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*Review Article*

# STEREOLOGY: A HISTORICAL SURVEY

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ABSTRACT

Stereology is the science of geometric sampling, with applications to the statistical analysis of microstructures in biology and materials science. Subsidiary disciplines are image analysis, quantitative microscopy, and radiology. This survey is organized chronologically within a series of topics which cover most aspects of stereology. Each topic is described informally to make it accessible to scientists of different disciplines.

Keywords: Anisotropy, connectivity, efficiency, global stereology, local stereology, particle counting and sizing, second order stereology, size and orientation distributions.

*Dedicated to the memory of Dr. Kiên Kiêu*

## INTRODUCTION

Our purpose is to present a brief historical survey of stereology, which is geometric sampling for estimating quantitative properties of spatial objects. In traditional sampling on discrete populations the sampling units were supposed to be accessible to observation, either directly, or from a database. In geometric sampling, however, the target object is a subset of (usually Euclidean) space, and the sample is the intersection between the object and a geometric test probe of known size and shape endowed with a well defined mechanism of randomness relative to the object. A test probe is usually a test system, namely a regular arrangement of test points, lines, planes, or slabs. In practice sampling is usually performed with the aid of microscopy, or non invasive radiology. The underlying theory is a blend of integral geometry, probability and statistics (Cruz-Orive, 2002), and the motivation is the application to biomedical and material sciences. It is therefore a multidisciplinary science represented in several books, and in a wide variety of journals. Official journals of the International Society for Stereology (ISS), recently renamed International Society for Stereology & Image Analysis (ISSIA), are Image Analysis & Stereology (IAS, Ljubljana) and Journal of Microscopy (Oxford).

Biomedical applications are usually concerned with bounded objects (*e.g.*, human brain), whereas material sciences usually deal with relatively small portions of practically unbounded spatial structures (*e.g.*, a rock, or a carbide). The corresponding approaches are called design- and model based, respectively. In design based stereology (or 'design stereology', for short) the object is assumed to be fixed and bounded, and sampling is performed with properly randomized test probes. In model based stereology (or 'model stereology'), however, the randomness is incorporated into the structure by means of a (usually stationary or 'homogeneous') random set model, whereby probe sampling is simplified. This distinction helps to understand the evolution of stereology. Model based is not the same as assumption based stereology, which relies on model shapes (*e.g.*, the sphere), and thereby leads to biased methods in general.

It also helps to distinguish between global and particle stereology. In the design context, global stereology deals with total quantities (such as the total number of neurons in a specific cerebral compartment). In the model context, global stereology deals with a ratio (such as the relative volume occupied by the tungsten grains in a carbide) which corresponds to the intensity of the random set model. On the other hand, particle stereology is concerned with mean properties of individual particles (*e.g.*, mean neuron, or grain, volume). A 'particle' is a connected compact domain separated from other particles.

Motivated by their common interests, a few scientists, coordinated by Hans Elias (who had met Ewald R. Weibel before) and Herbert Haug, coined the term 'stereology' (from the Greek '$\sigma\tau\varepsilon\rho\varepsilon\acute{o}\varsigma$', meaning 'solid') and founded the ISS on May 11–12, 1961 in Feldberger Hotel (Schwarzwald, Germany), see Elias (1962, 1963). Interesting details are given by Bach (1963) and in the history chapter of Miles (1987).

Geometrical probability and integral geometry constitute the mathematical background of stereology – hence a brief historical account is given in the

next section. On the other hand, stereological methods were largely motivated by the applied sciences, and they often used ad hoc rediscoveries of known mathematical results. This was happening well before the foundation of the ISS, almost independently in different countries, at different times, in different disciplines, and published in various languages.

The survey is organized by topics, chosen to cover most aspects of stereology. Informal explanations are given to make the ideas accessible to readers not familiar with stereology. For reasons of space many qualifying references, and figures, have been omitted. Papers bearing figures occasionally cited in the text are easily available in the web, or from the author on request.

# GEOMETRICAL PROBABILITY AND INTEGRAL GEOMETRY

## THE BIRTH OF GEOMETRICAL PROBABILITY

It is generally admitted that Buffon's needle problem, proposed and solved by the French naturalist George Louis Leclerc, Comte de Buffon (1707–1788), (Buffon, 1777, see Miles and Serra, 1978), encapsulates the art and spirit of stereology. As pointed out by Hykšová *et al.* (2012), however, toward 1664–1666 Isaac Newton (1643–1727) apparently formulated the principle that a 'random' point hitting a domain of area $A > 0$ will hit a subdomain of area $a \leq A$ with a probability equal to $a/A$. This may not be surprising inasmuch as probability was developing at Newton's time, notably with Blaise Pascal (1623–1662), and with the early members of the Bernoulli family. Nonetheless, Newton's leap from a discrete sample space (pertinent to dice and card games) to a continuous one, cannot be underestimated.

One of the merits of Buffon's problem is that it incorporates both a uniform random location of the centre of the needle and, independently, a uniform random orientation (namely an isotropic orientation) of the needle. An equivalent problem, suggested by Robert Deltheil (1890–1972), (Deltheil, 1926, p. 61) reads as follows

> "A needle of length $l$ is arbitrarily fixed inside a disk of diameter $h > l$ in the plane. A straight line in the same plane hits the disk at random. Calculate the probability that the straight line hits the needle".

If we knew "how many" random lines hit the needle, and how many hit the disk, then the ratio of both numbers would solve the problem. Morgan W. Crofton (1826–1915) discusses the important challenge of extending the traditional counting measure to some sort of continuous geometric measure (Crofton, 1868). Thus, to a straight line $L_1^2$ of normal coordinates $(p, \phi)$ in the plane, (where $p \in (-\infty, \infty)$ is the distance of the line from a fixed origin $O$, and $\phi \in [0, \pi)$ is the orientation angle), he associates a density $\mathrm{d}L_1^2 = \mathrm{d}p\ \mathrm{d}\phi$. With this tool he shows that the measure (*i.e.*, the "total number") of all straight lines hitting a convex set of boundary length $B$, is precisely $B$. For a needle of length $l$ the hitting measure is $2l$, (because the needle has to be regarded as a flattened convex set of perimeter $2l$), whereas for a disk of diameter $h$, the corresponding measure is $\pi h$. Thus, the required probability becomes $2l/(\pi h)$, which is Buffon's result.

Far from settling the issue, Crofton's results aroused controversy among mathematicians in the following years. For instance, one of the so called 'Bertrand's paradoxes', (Bertrand, 1889), implies the following. In the preceding example, Crofton's density $\mathrm{d}p\ \mathrm{d}\phi$ for a random chord of the disk means that, for each orientation $\phi$ of the chord, its distance $p$ from $O$ is uniform random along the diameter of the disk. Instead of this, suppose that the midpoint of the chord is uniform random in the interior of the disk. Then the support line of this chord is also 'random', but its density is $p\ \mathrm{d}p\ \mathrm{d}\phi$ instead of $\mathrm{d}p\ \mathrm{d}\phi$. As a consequence, the measure of all the lines hitting the convex set is no longer $B$ – it even depends on the location and orientation of the set inside the disk. Thus, which is the right answer?

## THE BIRTH OF INTEGRAL GEOMETRY

Élie J. Cartan (1869–1951) realizes the importance of establishing measure densities that are motion invariant, namely invariant with respect to translations and rotations (Cartan, 1896). In this way, the results should not depend on the choice of the reference frame. Fortuitously or not, Crofton's choice $\mathrm{d}p\ \mathrm{d}\phi$ is the (unique) motion invariant density for straight lines in the plane – it is therefore not surprising that, using this density, the measure of all the lines hitting a convex set is the perimeter length $B$, which is clearly a motion invariant property of the convex set.

Soon after, integral geometry emerges as a solid mathematical discipline whose basic purpose is to obtain motion invariant densities for geometric objects, thereby establishing a solid foundation of geometrical probability. The Hamburg School of Wilhelm Blaschke (1885–1962) exerts a great influence through its disciples, among which there was Luis Antonio Santaló (1911–2001), who showed an interest in stereology in later years. The Swiss

mathematician Hugo Hadwiger (1908–1981) created a relatively independent school (Debrunner *et al.*, 1982). Classic books are Blaschke (1936–37), who introduced the term 'integral geometry', Hadwiger (1957), Santaló (1976), and Schneider and Weil (2008). See also Naveira and Reventós (2009).

### CROFTON FORMULAE

Typical results of integral geometry are the so called Crofton formulae. For instance, consider a bounded planar curve $Y$ of finite length $B$. The measure of the number $I(Y \cap L_1^2)$ of intersections determined in the curve by all the motion invariant straight lines hitting it is

$$\int I(Y \cap L_1^2)\, \mathrm{d}L_1^2 = 2B\,. \tag{1}$$

If $Y$ is the boundary of a convex set, then $I(Y \cap L_1^2) = 2$, whereby the preceding formula yields the aforementioned hitting measure $B$. Convex sets were the main interest of Crofton (1868), Czuber (1884), etc., because explicit hitting measures yield attractive results. Eq. 1 is useful to estimate curve length, but sampling and estimation were established only in the 20th century. As detailed by Hykšová *et al.* (2012), it is noteworthy that Joseph-Émile Barbier (1839–1889) anticipated results like Eq. 1, its three dimensional (3D) version for surface area, and other important ideas (Barbier, 1860).

### THE CAVALIERI PRINCIPLE

A very simple but important Crofton formula arises as follows. Consider a bounded set $Y \subset \mathbb{R}^3$ of volume $V$, and let $A(p)$ denote the area of the intersection of $Y$ with a plane at a distance $p \in (-\infty, \infty)$ from a fixed origin $O$. Then, for any given orientation of the plane,

$$\int A(p)\, \mathrm{d}p = V\,. \tag{2}$$

The preceding formula is well known from elementary calculus. It may be regarded as a Crofton formula, however, inasmuch as $\mathrm{d}p$ is a translation invariant density. The discretized version of Eq. 2, namely the decomposition of a solid into thin slices to compute its volume, was known to Greek mathematicians, notably Archimedes of Syracuse (c. 287 – c. 212 BC), who applied it to regular solids, see for instance Tobias (1981).

Consider two solids of volumes $V_1$, $V_2$. If $A_1(p) = A_2(p)$ for all $p \in (-\infty, \infty)$, then $V_1 = V_2$. This is *Cavalieri's Principle*, formulated by the Italian mathematician Bonaventura Cavalieri (1598–1647), a disciple of Galileo, in his book Cavalieri (1635). The real merit of Cavalieri is that, unlike ancient mathematicians, he obtains a theorem for arbitrarily shaped objects. Unconsciously, one often fails to realize that section area and object volume have nothing to do with shape.

