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Particle Number and Sizes Estimated from Sections —A History of Stereology

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Abstract. The stereological methods for counting and sizing of particle hidden in an opaque specimen are reviewed. The methods may be separated into two types according to the measurement principle: 1) the methods of indirect estimation on independent sections based on the assumption that the particles have the same known and simple shape, and 2) the methods of direct estimation on physical or optical section pairs without any assumptions concerning particle shape; the advantages and disadvantages of which are discussed together with the historical background.

1. Introduction

Historically, stereology began with the French naturalist George Buffon and his publication (1777) of the now famous "Buffon needle problem" first solved by him in 1733. Stereology a word, however, coinced at the founding meeting of the International Society for Stereology (ISS) in 1961, has had its greatest impact and development in astronomy, geology, metallurgy, and microscopy, where analyses of three-dimensional structures are made by reference to two-dimensional polished surfaces or thin sections or projections. Stereology is defined at this meeting as: "stereology deals with a body of methods for the exploration of three-dimensional space, when only two-dimensional sections through solid bodies or projections on a surface are available (Underwood, 1970)." Contributions from statistics, statistical geometry, and topology have all played a role in the development of stereology.

A common problem in stereologist is that of estimating the size distribution of particles embedded in a three-dimensional specimen. The pioneering efforts by Wicksell (1925), and the following years, initiated the search for indirect methods of estimating particle numbers and the number distribution of particle size. In order to obtain these distributions, however, an *a priori* modeling of particle shape is always necessary. Departing from relatively simple geometrical shapes leads to mathematically intractable models, or even to indeterminate ones, so that, in many cases, the stereologist is felt with the unpleasant choice between an unrealistic particle model, a mathematically complex one, or no solution at all. However, on sections (or lines or points) a particle will appear with a chance proportional to its size because large particles have a greater chance of being hit by the prove than small ones. The introduction of three-dimensional probe with the publication of the disector method (Sterio, 1984) makes to change things really and then of a chain of increasingly powerful methods for unbiased sampling and sizing of arbitrary particles. Arbitrary shape particles of any size and shape, which may be point-like, are therefore sampled with a uniform chance only with a three-dimensional probe.

In this paper we will review the methods estimating particle number and sizes obtained from the measurements on the sections and provide detailed discussion together with some evaluation of the methods. Some of them are standardized or well-known stereological methods using a two-dimensional prove. Then, we show that the number and sizes of arbitrary particles can indeed be estimated using a three-dimensional probe. This paper considers references have been chosen for purposes of illustration.

2. Fundamental Formulae

For random plane section of 3-dimensional specimens, the well-known fundamental formulae of stereology are

$$V_V = A_A = L_L = P_P \qquad (1-\mathrm{a})$$

$$S_V = (4/\pi)L_L = 2P_L \qquad (1-\mathrm{b})$$

$$L_V = 2P_A \qquad (1-\mathrm{c})$$

$$K_V = C_A \qquad (1-\mathrm{d})$$

The principal symbols are used and give the combined notation in common usage in stereology. The term S_V, for example, refers to surface area/unit test volume and represents a fraction S/V_T. The numerator is the microstructual feature and the demoninator is the test quantity. Note that the equations relate the volume-based terms (volume density V_V; surface area density, S_V; length density L_V; curvature density K_V) in the space and the area-based terms (area density, A_A; length density, L_A; point density P_A; curvature density, C_A) on the section to the counting measurements (P_P, P_L, P_A). See, for example, Weibel and Elias (1967), DeHoff and Rhines (1968), Underwood (1970), and Miles and Davy (1976).

3. Indirect Methods for Simple Convex Particles

Consider a system of convex particles distributed randomly in a space. From the numerical density N_A of particle profiles determined experimentally by counting on a section, the particle numerical density N_V is

$$N_V = N_A / \overline{D} \tag{2}$$

where $\overline{D}$ is the mean tangent (caliper) diameter obtained by averaging over all orientations. This principle is usually ascribed to DeHoff and Rhines (1961), but it had already been given by Abercrombie (1946). We have to determine $\overline{D}$ analytically for the particles with simple shapes. By an observation of particle projection (for example, electron micrograph of thick section), for a polidispersed system of spheres, it is clear that the mean tangent diameter $\overline{D}$ is measured from the mean of the projected heights of the system of randomly oriented particles.

Another principle allowing an estimate of particle numerical density, which is described by Weibel and Gomez (1962), is used a different mathematical approach. Using V_V the areal fraction occupied by the profiles in a section, the numerical density of particles is given by the formula

$$N_V = \frac{K}{\beta} \times \frac{(N_A)^{1.5}}{(V_V)^{0.5}} \tag{3}$$

The factor $K(>1)$ is a dimensionless coefficient which depends on particle size distribution. The coefficient β depends on the shape of particles.

