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Stereology of Arbitrarily Shaped Particles: Unbiased Estimation of Number and Sizes

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A number of unbiased stereologic methods has recently been developed for estimating the number and sizes of arbitrary particles from sections without assumptions about the shape of the particles -under the sole requirement that one can always identify a particle from its profiles on one or more sections through it. One of these methods -the fractionator - is remarkable in the sense that it is independent of and unbiased by the dimensional changes (swelling, shrinkage, section compression etc.) usually induced by the chemical and physical handling of biological specimens.

On a single section it is possible to estimate the mean volume $\bar{v}_V$ in the volume weighted or sieving distribution of particles

$$\bar{v}_V = \frac{¶}{3} \cdot \overline{\ell_0^3} \qquad (1)$$

where ℓ_0^3 is the (length)^3 of an intercept in a random direction through a random point hitting the particle. The principles and details of the very fast estimation is given in Gundersen & Jensen, 1985. The estimation principle, closely related to the 4-linc by Miles (1985), is the only one leading to unbiased estimates of any natural size of particles on an ordinary, independent section. If one is willing to make the assumption that the particles are spheres some extraordinarily simple solutions are available for both line sampled and point sampled intercepts. For arbitrary powers of intercept length, ℓ^k, there exist direct relationships between moments of sphere sizes and moments of observed intercepts, Eqs. 4.5 and 4.6 in Gundersen & Jensen (1983). For line sampled intercept (length)^2 and point sampled intercept (length)^3 direct relations between the distributions of sphere size and observed

intercepts are also known (Eqs. 4.12 and 4.13 in Gundersen & Jensen, 1983) as well as very simple graphical unfolding procedures. It should not be overlooked, however, that the assumption of a spheroidal shape in practice introduces a bias of unknown magnitude in the estimates - and that Eq. 1 above, like the rest of the relations in this report, is valid without any shape assumptions.

On two parallel sections a known distance h apart one can estimate particle number using the disector (Sterio, 1984):

$$N = \frac{\bar{Q}}{P} \cdot \frac{V(ref)}{v(dis)} \qquad (2)$$

where Q^- is the number of particles intersected by one plane within a frame of area a(fra) but not intersected by the other plane. P is the number of test points which hit the reference space, v(dis) = h·a(fra) is the volume of the disector and V(ref) is the volume of the reference or containing space. For use at the electron microscopic level there exists a simple modification of the disector principle whereby the need to known the distance h (or the section thickness t) in the difficult range $\leq$ 100 nm is completely avoided (Gundersen, 1986). Using ordinary, well known stereologic estimators of total quantites Z: Height, Surface, or Volume on one of the sections provides estimates of the corresponding mean size $\bar{z}_N$

$$\bar{z}_N = \frac{Z}{N} \qquad (3)$$

when combined with Eq. (2). The estimator of $\bar{v}_N$, the mean volume in the number distribution of particle volume, may be combined with Eq. (1) to an estimate of the spread or Standard Deviation $SD_N(v)$ in the number distribution of particle volume

$$SD_N(v) = \left[\bar{v}_N(\bar{v}_V - \bar{v}_N)\right]^{\frac{1}{2}} \qquad (4)$$

On a stack of parallel sections with a total height in relation to the largest particle and with known distances between the sections a general, two-step-procedure exists for sampling and size-estimation of individual particles. In the first step, sampling of particles from a predetermined section in the stack may be performed using any of 4 probes: 0-dimensional points, 1-dimensional lines, 2-dimensional frames, or 3-dimensional disectors whereby individual particles are sampled from the distribution in measure Z, where Z is Volume, Surface, Height, or Number corresponding to the probe used, the dimensions of the probe and of Z must add up to 3. The size estimation of the individual, sampled particles in the second step is performed using the same probes

as mentioned under Eq. (3). Now, the size $z_{i,Z}$ of the i'th particle taken from the distribution in Z is