### PROBE AND OBJECT INTERACTION: DIMENSIONAL CONSIDERATIONS

For an object $Y$ hit by a probe $T$ in $\mathbb{R}^d$, the following dimensional relationship

$$\dim(Y \cap T) = \dim(Y) + \dim(T) - d\,, \tag{3}$$

holds up to a set of positions of of zero measure. Because $\dim(Y \cap T) \geq 0$, it follows that the inequality

$$\dim(Y) + \dim(T) \geq d \tag{4}$$

must always hold. Eq. 3 was presented as an axiom in Sommerville (1958, p. 10), see also Weibel (1967) – for a proof see Gual-Arnau *et al.* (2010, Appendix A).

If Eq. 4 holds and either $\dim(Y) = d$, or $\dim(T) = d$, then in order to relate $Y$ and $T$ by a Crofton formula it suffices that $T$ has a fixed orientation relative to $Y$, see, *e.g.*, Eq. 2. However, if Eq. 4 holds but $\dim(Y) < d$ and $\dim(T) < d$, then the density of $T$ has to be both translation and rotation invariant, that is, orientation matters, as in Eq. 1.

## THE INCORPORATION OF SAMPLING: DESIGN BASED STEREOLOGY

### SAMPLING IN A CONTINUOUS DOMAIN. TEST SYSTEMS

Apparently, Buffon's motivation behind his needle problem was to establish rules for fair gambling. A player throws a rod of length $l$ at random over a board bearing an array of parallel lines a distance $h \geq l$ apart. If the rod hits a line, the player obtains a money prize $m$, say. How much should he/she pay for playing? Buffon argues that the mean gain will be $m$ times the probability of winning, namely $2lm/(\pi h)$ and, therefore, for the game to be fair this should be the price of the bet.

With the progressive development of sampling and statistics from the beginning of the 20th century, Buffon's needle problem inspired estimation problems. Scientists soon realized that theoretical predictions obtained with motion invariant densities could be confirmed by real experiment. For convenience, recall the equivalent problem described in the preceding subsection. Hit the disk with $N$

random straight lines (called test lines in this context), and count the number $n \leq N$ of times the needle is hit. Then $n/N$ is an unbiased estimator (UE) of the hitting probability. Thus, knowing any three of the four quantities $2, l, \pi, h$, the fourth one can be estimated without bias. In particular, the estimation of $\pi$ by $2lN/(nh)$ was popular in the 19th century, see Gridgeman (1960) for a good review.

The idea of defining proper probability measures for random probes was fundamental for the progress in stereology. Key contributors here were Roger Edmund Miles and his student Pamela Joy Davy, (Miles and Davy, 1976, 1977; Davy and Miles, 1977). In turn, R.E. Miles had been a student of Patrick A.P. Moran, also an Anglo-Australian mathematician. The latter was a coauthor of the book Kendall and Moran (1963); this little book is an early authoritative combination of integral geometry, probability and statistics, together with applications that were the germ of stereology.

The probability element associated with a test line hitting a disk is

$$\mathbb{P}(\mathrm{d}p,\, \mathrm{d}\phi) = \frac{\mathrm{d}p\, \mathrm{d}\phi}{h\pi},\ p \in [-h/2, h/2],\ \phi \in [0, \pi)\,, \tag{5}$$

namely the motion invariant density normalized by the measure of all the test lines hitting the disk. This probability element implies that $\phi$ is uniform random (UR) in the interval $[0, \pi)$, namely isotropic random (IR) in the unit semicircle, whereas $p$ is independent and UR in the interval $[-h/2, h/2]$. The test line is thereby said to be isotropic uniform random (IUR) hitting the disk – a term introduced by R.E. Miles.

More generally, consider a planar curve $Y$ of finite length $B$ contained in a disk of diameter $h$. The sampling experiment consists of hitting the disk with a IUR test line $L_1^2$. Then $I(Y \cap L_1^2)$ is an integer valued random variable whose expectation or mean value with respect to the probability element given by Eq. 5 is easy to compute using Eq. 1, namely,

$$\begin{aligned} \mathbb{E}\left\{I(Y \cap L_1^2)\right\} &= \int I(Y \cap L_1^2)\, \mathbb{P}(\mathrm{d}p,\, \mathrm{d}\phi) \\ &= \frac{2}{\pi} \cdot \frac{1}{h} \cdot B\,. \end{aligned} \tag{6}$$

It follows that

$$\widehat{B} = \frac{\pi}{2} \cdot\ h \cdot I(Y \cap L_1^2) \tag{7}$$

is a UE of $B$. If the test line misses the curve, however, then $\widehat{B} = 0$, hence the method is more academic than practical. In practice a test system of lines is more convenient, and usually more efficient than independent test lines. The array of parallel lines used in the original Buffon experiment is in fact a test system – thus, Buffon also anticipated this concept.

Test systems are extensively used in stereology – hence a few details may be opportune. Fix a rectangular frame $Ox_1x_2$ in the plane. Instead of a test line, consider a bounded test probe such as a needle $T_1^2$ of length $l > 0$ with unit vector $(x, \omega)$, where $x \in \mathbb{R}^2$ is an endpoint of the needle and $\omega \in [0, 2\pi)$ is the orientation angle. The Crofton formula analogous to Eq. 1 is called the Poincaré formula, namely,

$$\int I(Y \cap T_1^2)\, \mathrm{d}T_1^2 = 4lB\,, \tag{8}$$

(*e.g.*, Santaló, 1976), where $\mathrm{d}T_1^2 = \mathrm{d}x\, \mathrm{d}\omega$ is the unique motion invariant density for bounded probes in the plane, called the kinematic density. Here $\mathrm{d}x = \mathrm{d}x_1\, \mathrm{d}x_2$ is the area element in the plane, and $\mathrm{d}\omega$ is the arc element in the unit circle. Actually, the way Buffon solved the needle problem implies that he used the kinematic density for the needle.

Consider, as before, a planar curve $Y$ of finite length $B$ contained in a disk of diameter $h$. We want to estimate $B$ by hitting the disk with a IUR test needle $T_1^2$, and observing $I(Y \cap T_1^2)$. The probability element associated with the needle is the kinematic density $\mathrm{d}x\, \mathrm{d}\omega$ normalized by the measure of all test needles hitting the reference disk. Thus, apart from the inherent inefficiency of the sampling design (because the needle may often miss the curve), the computation of the normalizing constant is an additional snag.

Instead, consider a partition of the plane into congruent regions or tiles $\{J_i = J_0 + t_i,\ \ i \in \mathbb{Z}\}$ generated by a family of translations $\{t_i\}$ of a bounded fundamental tile $J_0$ of area $a > 0$. Consider also a bounded fundamental probe $T$ (*e.g.*, a test point, a curve, or a quadrat) contained in $J_0$. To focus on the present example, suppose that $J_0$ is the square $[0, h)^2$ and $T$ is the straight line segment $[0, l)$, $0 < l \leq h$. The set of probe translates $\Lambda_T = \{T_i = T + t_i\}$ is a (fixed) test system (of straight line segments in this case).

Eq. 8 is unchanged if the needle $T_1^2$ is fixed and the curve $Y(x, \omega)$ is mobile with the kinematic density $\mathrm{d}Y = \mathrm{d}x\, \mathrm{d}\omega$. It suffices to attach a unit vector $(x, \omega)$, called the associated vector (AV), rigidly to $Y$, with origin at a point $x$, called the associated point (AP) of $Y$, see, *e.g.*, Gómez *et al.* (2016, Fig. 2). Then,

$$\begin{aligned} 4lB &= \int_0^{2\pi} \mathrm{d}\omega \int_{\mathbb{R}^2} I(Y(x, \omega) \cap T)\, \mathrm{d}x \\ &= \int_0^{2\pi} \mathrm{d}\omega \int_{J_0} \sum_{i \in \mathbb{Z}} I(Y(x, \omega) \cap T_i)\, \mathrm{d}x \\ &= \int_0^{2\pi} \mathrm{d}\omega \int_{J_0} I(Y(x, \omega) \cap \Lambda_T)\, \mathrm{d}x\,. \end{aligned} \tag{9}$$

The preceding result means that, instead of integrating the point $x$ over the entire plane and scoring intersections with a single test probe $T$, as in the first Eq. 9, it suffices to integrate $x$ over $J_0$, provided that the intersections are scored with the entire test system $\Lambda_T$. The pertinent probability element becomes,

$$\mathbb{P}(\mathrm{d}x,\ \mathrm{d}\omega) = \frac{\mathrm{d}x}{a} \cdot \frac{\mathrm{d}\omega}{2\pi},\ x \in J_0,\ \omega \in [0, 2\pi)\ , \qquad (10)$$

much simpler than in the single bounded probe case: now $x$ is UR in $J_0$ and $\omega$ is IR and independent. Thus,

$$4lB = 2\pi a \cdot \mathbb{E}\{I(Y \cap \Lambda_T)\}\ , \qquad (11)$$

from which a UE of $B$ is,

$$\widehat{B} = \frac{\pi}{2} \cdot \frac{a}{l} \cdot I(Y \cap \Lambda_T)\ . \qquad (12)$$

If $l = h$, then $\Lambda_T$ becomes Buffon's test system of parallel lines a distance $h$ apart, and Eq. 12 is analogous to Eq. 7 because $a/l = a/h = h$. If the fundamental probe $T$ is the union of the base and the left hand side edge of $J_0$, then $\Lambda_T$ is a square grid, as proposed by Steinhaus (1930), with $a/l = h^2/(2h) = h/2$.

Eq. 9 holds for an arbitrary polygon $J_0$ of area $a$ that can tile the plane, and an arbitrary fundamental curve $T \subset J_0$ of length $l > 0$. In particular, if $J_0 = [0, 2h) \times [0, h)$ and $T$ is the union of two half circles of diameter $h$, then $\Lambda_T$ is the Merz grid, see, *e.g.*, Howard and Reed (2005, Fig. 12.5). The advantage of this grid is that the curve $Y$ may be UR with a fixed orientation relative to the grid.

In computer assisted stereology the curve may be fixed and the grid IUR: the AP of $T$ is shifted to a UR point $x \in J_0$, dragging the entire test system with it, and then the latter is rotated isotropically at random about $x$.

