volume fraction V_V is estimated by point-counting; the number density is then $N_V = V_V / \overline{v}_N$. As the selector requires the single particles to be exhaustively sectioned it is a quite time-consuming method to obtain numbers, but the mean particle volume is also estimated (without the need of an internal point - e.g. a nucleolus). The most important strength of the selector may be the very stable estimate of the coefficient of variation of the number-weighted mean particle volume $CV_N(v)$. As $CV_N(v) = \sqrt{(\overline{v}_V / \overline{v}_N - 1)}$ and both the number-weighted and the volume-weighted mean volume of the particles can be estimated from the same data, the selector seems to be the most efficient estimator of this coefficient of variation. Finally, the *Horvitz-Thompson type* of estimators should be mentioned (Horvitz and Thompson, 1952; Overton and Stehman, 1995). It is a group of estimators where the sampling is non-uniform but where the probability of the sampling of single particles can be calculated form measurements made in the section. Examples are "The Nucleated Bag" using IUR-sections through a fixed reference point in the structure, (Gundersen et al., 1988b; Bagger et al., 1993) and

"FAVER" (Fixed Axis VErtical Rotator) using vertical sections (Baddeley et al., 1986) through a specific fixed vertical axis in the structure (Evans and Gundersen, 1989; Gundersen and Boyce, 1995).

The total volume of particles per volume of reference space V_V, total particle surface area per volume S_V and total particle height per volume H_V (i.e. the summated height of the particles in the direction perpendicular to the sectioning plane) are simply estimated from independent single sections using well-known classical stereological estimators (Underwood, 1970; Karlsson and Cruz-Orive, 1997). Dividing one of these estimates by the estimate of N_V provides a set of ratio-unbiased estimates of the number-weighted (i.e. the simple) means of the particle size parameters: mean particle volume $\overline{v}_N$, mean particle surface area $\overline{s}_N$ and mean particle caliper diameter ('mean height') $\overline{h}_N$. It should be noticed that it is only the mean of the size parameters, which is possible to estimate this way. The size distribution of particles related to one of the size parameters should if possible only be estimated in one of two ways: Either by using a single section (IUR or vertical) through an internal identifiable point in the sampled particles (e.g. the nucleoli of cells), or by exhaustive sectioning of the particles of interest.

MODEL-BASED SAMPLING OF NUMBER

Older methods of number estimation based on unfolding or simple profile counts in a single section are always more or less biased and are by our opinion to be considered obsolete. The profiles seen in a single section represent transects through particles – but what are seen are only profiles of particles, *not* particles, because particles are 3-dimensional (3-D) objects. The number of profiles observed in a single section is related to the number of particles in the material. But the profile count also depends of the height of the particles, their orientation (isotropy or anisotropy of the material) in relation to the sectioning plane and of the more or less complex 3-D-shape of the single particles – see Figure 2. Examples of very complex shaped particles are the podocytes in the kidney and the so-called dendritic iron. In both cases a single particle may produce hundreds of profiles in a single section (illustrating that the number of particles in some cases is of less value compared with e.g. the amount of volume and