Mean quantities of particles are frequently of interest. They include the mean volume of particles, $\overline{V}$ ($=V_V/N_V$); the mean surface area, $\overline{S}$ ($=S_V/N_V$); the mean length, $\overline{L}$ ($=L_V/N_V$) and the mean curvature $\overline{K}$ ($=K_V/N_V$). Unfortunately, these particle mean quantities are not determinate since estimations of N_V are partly influenced by particle size and shape (DeHoff and Rhines, 1961; Weibel, 1963; Loud, 1968). It, however, is often possible to base N_V estimates on an assumption of geometrically defined particle (e.g., sphere, cube, rod, disc), but if the real particles depart significantly from the ideal, then steps must be taken to compensate for the resulting estimation bias. In practice, one established approach involves attempting to define particle shape more precisely by observation with histological insights and measurement of particle profiles appearing on thin section (e.g. Weibel *et al.*, 1968). This approach is not universally successful.

A relationship for obtaining the size distribution of spherical particles (sphere and spheroid) of same shape (DeHoff, 1962; Miyamoto, 1984) is

$$N_V(j) = \Delta^{-1} \sum^{m} p^{ji} N_A(i) q \tag{4}$$

where $N_V(j)$ represents the numerical density in the j-th class interval; j is an integer with any value from 1 to m. The largest particle size corresponds to a value of $j = m$. The $N_A(i)$ are the numerical density of section profiles, grouped from $i = 1$ to m according to size groups increment Δ established by $A_{\max}$ (the largest profile size)/m. The coefficients p^{ji} is the ji-th element of the inverse of a matrix whose ij-th element is size correctors p_{ij}. The correctors are given by Cruz-Orive (1978). The coefficient q is the specific shape factor which is the function of spheroidal axil ratio. In the case of particles being sphere, $q = 1$. Many literatures were produced over the last six decades on such a model (e.g., Underwood, 1970; Weibel, 1980).

Particles exhibiting variation about shape as well as size require plural number of variables for describing the size and shape distributions. For example, a description of a variable triaxial ellipsoid requires three variables, whereas the distribution will be a three-dimentional function in terms of three variables. It seems intuitively clear that a n-dimensional particle distribution can be identified from the corresponding profile distribution only if the latter has a dimension greater than, or equal to n. The problem of identifying a three-variate distribution describing variable triaxial ellipsoids from plane sections would be indeterminate, because all the resulting profiles are ellipses, which can be fully described by a bivariate distribution only. On the other hand, the problem of identifying a bivariate distribution describing size and shape variable spheroid from the corresponding bivariate profile distribution is determinate if the spheroids are prolate or oblate type. In this case, the coefficients are separable into size coefficients p^{ik} and shape coefficients q^{lj}. The formula (Cruz-Orive, 1978; Miyamoto, 1987) is

$$N_V(i,j) = \Delta^{-1} \sum^{m} \sum^{n} p^{ik} N_A(k,l) q^{lj} \tag{5}$$

where size coefficients p^{ik} are same as p^{ji} of Eq. (4) and shape coefficients q^{lj} depend on for prolate or oblate spheroid case. The main drawback of this approach is that it might require very large profile samples (Weibel, 1980). The fundamental principle of this approach, however, is ascribed to Wicksell (1926).

Estimation of particle number, particle size, or particle shape distribution in a volume causes many problems. A plane section probe of an aggregate of particles will "hit" each object with a probability proportional to the object size. We have to recognize that the plane section measurement brings us a biased sample data.

4. Direct Counting Methods for Arbitrary Particles

The disector "which was described by Sterio (1984)" is a 3-dimensional counting rule and its integral test system for obtaining unbiased estimates of the number of particles in a specimen. The system has a 2-dimensional sampling frame and parallel section plane at a distance *h*, which is smaller than the minimum particle height. Particles are counted if their sampled profiles are not present in the look-up section (Fig. 1). The probability that a particle is hit by a section but is not hit by the parallel section is the same for both large and small particles. This probability is known and can be used to make a direct estimator of the total number of particles in a given space,

$$N = \frac{\sum^{j} Q^{-}}{h \sum^{j} a(\text{fra})} V(\text{ref}) \qquad (6)$$

where Q^{-} is the number of particles which are seen in a reference section but not seen