The generalized version of Eq. 9 to test systems of arbitrary dimension started with Santaló (1939), who called them 'unbounded figures'. After a series of papers, a polished version appeared in Santaló (1953) under the name 'lattices of figures', see also Santaló (1956, 1976, Ch. 8). Apparently Barbier (1860, p. 278) also anticipated the concept of test system. The incorporation of associated probability elements leading to precise sampling rules, however, is relatively recent (Miles, 1978a; Jensen and Gundersen, 1982; Cruz-Orive, 1982).

As a natural extension of Buffon's test system of parallel lines, consider a test system of parallel planes of a fixed orientation. Application of Eq. 9 to Eq. 2 yields a UE of the volume $V$ of a bounded object $Y$, called the *Cavalieri estimator*, (in honour of B. Cavalieri, see Cruz-Orive, 1987a), namely

$$\widehat{V} = h \cdot \sum_{k \in \mathbb{Z}} A(z + kh)\ , \qquad (13)$$

where $z$ is a UR variable in the interval $[0, h)$, and $h$ is the distance between planes. The first rigorous version of the Cavalieri estimator appears in Moran (1950) in the context of Monte Carlo integration. In fact, many stereological methods may be regarded as Monte Carlo integration methods. The essential unbiasedness condition (emphasized in the preceding paper) that $z$ should be UR in $[0, h)$ was also given by Matheron (1971, p. 21), and in a stereology context by Thioulouse *et al.* (1985) and by Gundersen and Jensen (1987). Thus, Cavalieri sampling is equivalent to systematic sampling along an axis with a random start.

Multidimensional versions of Eq. 1 and Eq. 8 are derived in the aforementioned integral geometry books. Miles (1972) gave generalized ratio estimators. General versions of Eq. 6, of its counterpart for bounded probes, and of Eq. 9, are given for instance by Voss and Cruz-Orive (2009), Eqs. A9, A21 and A28, respectively.

## DISCRETE SAMPLING

Test systems are not the only sampling tools available in design stereology. Consider the estimation of the volume $V$ of an object $Y$. Instead of using the Cavalieri method, we may split $Y$ exhaustively into fragments (of arbitrary size and shape), generally called 'blocks', of unknown volumes $\{V_1,\ V_2, ...,\ V_M\}$ adding up to $V$. (In sampling theory the term 'cluster' is customary instead of 'block', see, *e.g.*, Cochran, 1977). The idea is to replace a continuous sampling domain (such as the interval $[0, h)$ of the Cavalieri design) with the discrete one $\{1,\ 2, ...,\ M\}$. A general UE, useful in this context, is the Horvitz-Thompson estimator,

$$\widehat{V} = \sum_{i \in \mathscr{S}} \frac{V_i}{\pi_i}\ , \qquad (14)$$

(Horvitz and Thompson, 1952), where $\pi_i > 0$ is the probability (known a priori) that the $i$th block is included in the sample, and $\mathscr{S}$ represents the subset of indexes corresponding to the sampled blocks. In UR sampling the $\{\pi_i\}$ are all identical, but UR sampling is not unique.

*Simple random sampling of blocks without replacement*. This is UR sampling (also known as the 'lottery method') in which $\pi_i = n/M$, where $n \in \{1,\ 2, ...,\ M\}$ is the fixed sample size. Thus, $\widehat{V} = M \cdot \overline{V}$, where $\overline{V}$ is the mean volume of the sampled blocks.

*Systematic block sampling*. This is UR sampling in which every $k$th block, (where $k > 2$ is a fixed natural number called the sampling period), is selected with a UR start $z$ among $\{1,\ 2,...,\ k\}$. Thus $\pi_i = 1/k$, and therefore

$$\widehat{V} = k \sum_{i \in \mathbb{Z}} V_{z+ik}\ , \tag{15}$$

(namely the sampling period times the sample total), is a UE of $V$; for convenience we set $V_i = 0,\ i \notin \{1,\ 2,...,\ M\}$. Note that, unlike simple random sampling, here it is not necessary to know the population size $M$. The sample size is random unless $M$ is a multiple of $k$, with mean value $M/k$.

*The fractionator* is multistage systematic block sampling. Thus, at the $i$th sampling stage (with period $k_i$), the sampled blocks are split again into smaller blocks which are sampled with period $k_{i+1}$, etc. If $s$ stages are used, then the unbiased fractionator estimator of $V$ reads,

$$\widehat{V}_s = k_1 k_2 \cdots k_s \cdot Q_s\ , \tag{16}$$

where $Q_s$ represents the total volume of the blocks sampled at the last stage. The fractionator can be applied to whatever measurable quantity defined on an object. The last stage quantity $Q_s$ will usually have to be estimated from sections by stereological methods. In its original version (Gundersen, 1986), and in most further applications of the fractionator, the target was particle number – for a detailed application see for instance Ogbuihi and Cruz-Orive (1990). Artacho-Pérula *et al.* (1999), however, applied it to estimate total capillary length in skeletal muscle, whereas Wulfsohn *et al.* (2010b) showed how to estimate total leaf area in plants. To increase precision, Gundersen (2002) proposed the smooth fractionator: prior to sampling at each stage, the blocks are rearranged according to their apparent size into an approximately symmetric pattern, as smooth as possible and with a single peak. A similar procedure was proposed by Murthy (1967, p. 165) under the name 'balanced systematic sampling' ('bss'). If the target quantity in a block is proportional to its apparent size, then bss can estimate the target very accurately – even exactly under special conditions (Tinajero-Bravo *et al.*, 2014). Systematic sampling with probability proportional to size – called the proportionator in stereology – is also an interesting idea, see Andersen *et al.* (2015) and early references therein.

## MODEL BASED STEREOLOGY

Here the structure of interest is modelled by a manifold process $Y$ with realizations in Euclidean space (*e.g.*, a process of points, curves, surfaces, or volumes). Loosely, for any bounded test probe $T$, the intersection $Y \cap T$ is piecewise smooth with a finite measure which is a random variable. For a formal description see Mecke (1981). The relevant theory is stochastic geometry – early developments can be seen for instance in Harding and Kendall (1974), and Matheron (1975). More recent references relevant to stereology are Baddeley (1999), Stoyan *et al.* (1995) and Schneider and Weil (2008).

To facilitate estimation the process is assumed to be first order stationary or 'homogeneous'. For instance, if $Y$ is a volume process and $T_x$ is a translate of a probe $T$ of volume $V(T)$ by a vector $x$, then the mean value of $V(Y \cap T_x)$ does not depend on $x$. More precisely,

$$V_V = \frac{\mathbb{E}\{V(Y \cap T)\}}{V(T)} \tag{17}$$

is a constant called the intensity of the process – in this case the volume fraction occupied by $Y$ in the entire space.

The intersection of a stationary volume process $Y$ with a plane is a stationary planar area process of intensity $A_A$, say. Thus, if $T$ is a bounded planar probe of area $A(T)$ in space, then by the first order stationarity of $Y$ we have,

$$V_V = \frac{\mathbb{E}\{A(Y \cap T)\}}{A(T)} = A_A\ . \tag{18}$$

As a consequence,

$$\widehat{V}_V = A(Y \cap T)/A(T) \tag{19}$$

is a UE of $V_V$ irrespective of the location of $T$. This is essentially the *Delesse principle*, which is regarded as the first genuine result of stereology. By an argument based on Eq. 2, the French geologist Achille E.O.J. Delesse (1817–1881) established that the mean area fraction of a mineral in a polished section of a rock is equal to the volume fraction of the mineral in the rock (Delesse, 1847).

Ratios are the main meaningful first order properties in model stereology. To make estimation possible the process should also be ergodic – roughly, this means that for any fixed $T$ the mean contents of $Y \cap T$ over realizations of $Y$, remains constant, *i.e.*, the target intensity does not change. Further, if $Y$ is not isotropic, and neither $Y$ nor $T$ are of full dimension, then to preserve unbiasedness the probe $T$ should be isotropically rotated.

Model stereology does not necessarily apply to material sciences only. The fine structure of lung parenchyma (see for instance Weibel *et al.*, 1981) may be studied in a model based setting – Miles (1978b)

called this the extended case, revisited by Cruz-Orive (2009). To keep variation within useful bounds, however, the size of $T$ should be large enough to encompass the 'typical features' of the target structure (Lantuejoul, 1991, 2002). For instance, to estimate the volume ratio of tissue septa in lung parenchyma, the quadrat size should ideally be no less than the alveolar size.

# GLOBAL STEREOLOGY

## THE GENERAL DESIGN

The Delesse principle just described had a profound influence in the development of stereological methods over decades. In early years the 'classical stereological equations' were inspired by material science problems, thereby involving ratios almost exclusively. The relevant books of Saltykov (1958), DeHoff and Rhines (1968), Underwood (1970), and references therein, illustrate this. Even Miles (1972) was mainly concerned with ratios. In the biological context, stereological equations were still derived with reference to a 'cube of material'. Only a deep understanding of the real biological problems, often motivated by the need to correlate structure and function, helped some biologists to regard ratios as mere vehicles to estimate the relevant parameters, namely global quantities pertaining to complete organs. A good illustration of this is Chapter V from Weibel (1963). Shortly afterwards, the classical multistage design was made more explicit, (Weibel 1969, p. 271).

In organs such as lung, the target object is the last of a nested sequence of subsets,

$$\begin{aligned} &Y_0 \supset Y_1 \supset \cdots \supset Y_s \supset Y \\ &\text{lung} \supset \text{parenchyma} \supset \text{septa} \supset \text{capillaries} \,, \end{aligned} \tag{20}$$

which have to be observed at increasing magnifications. The target is the absolute, global quantity $\gamma(Y)$, where $\gamma$ may stand for total volume, surface area, length, or number. This target may be expressed by a product of ratios as follows,

$$\begin{aligned} &\gamma(Y) = V_0 \cdot R_1 \cdot R_2 \cdots R_s \cdot \frac{\gamma(Y)}{V_s} \,, \\ &R_i = V_i / V_{i-1} \,,\; V_i = V(Y_i) \,. \end{aligned} \tag{21}$$

The preceding scheme is the basis of a nested, 'cascade' sampling design (Cruz-Orive and Weibel, 1981, Weibel *et al.*, 1981).