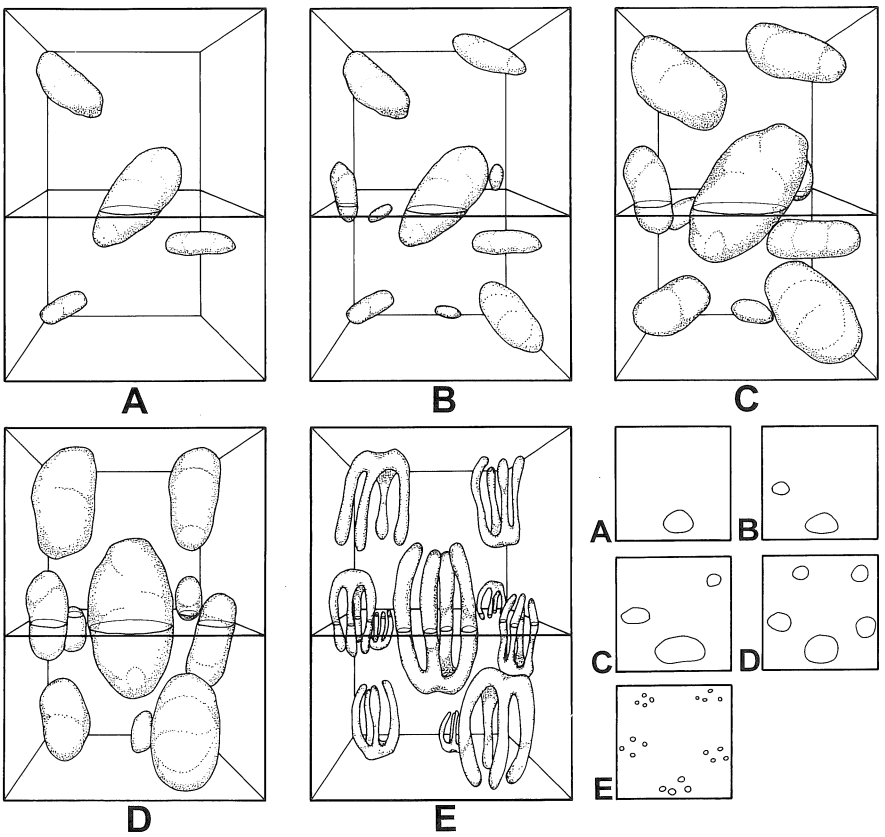

Figure 2. An illustration of the problem of number estimation from single sections. Estimation of number of particles (total or density) from single sections is always dependent on assumptions about the shape, size and orientation of the observed particles. *A* - The number of profiles in the section depends of the number of particles. *B* - If the number of particles increases, the profile number increases. *C* - If the size (i.e. the height) of the particles increases – so does the number of profiles. *D* – If the particles are oriented anisotropically, change of the orientation of the section plane will change the number of profiles observed. *E* – if the particles develop a more irregular shape, the profile number may increase. Finally it should be noted, that the serious practical problem of lost caps and tails is avoided using the disector in contrast to single sections.

surface). It should also be mentioned that the individual profile areas on one random section through a 'real' structure in no definable way represents the size of the individual 3-D particles. This will only be the case if a perfect model of the 3-D-shape-and-size of the particles exists – an unlikely and non-verifiable situation. Especially in the areas of mineralogy and materials science (but also in the area of biology), the size distribution of particles (grains) reported (by "non-stereologists") often is the distribution of grain profile areas sampled in single sections. In this way the sampling of the particles is height-weighted and the area of the profiles on a random section is not simply related to any of the well-defined 3-D-size parameters of particles. The interpretation of this so-called 'size distribution' is therefore dubious and this estimation of profile areas sampled from single sections should be avoided.

THE USE OF N_V

The world of applied stereology is divided in two major areas: biology and "non-biology", the latter covering materials science, mineralogy, geology etc. The practical problems may differ substantially between the two areas so they will be treated separately in the following.

In the **biological** world, the objects investigated are organic structures observed either in histological sections or in scans obtained using one of the modern image creating techniques (e.g. CT, MRI, PET, ultrasound etc.). Looking at histological sections, these are nearly always reasonably transparent and can be cut as thick sections, making the use of the earlier mentioned efficient optical disector possible – see Figure 3 *left*. The tissues are relatively complex, and most often the particles of interest are cells. Cells (with the exception of erythrocytes) always have internal structures. Especially, the cells often have a nucleus containing exactly one nucleolus. It is possible to use the very small nucleolus as a unique internal reference point of the cell making the sampling easy. Especially the local (i.e. single particle) size estimations are made simple. As the cell is always sampled in a central section containing the reference point it is possible to use the recently developed group of local size estimators, some of which depends heavily on optical sectioning (Jensen and Gundersen, 1993; Tandrup et al., 1997). It is therefore relatively easy to obtain an