$$z_{i,Z} = M(pro) \cdot \sum_{j=1}^{m} Q_{j,i} \qquad (5)$$

where M(pro) is the known measure of the (3-k)-dimensional orthogonal complement to the k-dimensional probe (Miles, 1972) and $Q_{j,i}$ is the number of times that the probe hits the i'th particle on the j'th of the total of m sections through the particle. Z and z need not correspond, but the application of the probes in the two steps must be independent. Details are given in Gundersen (1986), note that all estimators require only counting (or classification in Eq. 1), no measurements are performed. Taking a sample of n individual particles for size estimation in Eq. (5) provides the distribution in measure Z of size z of individual particles, the 4 sampling probes and the 3 probes for size estimation enables 12 distinct distributions. Each of these of course has a mean particle size $\bar{z}_Z$ and there is a straightforward, unbiased estimator of them all

$$\bar{z}_Z = \frac{1}{n} \sum_{i=1}^{n} z_{i,Z} \qquad (6)$$

All the estimations in Eqs. 1 to 6 are simple to perform by ordinary manual methods: 'point counting'. If the sections can be handled by automatic image analyzers the implementation of the methods in these machines is equally simple. As it is well known, this is still only possible for certain uncomplicated specimens in materials sciences and mineralogy, where planes of polish usually are substituted for sections. One can, however, easily imagine analyzing system which could handle such materials (Gundersen, 1986). All the necessary components of such a system do exist today, but only separately.

In all the above methods it is necessary directly or indirectly to measure something on a section or on the specimen: the length of an intercept in μm, the magnification as a ratio between 1 μm on the section and 1 μm on the image, the distance h in μm, the area a(fra) in μm^2, and the volume of V(ref) in mm^3. Especially in biological specimens the complete set of procedures in general use for preparing the specimen for stereological analysis: fixation, dehydration, embedding, and sectioning lead to changes in the dimensions of the structures. The direction of the changes - shrinkage or swelling - and their magnitude vary from step to step and depend on the particular

process which is used. It is, however, generally true that the end result is a dimensional change of the specimen and the structures, often a shrinkage and most pronounced in the easiest and most widely used procedures, especially paraffin embedding which in most soft tissues leads to a shrinkage in volume of 30 to 50 per cent. Unless the dimensional change of the structure or the reference space is quantified or measured and then corrected for, all the above estimators like all stereological estimators known until now give biased estimates with respect to the real specimens. If dimensional changes are ignored, in comparative studies, for example, one does run the real risk that the bias depends on the condition under study, spectacular examples of this are well known, see e.g. Haug (1985). It is unfortunately quite laborious to quantify the dimensional change of the reference space and no general methods are available for the estimation of shrinkage or swelling of structures in the specimen. Correction of the bias in all estimators except the one in Eq. 2 therefore relies on the assumption of homogeneity among all structures with respect to dimensional changes.

A new stereological estimator of the number of particles in a specimen, the fractionator (Gundersen, 1986) has been developed which completely circumvents all these problems - it also provides a simple and fast estimate of the dimensional change of the specimen. The principle of the fractionator is most unusual in a stereological context but it is also exceedingly simple and robust, it is illustrated in Fig. 1, and consists of the following steps in the general version.

1) Cut the containing object with N particles into a number of pieces in almost any arbitrary way but independent of the (generally interior, microscopic and invisible) particles.
2) Take an arbitrary, fixed and known fraction 1/p1 of the pieces, selected at random. If necessary, the sampled pieces are further cut into smaller pieces, among them a fixed and known fraction 1/p2 of the small pieces are sampled at random. It is unconditionally true that the expected or average number N' of particles in the aggregate of all pieces sampled in the last step is $N/(p1 \cdot p2)$.
3) All the small sampled pieces are now sectioned exhaustively in any arbitrary way which ensures that no particle will be undetectable if all sections were inspected. An arbitrary, fixed and known fraction 1/p3 of all sections is now sampled at random,

and whenever a section is sampled the 'next' section in an arbitrary fixed direction is also taken as a 'look-up' section.