In early years many biological studies reported ratios instead of absolute quantities, often leading to contradictory conclusions. The term 'reference trap' coined by Braendgaard and Gundersen (1986) warned against the failure to multiply a ratio with the pertinent measure of the reference space (*e.g.*, $V_0$), see also Gundersen (1992). Haug (1985) showed that old brains shrink less than young brains after laboratory processing, whereby the ratio 'number of neurons per unit volume' appears to be larger in younger brains. Shrinkage corrections dispelled the theory of neuron loss with age.

Whereas in model stereology the denominator of ratio estimators such as Eq. 19 is a controlled, non random variable, in design stereology edge effects usually arise, whereby the corresponding denominator is a random variable. This caused confusion in early years – Mayhew and Cruz-Orive (1974) pointed out that ratio estimators are unbiased in the former, but only ratio unbiased – a term coined by A.J. Baddeley – in the latter case. In general, proper sampling prescriptions, statistical properties of the estimators, etc., had to wait until R.E. Miles and P.J. Davy set the foundations in the late 1970s. Cruz-Orive (1980a) proposed statistical models which Jensen and Sundberg (1986) showed to be valid at the best when the denominator of a ratio is a controlled variable — see also Baddeley and Jensen (2005). Cruz-Orive (2009) relates the multistage design with the fractionator.

As pointed out by Baddeley and Cruz-Orive (1995), minimum variance unbiased estimators will usually not exist – at least in model stereology – due to the incompleteness of the underlying sigma algebra. In design stereology, the Cavalieri estimator, for instance, may not converge in distribution, (García-Fiñana, 2006) – it is therefore likely that the central limit theorem does in general not apply to estimators based on systematic sampling. Thus, stereological methods are mainly non-parametric.

## PARTICLE NUMBER

As far as counting, a set $Y$ of $N$ particles may be treated as a set of $N$ points of dimension $\dim(Y) = 0$. Thus, by virtue of Eq. 4 it follows that $\dim(T) \geq d - \dim(Y) = d$, which means that particles (*e.g.*, grains, or cells) in space cannot be counted unless a three dimensional probe is used.

Thompson (1932) anticipated the Horvitz-Thompson estimator and applied it to count Langerhans islets in pancreas, noting that the probability that an islet is sampled (*i.e.*, hit) by a section plane is proportional to the caliper height of the island normal to the plane. He proposed to measure the caliper with a negligible error using serial sections.

Cruz-Orive (1980b) rediscovered the same procedure. This method was used by De Groot (1988) to count synapses by electron microscopy, but it was too time consuming, hence short-lived.

Thompson (1932, p. 26) also anticipated the associated point (AP) rule (Miles, 1974), stating that an unbiased definition of the number of islets sampled by a slab could be the number of islet centroids in the slab. As remarked by Gundersen (1986), Thompson *et al.* (1932, p. 37) removed the need to identify islet centroids and proposed to count "islets having a part in the master section but not in a given adjoining section", which is the disector principle (Sterio, 1984) – see also Miles (1972, Section 7). Bendtsen and Nyengaard (1989), however, revealed that the latter principle had been used by different authors from the late 19th century to count islets and kidney glomeruli.

A problem with the earlier methods was the lack of a simple and unbiased rule to cope with edge effects in the plane – thus, these methods had to be based on entire sections with natural boundaries only. The unbiased frame rule (Gundersen, 1977) facilitated the polished tool described by D.C. Sterio (an anagram of 'disector'). A three-dimensional generalization of the unbiased frame is the unbiased brick (Howard *et al.*, 1985). A practical simplification is the optical disector (Gundersen, 1986; Gundersen *et al.*, 1988) – for details and references see, *e.g.*, Howard and Reed (2005), and West (2012).

An efficient combination, originally designed to estimate neuron number using the nucleus, or the nucleolus, as the counting unit, is the optical fractionator (West *et al.*, 1991). The last step requires the ratio of optical to physical section thickness, where bias artifacts have to be taken into account (Dorph-Petersen *et al.*, 2001). The selector method (Cruz-Orive, 1987b) circumvents the need to measure section thickness, but it requires serial sections and it is time consuming, hence it was soon replaced with the nucleator (Gundersen, 1988) – both methods are briefly revisited in Section *Size estimators of local stereology*.

At the macroscopic, non stereological level, Wulfsohn *et al.* (2012) used the fractionator to estimate the total number of fruits in an orchard.

Motivated by the problem of counting and measuring biological organelles, S.D. Wicksell treated the more general problem of estimating or 'unfolding' the size distribution of a population of particles solely from plane sections. The problem is indeterminate unless the particles are assumed to have specific, simple geometric shapes. Wicksell (1925) first considered spherical particles; the model leads to an Abel integral equation, which he solved analytical and numerically. Because the kernel of the integral equation has a singularity, the subsequent numerical problem is ill-conditioned, and the estimation unstable near the origin. The problem presented challenges very attractive to mathematicians, and in the following decades hundreds, even thousands of papers were devoted to it. The problem was often known as the 'Swiss cheese' (here the 'particles' were voids), or the 'tomato salad' problem, and it became so popular that many scientists even identified the problem with stereology itself. For a selection of the literature see Cruz-Orive (1983). Wicksell (1926) considered ellipsoids, see also Cruz-Orive (1976), whereas Ohser and Mücklich (2000) treat various model shapes.

As far as counting only, if a stationary and isotropic particle process of number intensity $N_V$ is hit by a plane, then the intersection is a stationary and isotropic particle transect process of number intensity $N_A$, and $N_V = N_A/\mathbb{E}(H)$, where $\mathbb{E}(H)$ is the mean caliper height of the particles normal to the sectioning plane. The preceding equation (implicit in Wicksell, 1925, p. 89) was massively used also in biology, in a design based setting. The problem, of course, is that $\mathbb{E}(H)$ is not accessible from independent sections. Apart from the spheres assumption, additional ones relative to truncation (*i.e.*, unobservability or loss of small transects), over and underprojection under slab sectioning, etc., proliferated in the literature from the early 1940's – for a survey in the area of neuroscience see Haug (1986).

Thus, William R. Thompson's papers were unfortunately overlooked for over fifty years – the too strict interpretation of stereology as inference 'solely from sections' probably contributed to this (Cruz-Orive, 1987a, p. 47).

## CONNECTIVITY

The Euler-Poincaré characteristic $\chi(Y)$ of a particle $Y \subset \mathbb{R}^2$, with smooth boundary $\partial Y$, is a topological invariant, that is, its value remains unchanged under continuous shape deformations of $Y$. If $Y$ encloses no cavities, then $\chi(Y)$ is related with the connectivity number $p$ (namely the number of 'tunnels', 'handles', or 'extra connections' of $Y$) via $\chi(Y) = 1 - p$. If $Y$ is a finite union of separate particles, possibly enclosing cavities, then

$$\begin{aligned} \chi(Y) &= N(\text{separate particles}) \\ &- N(\text{extra connections}) \\ &+ N(\text{enclosed cavities}) , \end{aligned} \qquad (22)$$

where $N(\cdot)$ denotes number. If all the particles are simply connected ($p = 0$), then $\chi(Y) = N(Y)$. In the notation of Gundersen *et al.* (1993), if a

disector of a suitably small thickness sweeps $Y$ with a fixed orientation, then it will encounter $I$ 'islands' (corresponding to convex tangent points), $B$ 'bridges' (corresponding to saddle points), and $H$ 'holes' (corresponding to concave tangent points of cavities). The characterization theorem of Hadwiger (1957) states that

$$\chi(Y) = \frac{1}{2}(I - B + H) \,. \tag{23}$$

Nyengaard and Marcussen (1993) applied the preceding formula to glomerular capillaries, and Youngs *et al.* (1994) to trabecular bone. For further refinements see Ohser and Nagel (1996). Hadwiger's representation is equivalent to the classical one (DeHoff, 1968), based on a sweeping plane, in which the counts of convex, saddle and concave tangent events corresponded to $I$, $B$, and $H$, respectively. However, the latter representation was based on the integral of Gaussian curvature via the Gauss-Bonnet theorem, whereby it was believed that the sweeping plane had to be isotropic – see also Weibel (1980), Serra (1982), or DeHoff (1987). Note that Eq. 23 holds for any sweeping orientation, even though each of the individual terms $I, B, H$ may vary for different orientations.

By analogy with Gundersen's unbiased frame to count particles in the plane, Bhanu Prasad's shell method allows the estimation of $\chi(Y)$ by subsampling quadrats in the plane (Bhanu Prasad *et al.*, 1989), or boxes in space (Bhanu Prasad and Jernot, 1991). Thus, the shell method may be used to count simply connected particles in the plane with systematic quadrats whenever the only information available lies within the quadrats (that is, when the unbiased frame cannot be used). The implementation of the shell method in space, however, presents similar difficulties as that of the unbiased brick. Wulfsohn *et al.* (2010a) coped with edge effects using resources analogous to the optical disector to progress on the difficult problem of counting ventilatory units in lung.

## PLANAR AREA AND VOLUME

To estimate the section area of a mineral component in a rock (*e.g.*, the numerator of Eq. 19), Delesse (1847) proposes the cut-and-weight method apparently prevailing at the time.

Rosiwal (1898, Fig. 1, Fig. 2) shows synthetic planar domains with a square grid superimposed on them but, instead of counting test points, he proposes to use a two dimensional version of Eq. 13 to estimate planar area via total line intercept length. In a footnote of p. 146 he uses integral calculus to show that, besides the Delesse identity $V_V = A_A$ leading to areal analysis, one may also use $V_V = \mathbb{E}\{L(Y \cap T)\}/L(T) = L_L$, where $T$ is a test line segment, or a bounded grid of lines, of total length $L(T)$. This led to the graphical lineal analysis in a picture – a big step in efficiency with respect to the weighing technique.

Thomson (1930) proposes the 'areal method' using grids "so arranged that one square or one segment represented 1 per cent of the total area, thus giving results directly in percentage values", (p. 200). The term "one segment" corresponds to an annulus fragment from a circular grid (p. 200, Fig. 8) which is not a proper test system. Careful reading supplies no evidence that Thomson's areal method involved point (or grid corner) counting. Perhaps the idea was to count grid squares or 'segments' inside the mineral components of interest in a section ("the magnification used being adapted to the size of the particles", p. 200). To support this, in pp. 202–203 one reads: "All of the 16 showing differences greater than 3 per cent were of the small scattered, irregular type mentioned above, where the estimation of the number of squares occupied by any one mineral was of necessity but approximate in character. For such cases the lineal method is recommended, and for ease of calculation the square instead of the circular grid should be used.". The "differences" (namely the raw deviations, with no reference to coefficients of error), referred to checking the method on a synthetic mixture of minerals with known proportions.