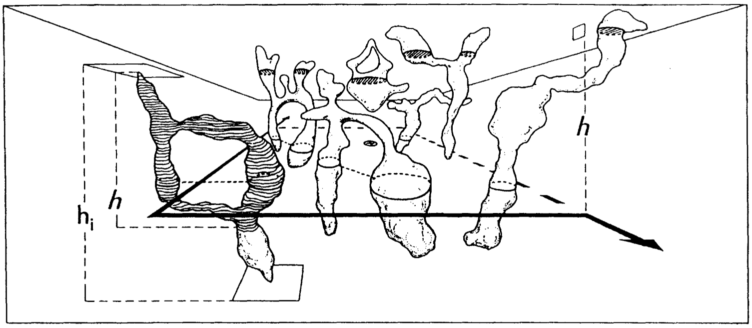

Fig. 1. The disector is a two parallel section planes of a known distance *h* apart with an unbiased counting frame of area *a* (fra) on the sampling or reference plane. Complete transects (one or more profiles in the same particle) are sampled if they are partly or totally inside the frame provided 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 remaining $Q^{-} = 2$ (cite from Sterio (1984)).

in the look-up section and V(ref) is the total volume of the specimen or reference space. a (fra) is the area of an unbiased, 2-dimensional frame (Gundersen, 1977) used for the sampling of particles to be counted, and the summation is carried out over j randomly positioned disectors in the reference frame.

It should be pointed out that the disector principle is not influenced by bias related to lost-caps and overprojection due to section thickness. Over- and under-counting on the frame edge is eliminated by the unbiased rule for the first time in direct stereological counting of particles, but shrinkage due to fixation and embedding and of section compression, etc. is still the source of potential bias.

As noted by Bendtsen and Nyengaard (1989), however, the simple counting principle based on section pairs can be regarded as re-discoveries of ideas already described several times since 1895 (Miller and Carlton, 1895; Boycott, 1911; Kittelson, 1917; Thompson, 1932; Rhines, 1967).

The bricking rule (Howard *et al.*, 1985) is basically a 3-dimensional extension of the 2-dimensional unbiased counting frame mentioned above. It can accomplish on the tandem scanning reflected light microscope (TSRLM) and permits the observation of thin optical sections in unprepared biological tissue. The concept is as follows. Consider an infinite 3-dimensional space which contains particles of arbitrary shape. Divide the entire space into rectangular parallelepiped or "brickes" of equal size and shape. Choose a particular brick which any surface of it is either "forbidden" or "not forbidden". A particle is counted if it intersects the brick, but does not intersect any forbidden surface. Note that the particle will be counted if it lies wholly inside the brick. The basic property of this counting rule is that each particle is counted by exactly one brick. The bricking rule is non destructive method conceived for 3-dimensional confocal-scanning light microscopy. It, however, requires complete 3-dimensional information about the particle should be counted or not.

The preceding two methods require the measurement of the reference space (for instance, to count with independent disectors, section thickness must be accurately measured). This is a clear disadvantage, because section thickness is not always easy to measure. The following method, however, does not suffer from that limitation, being therefore intensive to shrinking and swelling of the reference space.

The factionator (Gundersen, 1986) is an unbiased method for estimating the total number of particles of arbitrary shape in a bounded solid. The estimation method is direct, in the sense that it only requires counting particle in a few blocks but not measuring the corresponding reference space. The object containing N particles is cut exhaustively into a large number of sections as before. If, on all sections, one count the number Q^- of particles seen in one section and not in the previous section, all particles are counted once and $N = \sum Q^-$. One may count in a known and fixed fraction $1/f_1$ of all sections, sampled at random together with their neighboring, look-up sections. Since the sampled sections constitute $1/f_1$ of the whole nucleus, the number of particles counted in these sections is N/f_1 and $N = f_1 \cdot \sum Q^-$.

Finally, since the counting of particles in each section is performed on a large number of microscopic fields, one may select at random a known and fixed fraction $1/f_2$ of all fields on sampled sections and only in these fields count the number Q^- of particles which are not seen in the corresponding fields of vision in the look-up section. The final estimator becomes:

$$N = f_1 \cdot f_2 \cdot \Sigma Q^- \tag{7}$$

It is not necessary to know the magnification, the area of the sampling frame, the section thickness, the volume of the reference space, etc.