4) All particles which are seen in a sampled section but which are not seen in the look-up section are counted using an unbiased 2-dimensional counting rule, this number Q^- is summed over all sections: ΣQ^-. It is obviously true that $\Sigma Q^- = N'/p3$, provided that the particle fragments at the edges of the sections are properly handled. Since the artificial edges of the sections corresponds to the cuts made in the first steps independently of the particles it suffices to estimate the aggregate areal fraction $1/pa = \Sigma P(\text{interior})/\Sigma P(\text{section})$ of the sections which is interior to a sufficiently wide guard area and where no fragments are therefore present, see Gundersen (1986) for details.

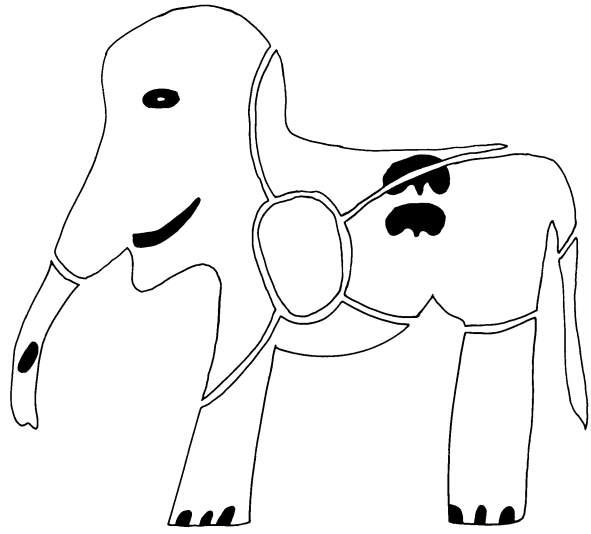

Fig. 1: An arbitrary object containing a number of arbitarily shaped particles. The object is cut into an arbitrary number of pieces of unknown shapes and sizes, the only restriction being that the cuts are made independent of the particles.

It follows that

$$N = p1 \cdot p2 \cdot p3 \cdot pa \cdot \Sigma Q^- \quad (7)$$

is an unbiased estimator of the total number of particles in the containing object. No assumptions need to be made regarding particle shape, dimensional changes of the reference space or of the particles, section compression or lost caps or Holmes' effect, nor is it necessary to know or measure anything of a non-zero-dimension.

If in step 3) above the average section thickness $\bar{t}$ is estimated (Sterio 1984, Gundersen et al. 1983) and the area a(p) corresponding to a test-point in the grid used in step 4) is known then

$$V'(ref) = p1 \cdot p2 \cdot p3 \cdot \bar{t} \cdot a(p) \cdot P(section) \quad (8)$$

is an unbiased estimator of the volume of the reference space after all dimensional changes have taken place. Knowledge of this volume before processing, V(ref), then provides a straightforward estimator of the relative net dimensional change of the reference space on a volumetric scale: V'(ref)/V(ref) - 1.

The above set of methods has resently been used in a number of worked out examples in the central nervous system (Brændgaard & Gundersen, 1986).

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Q: 1) Do you have any need for the distances between the sections in the disector method? I ask this, because I once made an experiment, in which observers compared sections of herring eggs. It turned out that they could not identify the sections from the same block if the distance between them was less than about 1/6 times the diameter of the eggs (Collan and Collan, Z. Wiss. Mikrosk. 1970).

2) Is there any method of getting the standard deviation of the sizes of particles in any individual sample by using the disector-method? (Y. Collan)

A: 1) From practical experience, I would say that the distance (h) between disector planes should be less than half the 'diameter' of a typical particle, maybe less in closely packed aggregates.

2) Yes, by continuing the disector and point sampled intercepts (from which one gets $\bar{v}_V = \frac{1}{3} \cdot \bar{l}_0$): $sD_N(v) = \left[\bar{v}_N(\bar{v}_V - \bar{v}_N)\right]^{\frac{1}{2}}$ is an estimator of the standard deviation of the ordinary number distribution of individual particle volume.

Q: I appreciated your intensive study in its field. (You have stressedthe unbiasedness of the estimator.) In the small sample case, however, the variances of the estimator are also important. Do you have some prospect to the further study of the variances? (I. Higuti)

A: The sample variances (CE of the estimate) can be estimated in all cases, formulae are given in the original references.