Glagolev (1933) uses integral calculus – similarly as Rosiwal (1898) – to show that $V_V = \mathbb{E}\{P(Y \cap T)\}/P(T) = P_P$, where $T$ is a bounded grid of $P(T)$ test points. In Fig. 9 and Fig. 10 he reproduces the aforementioned Fig. 1 and Fig. 2 from Rosiwal (1898), respectively, but now Glagolev explicitly proposes (apparently for the first time) to count corner points instead of measuring intercept lengths. Based on Henri Lebesgue's definition, the area of a planar set is commonly conceived as the limit of the sum of the areas of squares covering the set, as the size of each square tends to zero. This might have inspired E. Thomson's 'areal method'. On the contrary, A.A. Glagolev's definition of area is essentially statistical, namely the mean number of points of a UR test system hitting the set, times the tile area. Incidentally, Glagolev socialized with Andrei Kolmogorov, founder of modern probability.

In design stereology, the reference volume $V_0$ entering in Eq. 21 was measured in early times by the water displacement method (Weibel, 1963, p. 45). Thompson (1932, p. 21) suggests that $V$ may be approximated by a discretized version of the relevant integral in Eq. 2. Elias *et al.* (1971, p. 164) also indicate

that $V$ may be approximated by exhaustive slicing. The proper implementation of the Cavalieri design had to wait until the 1980's, see the references cited after Eq. 13.

## SURFACE AREA AND LENGTH UNDER ISOTROPY

The aforementioned dimensional conditions imply that surface area $S$ can be estimated with planar, or linear, probes, and curve length $L$ with planar probes, which are IUR relative to the object. The pertinent stereological equations were derived ad-hoc (without reference to equivalent, already known results of integral geometry), by material scientists, notably Saltykov (1946) and, independently, by Smith and Guttman (1953). Thus, the initial setup pertained to model stereology. Tomkeieff (1945) has often been cited in this context, but he only mentions two elementary results on mean linear intercept length.

Let $Y \subset \mathbb{R}^3$ represent indistinctly a stationary and isotropic surface process of intensity $S_V$, or a curve or fibre process of intensity $L_V$. The corresponding intersection $Y \cap T$ with a test plane $T$ is a stationary and isotropic process of trace curves of intensity $B_A$, or one of transect points of intensity $Q_A$, (from the German 'Querschnitt'), respectively. In the case of a surface process, if $T$ is a test line then $Y \cap T$ is a stationary process of intersection points of intensity $I_L$ on the line. The pertinent classical stereological equations read as follows,

$$S_V = (4/\pi)B_A,\ S_V = 2I_L,\ L_V = 2Q_A\ . \qquad (24)$$

The second Eq. 24 follows from the first because, by an argument analogous to that leading to Eq. 6, one has $B_A = (\pi/2)I_L$. On the other hand, the second and the third equations are dual of each other by interchanging the roles of process and probe.

The model based ratio estimators $\widehat{S}_V$, $\widehat{L}_V$ are unbiased because their denominators are non random, controlled measures. In the general design based case, however, (*e.g.*, Eq. 21), the target structure $Y$ is contained in a bounded reference set $X$, and the target ratio is $\gamma_V = \gamma(Y)/V(X)$, where $\gamma \in \{V,\ S,\ L,\ N\}$. As mentioned in Section *The general design*, now $\widehat{\gamma}_V$ will generally not be strictly unbiased but ratio unbiased, although the bias is usually unimportant in practice. For a stationary and isotropic volume process $Y$ with piecewise smooth boundary $\partial Y$, Saltykov (1967) considered the 'specific surface area' $S_V = S(\partial Y)/V(Y)$. On a planar section superimpose a multipurpose test system of test lines ($\Lambda_1$) and test points ($\Lambda_0$) with a ratio $l/p$ of test line length per test point. Then, the estimator

$$\widehat{S}_V = 2 \cdot \frac{p}{l} \cdot \frac{I(\partial Y \cap \Lambda_1)}{P(Y \cap \Lambda_0)}\ , \qquad (25)$$

is also ratio unbiased because the denominator is a random variable – the preceding formula is in fact typical of design stereology. Recall that, for $\gamma \in \{S,\ L\}$, unbiasedness, or ratio unbiasedness, requires that object and probe be IUR relative to each other. Mattfeldt *et al.* (1990) and Nyengaard and Gundersen (1992) described the 'orientator' and the 'isector' methods, respectively, to generate IUR sections. Gundersen (1979) considers the estimation of $V_V$, $S_V$, $L_V$ for non self-intersecting tubules, or solid cylinders, from IUR slab sections.

In the design based case $\gamma(Y)$ can often be estimated directly (*i.e.*, without resorting to ratios) by means of a three dimensional test system. We have seen that $V(Y)$ may be estimated with Cavalieri planes of a fixed orientation. In turn, $S(Y)$ and $L(Y)$ may be estimated with isotropic Cavalieri planes, and $S(Y)$ also with a 'fakir probe' of IUR parallel test lines (Cruz-Orive, 1993, proposed the fakir probe of a fixed orientation to estimate volume). Earlier, a IUR test system of test lines in three mutually perpendicular directions, called the 'spatial grid', had been proposed by Sandau (1987) – see also Cruz-Orive (1997). The latter probes may be implemented on a IUR stack of physical serial sections (Pache *et al.*, 1993). More efficient, however, is to use non invasive data acquisition supported by a proper software (Kubínová and Janáček, 2001; Kubínová *et al.*, 2003).

By analogy with the UR Merz grid to estimate curve length in the plane (Section *Sampling in a continuous domain*), curve length in space may be estimated with UR sphere probes (the 'spaceball' probe) aided by confocal microscopy, see Mouton *et al.*, 2002, and West, 2012).

## VERTICAL DESIGNS

A vertical plane (VP) is normal to an arbitrarily fixed plane (called the horizontal plane, HP) in such a way that the corresponding trace is a motion invariant line in the HP. At the 1st International Workshop in Stereology (DK-Aarhus, November 1981), E. Hasselager read a paper entitled "Stereological studies of the porcine placenta". The target was the exchange surface area and, apart from efficiency questions, it was suggested that sections normal to the relevant barrier would allow tissue recognition better than IUR sections. Weibel (1979, Section 3.6) also claimed this for most ordered tissues. The identity $S_V = (4/\pi)B_A$

requires IUR sections, however, and it was believed that using $S_V = 2I_L$ should require IUR test lines generated either directly in space, or on IUR planar sections. Motivated by this problem, A.J. Baddeley (personal letter of 21st March, 1982) noted that a motion invariant test line in space can always be embedded in a VP, and therefore surface area can always be estimated with test lines embedded in VPs. There are various approaches, see Baddeley (1984, 1985) and Baddeley *et al.* (1986).

1. *A posteriori weighted intersection counts.* Let $I(\theta)$ denote the number of intersections between the target surface and an invariant test line within a VP making a colatitude angle $\theta$ with the vertical axis (VA). Because the probability element of $\theta$ for a IR direction in space is $\mathbb{P}(\mathrm{d}\theta) = \sin\theta\ \mathrm{d}\theta$, to preserve unbiasedness the count $I(\theta)$ has to be weighted by a factor proportional to $\sin\theta$.

2. *A priori weighted test lines.* In a VP, sample a test line whose colatitude $\theta \in [0,\pi)$ has a probability density equal to $\sin\theta$, *i.e.*, take $\theta = \arccos(1-2U)$, where $U \sim \mathrm{UR}[0,1)$. Then the equation $S_V = 2I_L$ holds directly.

3. *A priori weighting with cycloids.* In a VP, use a test curve with length element $\mathrm{d}s(\theta) \propto \sin\theta\ \mathrm{d}\theta$, namely a cycloid with the minor axis parallel to the VA. Again $S_V = 2I_L$.

4. *Weighted rose of directions.* The intersection between the target surface $Y$ and a vertical plane is a vertical trace curve $C$, say. Let $\mathrm{d}y$ denote the arc element of $C$ at a point $y \in C$, and let $\psi := \psi(y) \in [0,\pi)$ denote the colatitude of the tangent to $C$ at $y$. Then, the integral of the functional

$$\lambda(C) = \frac{1}{\pi}\int_C \{\sin\psi + (\pi/2-\psi)\cos\psi\}\ \mathrm{d}y \quad (26)$$

over all vertical planes hitting $Y$, is equal to the surface area $S$ of $Y$, (Baddeley, 1985). For an application to digitized vertical sections see Cruz-Orive *et al.* (2014). For a connection with the rose of directions of $C$ see Section *Anisotropy* below.

Hilliard (1967, p. 225) showed a graph, and gave the coordinates of a few points, of a test curve which would estimate $S_V$ via $2I_L$ on a "longitudinal section" of a "specimen containing an axis of symmetry", with "the minor axis parallel to the axis of symmetry". See also Weibel (1979, Fig. 6.14). That curve was in fact a cycloid. Hilliard's unnecessary restriction was probably due to the fact that his main purpose was to characterize anisotropy by means of harmonic analysis. Earlier, Spektor (1960) derived and identified the test curve, but he also assumed that it would apply only to anisotropic structures, unfortunately. The principle is completely assumption free, and the vertical axis may be chosen as convenient depending on the structure.

Vertical sections are extensively used. Early applications involving physical sectioning include Cruz-Orive and Hunziker (1986) and Vesterby *et al.* (1987). Tissue recognition may not be easy on vertical sections in special cases. Dorph-Petersen (1999) handles this problem with reference to the human brainstem. Michel and Cruz-Orive (1988) consider total surface area estimation without intermediate ratios using vertical Cavalieri sections and cycloids. A vertical spatial grid, consisting of mutually perpendicular cycloid chains, was proposed by Cruz-Orive and Howard (1995). Virtual cycloids, suitable for optical sectioning on thick slices, were described by Gokhale *et al.* (2004), see also West (2012). Automatic vertical sectioning of computer renderings of human brain, automatic intersection counting with cycloids, automatic implementation of Eq. 26, and mathematical derivations, are given by Cruz-Orive *et al.* (2014).