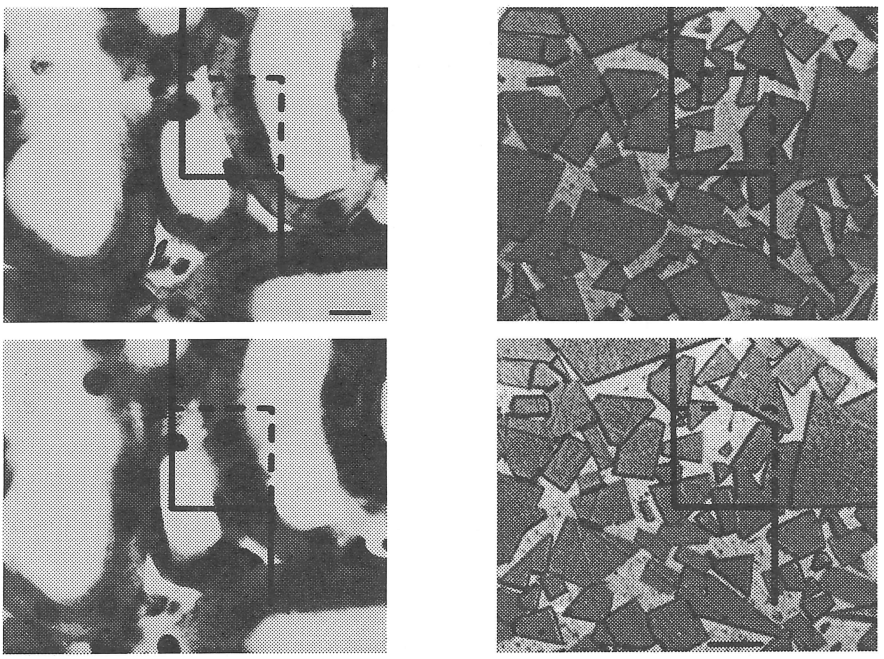

Figure 3. *Left* – an optical disector through kidney tissue. The top and bottom photos are focal planes 4 μm apart in the middle of a modified periodic acid-Schiff-stained glycholmethacrylate section of 35-μm thickness. In the bottom unbiased counting frame two cell nuclei come into focus and are counted. The bar indicates 10 μm (illustration modified from Nyengaard et al., 1993). *Right* – A physical disector through cemented carbide as an example of grains in a matrix. The top and bottom photos show the surface of the block before and after repolishing. The two surfaces are 0.5 μm apart. In the centre of the bottom unbiased counting frame two new grains are transected and are counted. The bar indicates 5 μm.

estimate of the size-distribution of the particles. In the biological world the N_V - the numerical density of the structures – provides relative information about the tissue, i.e. the concentrations of different structures (e.g. different cell types or organelles) in relation to each other and thereby reports about function. Usually the total volume of the organ or compartment is just as important as N_V. The product of these two estimates (or the use of the earlier mentioned fractionator, selector or Horvitz-Thompson based designs) provides an estimate of the total number of particles. This may be the most relevant estimate in most cases, especially as the volume and the number density both are influenced by shrinkage of the tissue during processing. In the biomedical area of stereology the observer should therefore primarily report the results in terms of total numbers instead of number densities.

The world of the "**non-biological" sciences** is faced with some special problems when compared with the biological world. The objects of interest are primarily inorganic structures observed in polished surfaces. The relevant structures are often space-filling grains or grains (or pores) embedded in a matrix (Kurzydlowski and Ralph, 1994). As the sections are non-transparent (with a few exceptions) the application of the very efficient methods taking advantage of optical sectioning is typically impossible. The observer is therefore forced to use a physical disector for unbiased estimation of numbers (Karlsson and Cruz-Orive, 1992) – see Figure 3 *right*. This requires the production of a polished and etched surface and then repolishing it to a slightly deeper level in order to get the second parallel sectioning plane (Patterson and Rhines, 1979). The technical problems involved in producing such physical disectors will hopefully attract attention from researchers in the near future in order to satisfy this urgent need. The level difference between the two planes can be measured simply by making some (e.g. four) Vicker's hardness indents in the first surface – see Figure 4. Photos of the surface are taken before and after the second polishing. The change in the size of the impressions between the two surfaces makes it possible to measure the change in level. The net result is at most a doubling of the workload but ensures the unbiasedness of the number density estimate. Without this extra effort, neither an unbiased estimate of N_V, nor any of the relevant mean particle size parameters can be obtained. Another main difference comparing non-biological