5. Direct Sizing Methods for Arbitrary Particles

Point-sampled intercepts (Gundersen and Jensen, 1985) enable the unbiased estimation of volume-weighted mean volume of arbitrarily shaped particle using independent sections. The estimator is given by

$$\overline{v_V} = \frac{\pi}{3} \overline{l_0^3} \tag{8}$$

where v_V is the mean particle volume form the volume weighted distribution of individual particle size. $\overline{l_0^3}$ is the (length)^3 of a random intercept through a test point which hits a particle, i.e., whenever one of the points of the integral test system hits a profile, the (length)^3 of the linear intercept through the point is measured in a predetermined direction. Through arbitrary particles more than one intercept may be generated within one transect, in which case $\overline{l_0^3}$ is a known linear function of these intercepts (Cruz-Orive, 1987).

The selector is the techniques by which particle size and number may be estimated if just the magnification is known. It is based on a combination of number-weighted particle sampling (with a disector of unknown thickness) and unbiased volume estimation of sample particles (point-sampled intercepts) and was first described by Cruz-Orive (1987). In a stack of section higher than the highest particle but otherwise of known and possibly varying section thickness, the first two sections are used as a disector for sampling n particles. Then the particles were sampled uniformly in a disector. These particles are followed through all the next sections, projected onto a systematic set of points, and complete intercepts are measured through every point hitting a sampled particle. All n sampled particles must be hit with a test-point at least once; if more than one intercept is measured in a particle one calculates the simple mean of the cubed lentghs for that particle, $\overline{l_{0,i}^3}$.

Since $v_i = (\pi/3) \cdot \overline{l_{0,i}^3}$ is an unbiased estimate of the volume of the *i*-th particle, it follows that

$$\overline{v_N} = (\pi / 3n) \cdot \sum_{i=1}^{n} \overline{l_{0,i}^3} \tag{9}$$

is an unbiased estimate of the mean volume of the particles from the number distribution of particle volume. It eliminates the need for knowing the disector-height h.

The nucleator, which is nick-named and considered by Gundersen (1988a), allows unbiased estimates of absolute structural quantities in arbitrarily shaped particles to be made from observations sampled in arbitrary points on independently isotropic probes. In any n-dimensional space, from an arbitrary and fixed point measure, the distance l to the boundary in any isotropic direction, it is followed that

$$content = c \cdot \overline{l^n}$$

where for $n = 1, 2, 3,...$ "*content*" is length, area, volume,... and $c = 2, \pi, 4\pi/3,...$ (Gundersen *et al.*, 1988b). For a 3-dimensional object, the relationship

$$\overline{v_N} = \frac{4\pi}{3} \overline{l_n^3} \tag{10}$$

provides an unbiased estimate of the ordinary mean particle volume in the number distribution without any further assumptions regarding, e.g., the shape of the particle. This means that the volume of the object can be estimated on just one section through the fixed point. The section must be either isotropic or fulfill the requirements for a "vertical" section to enable us to measure in isotropic directions in 3-dimensional space. Note that in practice the point must be unique recognizable for it to maintain in a fixed position independent of the direction of the Section. These conditions can all be met in mononucleated cells and, even more efficiently, in cells with just one nucleus.

6. Discussion

From the foundation of ISS in 1961 until recently, the stereologists were repeatedly startled at predecessor sagacities and pioneering efforts at least three times. The method for estimating the numerical density of particles from the profile numerical density in the section and mean particle diameter using Eq. (2) is regarded as re-discoveries of ideas ascribed to the study on cell nuclear population by

Abercrombie (1946). Until the seventies, the unfolding methods for spheres and spheroids (Eq. (4) and Eq. (5)) were considered as one of the most important and inherent subject of the society, but the original unfolding theory had already been described by Wicksell (1925, 1926). He took the question up from an anatomist Hellman as a "copuscle problem." A principle for counting particles by means of randomly selected section pairs was described by Sterio (1984), but the same idea had already been discovered by Miller and Carlton (1895) on counting glomeruli in section pairs.

All the mentioned predecessors dealt mainly with biological specimens and published the outcomes to a journal of biological or medical realm. Thereafter, these ideas were not followed by neither the later biological scientists nor the stereologist. On the other hand, the method for unfolding particle distribution had begun to be considered among metallurgists (e.g., Scheile, 1935) independent of Wicksell's paper. The unfolding method succeeded study by the later metallurgists and became a prevalent subject of metallurgist and biologist in ISS. These circumstances may partly explain why the priority of particle counting and unfolding method went unnoticed, and the general appreciation of particle counting principle based on section pairs took so long to become accepted among ISS stereologists. In addition, the stereologists from the beginning were probably, quite particular about the terms "two-dimensional section ... or projections ..." as in the definition of stereology, then restrained to their studies. Besides, it is hard to tell if the authors put great emphasis on methodology and description.

The application of the principle, however that may be, has been accelerating after the recognition of the method validity especially Sterio's paper.

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