A dual vertical design is that of vertical projections to estimate curve length in space. Here the probe is a cylindrical surface whose generator is a cycloid with the major axis parallel to the VA. The principle was discovered by Gokhale (1990) for vertical slices of a known thickness $t > 0$. Probe and target curve are projected together onto a vertical observation plane parallel to the slice and, in the absence of overlapping, the identities $L_V = 2Q_A = (2/t)I_L$ hold. For an application to capillary length see Batra *et al.* (1995). Cruz-Orive and Howard (1991) extended the method to estimate the finite length of a bounded curve using total vertical projections. Roberts *et al.* (1991) applied the method to estimate the length of blood vessels, Howard *et al.* (1992) to neuron dendrite length, and Wulfsohn *et al.* (1999) to plant root length in a transparent medium.

# INDIVIDUAL PARTICLE SIZING

## WEIGHTING BY NUMBER, AND BY SIZE

In a design based setting, the problem is to estimate the mean of an individual particle size parameter $X$ (which may stand for $V$, $S$, and occasionally for mean caliper length $H$) over a population of particles. The arithmetic mean $\mathbb{E}(X)$ is number weighted. In

stereology the $W$-weighted mean,

$$\mathbb{E}_W(X) = \sum_{i=1}^{N} W_i X_i \Big/ \sum_{i=1}^{N} W_i \,, \qquad (27)$$

plays an important role (Karlsson and Cruz-Orive, 1997). Here $W = 1, H, S, V$ according to whether the sampling probe is a UR disector, a IUR test plane, a IUR test line, or a UR test point, respectively. In practice IUR test systems are used. A particle is sampled with a random multiplicity $M$, namely the number of times the particle is hit by the test system, and the fundamental equations of stereology imply that $\mathbb{E}(M) = c \cdot W$, where $c$ is a known constant. As before, let $\mathscr{S}$ represent the subset of indexes corresponding to the sampled particles. Then,

$$\overline{X}_W = \sum_{i \in \mathscr{S}} M_i X_i \Big/ \sum_{i \in \mathscr{S}} M_i \,, \qquad (28)$$

is a ratio unbiased estimator of $\mathbb{E}_W(X)$. Further, each $X_i$ may be estimated by the mean of $M_i$ unbiased estimators $\{\widehat{X}_{ij},\ j = 1, 2, \ldots, M_i\}$ which should be independent from the sampling probe, hence from $M_i$. For instance, if we sample with a IUR test system of points and the $i$th particle is hit by $P_i$ test points, then from each hitting point we generate a IR chord to estimate its volume, see next section. Thus, a UE of $X_i$ is $\widehat{X}_i = \sum_{j=1}^{M_i} \widehat{X}_{ij}/M_i$, and therefore the final, ratio unbiased estimator of $\mathbb{E}_W(X)$ is,

$$\overline{x}_W = \sum_{i \in \mathscr{S}} \sum_{j=1}^{M_i} \widehat{X}_{ij} \Big/ \sum_{i \in \mathscr{S}} M_i \,, \qquad (29)$$

namely a ratio of sums over all the primary hits generated by the sampling probe in the sampled particles.

Particle sampling developed along the years following the publication of the disector (Sterio, 1984), *e.g.*, Gundersen and Jensen (1983, 1985), Jensen and Gundersen (1985), Gundersen (1986, Section 3), Cruz-Orive (1987b), Jensen (1987, 1991, 1998), and Baddeley (1999).

## SIZE ESTIMATORS OF LOCAL STEREOLOGY

In practice the usual target in particle stereology is the population mean volume $\mathbb{E}(V)$. If the probes are disectors, then $M_i = 1$ for every sampled particle, and Eq. 29 yields the ratio unbiased estimator $\overline{v} = \sum_{i \in \mathscr{S}} \widehat{V}_i / n$, where $n$ is the (random) number of sampled particles and $\widehat{V}_i$ is a UE of the volume of the $i$th sampled particle. For arbitrarily shaped particles, an option is to estimate $V_i$ with Cavalieri sections (independent from the disector itself, see Gundersen, 1986, Fig. 3.3), which is time consuming. The situation is simplified if each particle contains a single nucleus, or better a nucleolus, which can be used as the sampling unit in combination with the optical disector. This possibility motivated the development of local stereology (term coined by Jensen, 1998) which deals with IR test probes containing a fixed point, or a fixed axis, in space.

Except the invariator, the following methods may be implemented on vertical sections using sine weighted rays, or intercepts.

*The nucleator.* The volume of a convex particle $Y$ may be expressed by the nucleator formula $V = (4\pi/3)\mathbb{E}(l_+^3)$, where $l_+$ is the length of a IR radius vector emanating from an arbitrary point $O$ inside $Y$, (Santaló, 1976, Eq. 12.63). For a non convex particle the formula is easily adjusted, see Cruz-Orive (1987b, Eq. B.3). For simplicity, convexity is assumed in the following paragraphs without loss of generality. The corresponding volume estimator $\widehat{V}_i$ of the $i$th disector sampled particle yields an efficient estimator of $\mathbb{E}(V)$ via the aforementioned formula for $\overline{v}$. The practical implementation is due to Gundersen (1988), see also Tandrup (1993).

*Point sampled intercepts.* If $O$ is UR inside $Y$, then the point sampled intercept formula reads $V = (\pi/3)\mathbb{E}(l_0^3)$, (Miles, 1979, Gundersen and Jensen, 1983, 1985), where $l_0$ is the length of a IR intercept through $O$. Combination with Eq. 29 yields a ratio unbiased estimator of the volume weighted mean particle volume $\mathbb{E}_V(V)$, see the preceding references. For early applications see Howard (1986) and Brüngger and Cruz-Orive (1987).

*The selector.* Cruz-Orive (1987b) proposed the selector method to estimate particle number per unit volume via $\widehat{N}_V = \widehat{V}_V / \overline{v}$. Particles are sampled with disectors of unknown thickness (called 'selectors' in the paper). The volume of each sampled particle is estimated as $\widehat{V} = (\pi/3) \cdot \overline{l_0^3}$ with point sampled intercepts on an independent series of Cavalieri sections an unknown distance apart, and then $\overline{v}$ is computed as in the disector case. The method is time consuming, but it may be applied to arbitrary particles without a nucleus, or nucleolus, and it avoids section thickness measurement. For an application see McMillan and Sørensen (1992).

*The surfactor* is intended to estimate mean particle surface area (Jensen and Gundersen, 1987, 1989). For disector sampled particles bearing a nucleolus, the surfactor estimates $\mathbb{E}(S)$. The required

measurements on a IR section through the nucleolus (henceforth called a pivotal section) are (i) IR ray length $l_+$, and (ii) angle between the ray and the tangent to the particle boundary trace at the intersection point. For point sampled intercepts the surfactor estimates $\mathbb{E}_V(S)$ via $l_0$ and the corresponding angles – see also Karlsson and Cruz-Orive (1997, Section 5).

*The optical rotator* uses optical sectioning within a slab probe, which may be either IR through a fixed point in space, or IR around a fixed vertical axis. It may be used to estimate particle volume and surface area (Tandrup *et al.*, 1997).

*The invariator design* (Cruz-Orive, 2005, although the name 'invariator' was coined in Cruz-Orive, 2009) is based on a IR pivotal plane through a fixed pivotal point $O$ in space. The idea was inspired by a result of Varga (1935). According to Schneider and Weil (2008, p. 285) the underlying theory is implicit in a general formula of Petkantschin (1936). For general proofs see Gual-Arnau and Cruz-Orive (2009) and Auneau and Jensen (2010). The UEs of individual particle surface area and volume given below may be applied to estimate $\mathbb{E}(S)$, $\mathbb{E}(V)$ if the particles are sampled with disectors, or $\mathbb{E}_V(S)$, $\mathbb{E}_V(V)$ if point sampling is used, respectively.

1. *A priori weighted test lines.* On a pivotal plane superimpose a IUR test system of points of fundamental tile area $a$. Through each test point $P$ draw a test line normal to the axis $OP$: the result is a pivotal grid $\Lambda$. Let $Y \ni O$ be a particle of volume $V$ and surface area $S$. Then, the estimators

$$\widehat{S} = 2a \cdot I(\partial Y \cap \Lambda) \,,$$
$$\widehat{V} = a \cdot L(Y \cap \Lambda) \,, \qquad (30)$$

   are unbiased for $S$, $V$ respectively. For an application see Cruz-Orive *et al.* (2010).

2. *A posteriori weighting.* On the pivotal plane superimpose a IUR test system of parallel lines a distance $T$ apart. Then,

$$\widehat{S} = 2\pi T \sum_{k\in\mathbb{Z}} |r_k| I_k \,,$$
$$\widehat{V} = \pi T \sum_{k\in\mathbb{Z}} |r_k| L_k \,, \qquad (31)$$

   are also unbiased for $S$, $V$ respectively, where $r_k$ denotes the distance of the $k$th test line from $O$, and $I_k, L_k$ the number of intersections determined by the same test line with the pivotal trace of $\partial Y$, and the corresponding intercept length determined in the pivotal transect of $Y$, respectively, (Cruz-Orive and Gual-Arnau, 2015).

3. *The flower and the peak-and-valley formulae to estimate S.* The surface area of a convex particle $Y$ is 4 times the mean area (over isotropic orientations of the pivotal plane) of the flower of a pivotal section (Cruz-Orive, 2005). The flower (term coined by P. Calka in another context) of a planar convex set is the set enclosed by the graph of the support function. Thórisdóttir and Kiderlen (2014) and Thórisdóttir *et al.* (2014) have extended the result to arbitrary particles. Cruz-Orive and Gual-Arnau (2015) give a simplified formula called the peak-and-valley formula – for a general treatment see Gual-Arnau and Cruz-Orive (2016).

4. *The pivotal conjecture.* Conjecture 4.1 of Gual-Arnau *et al.* (2010) states that, conditional on a given pivotal section, the mean of all possible estimators of $V$ have a unique expression, and similarly for $S$. For an arbitrary particle, Cruz-Orive (2012) proved that the integral of any invariator volume estimator over a given pivotal section coincides with that of the nucleator. Also, for a convex particle the integral of the surfactor estimator over a given pivotal section coincides with the flower area of the section. The general conjecture remains open, however, because the examined estimators need not be the only possible ones.

*Pappus-Guldin volume estimators.* The volume $V$ of a bounded set $Y \subset \mathbb{R}^3$ can be represented from IR planar sections containing a fixed, arbitrary axis. Cruz-Orive and Roberts (1993) applied the estimation method (called the coaxial sections method) to human bladder. Independently, Jensen and Gundersen (1993) considered a vertical axis through a fixed pivotal point $O \in Y$, coined the term rotator, and applied it to neurons with a nucleolus, see also West (2012).