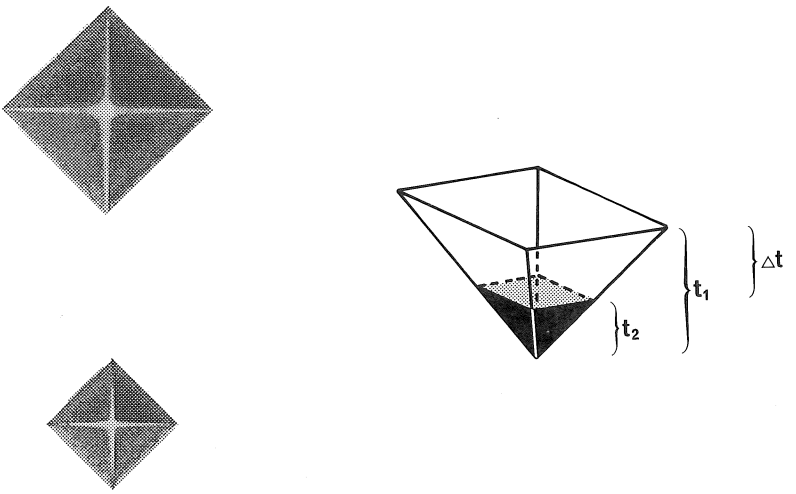

Figure 4. A method for measuring the height of the physical disector in polished surfaces. In Vicker's hardness indent a pyramidal diamond is pressed against the surface. *Left* - two photos of a surface with an impression from such a Vicker's hardness indent before and after repolishing. *Right* – a direct measure of the level change of the surface is obtained from the angels of the diamond and the change in area of the indent comparing the first to the second polish.

sciences to biomedicine is the often lack of a well defined or meaningful total reference space. As the observed structures are either parts of geological formations or man-made, the total number of e.g. grains is not defined. On the other hand, the number density of particles in this case is a relevant and well-defined parameter due to the lack of shrinkage. A third main difference is the homogeneity of the interior structure of the grains examined. In the case of space-filling granules the lack of a unique identifiable point-like internal structure (as the nucleus or nucleolus in the cell) makes sampling itself more difficult. The problem is caused by the demand for identification of the profiles in the sections. When comparing two sections, cases can occur where it is impossible to verify the exact relations of the profiles. In these cases as much as four sections may be needed for every disector – see Figure 5. The lack of a recognisable internal reference point and the impossibilities of optical sectioning make local size estimation quite difficult. The unbiased estimation of single particle size (volume, surface area or caliper diameter) required for the (number weighted) size distributions of the particles, can only be obtained by exhaustive sectioning of the sampled particles. The estimation of the size distributions of grains therefore result in a significantly increased workload. As mentioned earlier, unbiased estimates of the simple means of the particle sizes ($\overline{v}_N$, $\overline{s}_N$ and $\overline{h}_N$) can be obtained from section pairs or in the worst case four sections.

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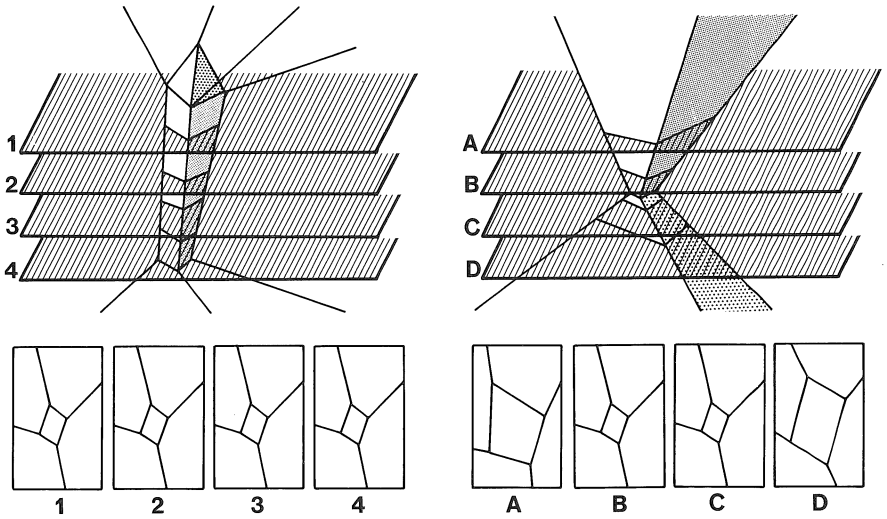

Figure 5. The study of space filling grains being typically polyhedral and often convex may sometimes require more than two sections in the 'disector', as shown here. To the left is shown sections through one thin grain and on the right, the sections transect two different grains. The two section series are indistinguishable looking only at the two middle sections. Looking at the whole section series to the right, the structure will show an hourglass-shaped configuration. This may be interpreted as two particles, showing an example of why the use of the disector in materials science sometimes may require more than two sections.

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