# SIZE AND ORIENTATION DISTRIBUTIONS

## PARTICLE SIZE

As discussed in Section *Particle number*, size distributions are inaccessible from information solely on independent planar or linear sections unless the particles are assumed to have a simple geometric shape such as the sphere.

For arbitrary particles, size distributions of interest are with respect to number, or to volume (Gundersen and Jensen, 1983, Gundersen, 1986, see also Howard and Reid, 2005, Section 9.3). The former requires UR

disector sampling, the latter UR point sampling. In a second step, the relevant size (usually the volume) of each sampled particle has to be measured as accurately as possible – otherwise the error arising from the individual volume estimation will be confounded with the natural variation among particles. A method of choice is Cavalieri's, see for instance Sørensen (1991). Nowadays non invasive scanning methods should simplify the task. Local stereology methods require isotropy and may not be accurate enough for that purpose. If only the mean and the coefficient of variation of particle size are needed, then it is not necessary to estimate the size distribution because $\mathrm{CV}^2(V) = \mathbb{E}_V(V)/\mathbb{E}(V) - 1$, (Gundersen and Jensen, 1985).

## MEMBRANE THICKNESS

For estimation purposes a membrane or 'sheet' is the portion of space comprised between two smooth, not interpenetrating surfaces called faces. The local sheet thickness $\tau(y)$ at a point $y$ of a face is the distance of the other face from $y$. A conventional definition of mean sheet thickness is $\mathbb{E}(\tau) = 2V/S$, where $V$, $S$ are the total sheet volume and the total surface area of the faces, respectively. For a IUR test line hitting the sheet, $S_V = 2I_L$, and because $\mathbb{E}(I) = 2\mathbb{E}(N)$, where $N$ is the number of intercepts, we have $\mathbb{E}(\tau) = (1/2)\cdot\mathbb{E}(l)$, where $l$ is the intercept length (Weibel and Knight, 1964, Gundersen *et al.*, 1978). Relevant to membrane diffusion is the harmonic mean thickness $\mathbb{E}(1/\tau) = (3/2)\cdot\mathbb{E}(1/l)$. In the latter two papers the preceding identity was derived using a sheet model in which the face elements in the neighbourhood of a point $y$ of a face are essentially planar and parallel a distance $\tau(y)$ apart. This model also allows to unfold the distribution of $\tau$ from that of $l$, (Gundersen *et al.*, 1978, Cruz-Orive, 1979). Further, the trace of a sheet with a IUR plane is a curved stripe of local thickness $r(y)$. The latter author, and independently Jensen *et al.* (1979) also solved the unfolding problem in this case. From the moment relations derived in the last two papers, $\mathrm{Var}(\tau)$ can be estimated directly from planar sections – but not from linear sections – without unfolding, whereas $\mathrm{Var}(1/\tau)$ can be estimated directly in either case. See also Weibel (1980, Ch.9).

## ANISOTROPY

For a planar smooth curve $Y \subset \mathbb{R}^2$ of length $B$, let $\psi = \psi(y) \in [0,\pi)$ be the angle of the tangent to $Y$ at the point $y \in Y$ with a fixed axis, as in Eq. 26, and let $\delta(\psi) = (\psi, \psi + \mathrm{d}\psi]$ denote the arc element at $\psi(y)$. The rose of directions of $Y$ is given by the probability element $\mathbb{P}(\mathrm{d}\psi)$ of $\psi$, namely

$$\mathbb{P}(\mathrm{d}\psi) = L\{y \in Y :\ \psi(y) \in \delta(\psi)\}/B\,. \qquad (32)$$

For a smooth curve $Y \subset \mathbb{R}^3$ of length $L$, the direction of the tangent at a point $y \in Y$ is represented by a point $u = u(y) \in \mathbb{S}^2_+$ of the unit hemisphere $\mathbb{S}^2_+$. Let $\delta(u)$ denote the area element of $\mathbb{S}^2_+$ at the point $u(y)$. Then, the rose of directions of $Y$ is given by

$$\mathbb{P}(\mathrm{d}u) = L\{y \in Y :\ u(y) \in \delta(u)\}/L\,. \qquad (33)$$

Finally, the rose of directions for a smooth surface $Y \subset \mathbb{R}^2$ of area $S$ is defined similarly with $u(y)$ representing the orientation of the normal to $Y$ at a point $y \in Y$.

Details on the foregoing definitions can be found in Weibel (1980, Ch. 10), see also Serra (1982), or Stoyan *et al.* (1995) for the model based approach. For the non-parametric inference of the rose of directions we may consider the first three possibilities below – the fourth one is semiparametric.

1. *Direct methods.* The rose of directions of a bounded and smooth planar curve can be estimated directly with arbitrary accuracy by scoring tangent counts with a sweeping line at systematic angles with an arbitrarily small period. Systematic sampling with thin Cavalieri disectors is an alternative to exhaustive sweeping and counting. The extension to 3D with sweeping planes, or systematic disectors, is possible at least in theory. Ohser (1981) proposed to measure $\psi(y)$ at typical points sampled in the curve with test circles (the Merz grid may be used). Mattfeldt *et al.* (1994) used confocal laser microscopy to sample glass fibres with parallel virtual planes a known distance apart. Following Stoyan (1985) the empirical fibre orientation distribution was reconstructed using the pertinent corrections, because a fibre element is sampled with a probability proportional to the length of its orthogonal projection onto the normal to the sampling plane.

2. *Rose of intersections methods.* The rose of intersections of a planar curve, or of a surface in 3D, at a given orientation, is the mean number of intersections determined by a test line with that orientation, per unit length of test line. For a curve in 3D a test plane is used. The roses of directions and intersections are related by an integral equation (usually refereed to as the 'cosine', or 'Buffon', transform), and the problem is to recover the rose of directions from the observable rose of intersections. Hilliard (1962, 1967) used Fourier and harmonic analysis in 2D and 3D respectively. Kanatani (1984) used tensor analysis. For detailed surveys see Stoyan *et al.* (1995) and Beneš and Rataj (2004).

3. *Unfolding methods.* For 3D curves, or surfaces, exhibiting axial, or lamellar, anisotropy, the rose of directions may be assumed to be symmetric about a fixed vertical axis. In this case the only target is the probability element $\mathbb{P}(\mathrm{d}\theta)$ of the colatitude angle $\theta$ of a relevant direction $u = (\phi, \theta) \in \mathbb{S}^2_+$. The natural probe is a vertical plane of section for a surface, or a vertical projection plane for a curve. In either case the result is a planar curve whose rose of directions $\mathbb{P}(\mathrm{d}\psi)$ is observable. The latter may be expressed in terms of $\mathbb{P}(\mathrm{d}\theta)$ by an integral equation which can be inverted analytically, and the empirical $\mathbb{P}(\mathrm{d}\theta)$ can be unfolded numerically. Scriven and Williams (1965) used this approach and applied it to copper specimens. Gokhale (1996) and Beneš *et al.* (1996) revised the method and applied it also in materials science.

4. *Semiparametric methods.* For structures exhibiting a single axis of anisotropy, an alternative option is to model $\mathbb{P}(\mathrm{d}\theta)$ by a parametric class of directional distributions. The model is 'semiparametric', however, inasmuch as the structure itself is not parametrized (Jensen *et al.*, 1985). Motivated by the problem of estimating onion root length, Baldwin *et al.* (1971) proposed to model $\mathbb{P}(\mathrm{d}\theta)$ by the Dimroth-Watson model (*e.g.*, Mardia, 1972, Weibel, 1980, Ch. 10). Mathieu *et al.* (1983) applied the model to estimate length density of skeletal muscle capillaries, and Mattfeldt and Mall (1984) to myocardial capillaries. The model depends on a single 'concentration parameter' $\kappa$ which can be estimated from vertical and horizontal sections only, and provides a measure of anisotropy and (generally not unbiased) estimators of surface area and length densities. Cruz-Orive *et al.* (1985) completed technical details and checked the model for capillary length and surface densities, and Cruz-Orive and Hunziker (1986) did it for chondrocyte surface area. This approach avoided the use of IUR sections, but it was gradually superseded by the vertical designs.

## SECOND ORDER STEREOLOGY

The mean of a random variable is a first order property, whereas the variance is a second order property. Likewise, the intensity $V_V$ of a stationary volume process $Y$ is a first order property of $Y$, see Section *Model based stereology*. Actually, the probability that the origin $O$ is covered by $Y$ is $V_V$. However, the probability $C(r)$ that two points a distance $r$ apart are covered by a second order stationary and isotropic volume process $Y$, is a second order property, called the scalar non centred covariance function of $Y$, which constitutes a partial descriptor of the shape of $Y$. Matérn (1960) and Matheron (1967) pioneered these concepts, see also Serra (1982). From $V_V$ and $C(r)$, other second order descriptors can be derived, *e.g.*, the $K$-function $K(r)$, which is $V_V^{-1}$ times the mean volume of $Y$ in a ball of radius $r$ centred a typical point of $Y$, and also the radial distribution function $R(r)$, the pair correlation function $g(r)$, etc. Initially the latter descriptors were developed mainly for point processes (Ripley, 1977). Similar functions can be defined for surface, or curve, processes (Ripley, 1981, Cruz-Orive, 1989a, Stoyan *et al.*, 1995).

For a volume process, $K(r)$ can be estimated from planar sections with the nucleator, see Cruz-Orive (1989a), or via $C(r)$, see Mattfeldt *et al.* (1993). Point processes in 3D, however, require 3D probes. Braendgaard and Gundersen (1986) analysed neuron centroid patterns by mapping their 3D coordinates via physical serial sections. Baddeley *et al.* (1987) analyzed osteocyte lacunae using non invasive scanning with laser microscopy. Proper edge corrections for block sampling were developed by Baddeley *et al*. (1993). To estimate the $K$-function of a point process, coordinate recording can be avoided by using a IR disector through a fixed point (Gundersen *et al.*, 1988, Evans and Gundersen, 1989). The latter method is part of a scheme of local stereology developed by Kiêu and Jensen (1993) to estimate $V$, $S$, $L$, or $N$, and the corresponding second order properties, with virtual probes hitting a IR slice through a fixed point. Stark *et al*. (2011) proposed the saucor method to gain efficiency in the estimation of the $K$-function of a point process, and applied it to estimate the radial number density of glial cells around neurons.

## EFFICIENCY

### PLANNING A STEREOLOGICAL DESIGN

A standard problem in design stereology is to look for a statistically significant difference between control and treated population means. The subsidiary question is how to optimize the group sample sizes, and the amount of stereological work invested within each primary sampling unit (animal, or whatever laboratory specimen), to achieve that goal. By the late 1970's the within-primary-unit, 'single organ' designs (Eqs. 20, 21) were increasingly well understood from the work

of R.E. Miles and P.J. Davy, but the ordinary statistics necessary to deal with groups of animals was not yet commonplace in stereological practice – this was felt at the early ISS Courses. Group size can be estimated for instance from Snedecor and Cochran (1980, Section 6.14). Shay (1975) considered a nested design (see Section 21.10 of the preceding book), and Gundersen and Østerby (1981) adapted these ideas to stereology. For a multivariate update see Cruz-Orive *et al.* (2004).

The target quantity $\gamma$, (*e.g.*, the total number of neurons in a brain compartment) will have a biological variance $\mathrm{Var}_b(\gamma)$ among brains. For each brain, a UE $\widehat{\gamma}$ of $\gamma$, with within brain error variance $\mathrm{Var}_w(\widehat{\gamma})$, is available by stereology. Let $\mathbb{E}_b\{\mathrm{Var}_w(\widehat{\gamma})\}$ denote the corresponding mean error variance between brains. Then,

$$\mathrm{Var}_b(\widehat{\gamma}) = \mathrm{Var}_b(\gamma) + \mathbb{E}_b\{\mathrm{Var}_w(\widehat{\gamma})\} \,. \qquad (34)$$

The group size required to detect a difference of at least $\delta$ between group means may be estimated by $2c^2 \cdot \mathrm{Var}_b(\widehat{\gamma})/\delta^2$, where $c^2$ is a constant depending on the significance level and on the test power adopted – usually $c^2 \approx 10$. Thus, $\mathrm{Var}_b(\widehat{\gamma})$ should ideally be less than $\delta^2$. If the biological variance is large, then by virtue of Eq. 34 little can be done except working with less variable brains. If the biological variance is small, but the within brain error variance is too large, then in order to decrease it the stereological sampling should be intensified within each brain – the question is where and how much (how many Cavalieri slices, disectors, or cells?). To answer these questions, theoretical predictors of $\mathrm{Var}_w(\widehat{\gamma})$ under systematic sampling can be helpful, see the next subsection; in the aforementioned papers sampling was assumed to be independent.

Up to the late 1970's, a controversial issue was the choice between manual and automatic, or semiautomatic, methods to measure sections. Mathieu *et al.* (1981) and Gundersen *et al.* (1981) used resampling experiments to show the higher efficiency of manual point and intersection counting in the cases studied. Incidentally, in Fig. 3 of the former paper the fundamental tile was not properly defined, hence Fig. 4 reveals bias for small test point and line densities. This does however not affect the main conclusions.

Contrary to intuition, point counting can be more precise than intercept length, or section area measurements, to estimate area, or volume. Jensen and Gundersen (1982) showed by resampling that the number of corners of a UR square hitting a disk may be more precise than the intersection area between the square and the disk for estimating the area of the disk. The point is that the square, and its four corners, are two unrelated test systems: the number of test points hitting the disk does not estimate the intersection area between square and disk, hence the Rao-Blackwell theorem does not apply (Baddeley and Cruz-Orive, 1995). Likewise, the number of end points of a test segment of length $l$ hitting a disk may be more precise than the corresponding intercept length to estimate the area of the disk. However, using a pair of test points a fixed distance $l$ apart is always less efficient than using a test segment of length $2l$. This is because now the pair of test points can slide freely inside the test segment, scanning it entirely, and therefore the number of test points in the disk can estimate the intercept length – thus the Rao-Blackwell theorem does apply in this case. This, and various other examples, are studied by Voss and Cruz-Orive (2009), who give exact expressions for the relevant variances. A general theory is still lacking for this topic, however.

## ERROR VARIANCE PREDICTION UNDER SYSTEMATIC SAMPLING

For a discrete, finite population, the variance of the UE of the population total under simple random sampling is well known, *e.g.*, Cochran (1977, Eq. 2.13). Under systematic sampling, however, the problem is non trivial because, unless the population is itself a random permutation, the sampled items are correlated to unknown degrees depending on the population pattern.

The most studied kind of systematic sampling is Cavalieri sampling along a fixed axis – the corresponding estimator has the form of Eq. 13. An exact representation of $\mathrm{Var}(\widehat{V})$ is easily available (Moran, 1950, Eq. 2), but not suitable for estimation. Because $\widehat{V}$ is a periodic function of period $h$, a more convenient formula can be written in terms of the Fourier transform of the measurement function $A$, (Moran, 1950, Eq. 4). As such, this formula is not useful either unless the function $A$ is known explicitly. Matheron (1965, 1971) wrote the preceding formula in terms of the Fourier transform of the covariogram $g$ of $A$, and made a significant progress by noting that the trend of $\mathrm{Var}(\widehat{V})$ as a function of $h$ was explained by the behaviour of $g$ near the origin. He therefore modelled $g$ near the origin by a polynomial of arbitrary degree. Gundersen and Jensen (1987) adopted a polynomial or degree 2, and derived an explicit variance approximation which was extensively used. For a survey of G. Matheron's transitive theory see Cruz-Orive (1989b).

Souchet (1995), Kiêu (1997) and Kiêu *et al.* (1999) related the degree of Matheron's polynomial model

with the shape (more precisely with the smoothness constant $q \in [0,1]$) of the function $A$, and improved Gundersen and Jensen's estimator (which implied the value $q = 0$) with a numerical coefficient depending on $q$. On empirical grounds supplied by Neil Roberts, Cruz-Orive (1993) had considered the alternative $q = 1$ for fairly smooth measurement functions, but Kiên Kiêu's theory significantly contributed to better understand the problem. Cruz-Orive (1993) and Gundersen *et al.* (1999) considered also local measurement errors. Gual-Arnau and Cruz-Orive (1998) extended the variance predictors to Cavalieri slabs for $q = 0,\ 1$, and Cruz-Orive (1999) incorporated local errors. Later, Cruz-Orive (2006) considered slabs, local errors, and an arbitrary $q \in [0,1]$. The main snag is that the estimation of $q$ from sample data is very unstable (García-Fiñana and Cruz-Orive, 2004), hence the practical problem remains basically open.

A related predictor, useful to estimate particle number with Cavalieri slabs, or with the fractionator, is the splitting design (Cruz-Orive, 2004, Cruz-Orive and Geiser, 2004, Maletti and Wulfsohn, 2006).

If the target is planar area, or volume, of a bounded object, useful variance approximations are available for test systems which have to be not only UR but also IR relative to the object (*e.g.*, isotropic Cavalieri lines, stripes, planes, or slabs; test points, quadrats, or boxes; fakir test lines, etc.). In this case the treatment is simplified because the relevant covariogram is the geometric covariogram of the object, which is linear in $h$ near the origin. The idea is also in Matheron (1971), and it was exploited by Gundersen and Jensen (1987) to predict the variance of the point counting estimator of area. The theory was extended by Kiêu and Mora (2006, 2009), who developed the package pgs of the statistical software R (http://www.r-project.org/) to compute the relevant coefficients – for a survey see Cruz-Orive (2013). The development of analogous, readily applicable variance predictors for surface area estimators remains an open problem.

If the distance $h$ between sections is not constant, then a sufficient condition that the Cavalieri estimator (Eq. 13) remains unbiased is that the distribution of $h$ is stationary, in which case $h$ may be replaced with its sample mean (Pache *et al.*, 1993, Appendix). The subject has attracted further attention over the last ten years, see Kiderlen and Dorph-Petersen (2017) and references therein.

The variance under systematic sampling on the circle was studied by Gual-Arnau and Cruz-Orive (2000) using a positive definite polynomial to model the entire covariogram. The predictors were applied and compared in Cruz-Orive and Gual-Arnau (2002) with the classical Matheron's predictors, with no significant improvement, see also Hobolth and Jensen (2002). Gual-Arnau and Cruz-Orive (2002) proposed a sampling design on the sphere, and derived error variance predictors, which have recently been shown to perform poorly (González-Villa *et al.*, 2017).

## RECENT TRENDS

In recent years advanced computing and image processing are playing an increasing role in stereology (Ohser and Schladitz, 2009). A trend is to model microstructures by random set models such as tessellations (Redenbach *et al.*, 2011), to estimate the empirical distribution of fibre orientation (Wirjadi *et al.*, 2016), *etc*. The idea is to obtain some information about shape and anisotropy in addition to the parameters supplied by classical stereology. One often misses, however, a cross check of the models with stereological estimators, which are known to be unbiased and model free.

The preceding techniques are not that common in biology. Point processes in 3D have been around for a long time (see Section *Second order stereology*) and the R package spatstat, first implemented in 2005, is a powerful tool (Baddeley *et al.*, 2015). The main snag may be that 3D data acquisition is expensive in ordinary experiments.

Volume tensor analysis, a concept related to principal component analysis of multivariate statistics, is also getting fashionable, and it can be implemented on sections – Rafati *et al.* (2016) describe a neuroscience application, see also Jensen and Kiderlen (2017). An early, related idea was suggested by Cruz-Orive *et al.* (1992).

Another computer oriented trend is to vectorize the boundary of real objects by fine triangulations, and to implement automatic Monte Carlo resampling on volume renderings of them, in order to check the performance of error variance predictors developed for systematic sampling over the years (Cruz-Orive *et al.*, 2014, Gómez *et al.*, 2016). The conclusions will lack generality because they are specimen dependent, but the technique may help to discard options (González-Villa *et al.*, 2017).

The unbiased counting frame (Gundersen, 1977, see Subsection *Particle number*) has been applied in a non stereological context to estimate the size of a population of particles (*e.g.*, human, or animal crowds, trees, etc.) on an essentially flat surface – ideally on an aerial photograph (Cruz *et al.*, 2015). Automatic methods to detect human faces have been tried, but

they have proved to be strongly biased in general. The ratio method based on the identity $N = N_A \cdot$ (Reference area) has also been tried by modelling the crowd by a realization of a stationary planar point process with number intensity $N_A$. The estimation of $N_A$ with arbitrary quadrats usually fails because crowd pictures exhibit perspective artifacts and cannot be assumed to correspond to a stationary process. Also, empty quadrats have to be taken into account and, last but not least, no objective way exists to define the reference area. In the latter paper the direct identity $N = (a/a') \cdot \mathbb{E}(Q)$ is used, where $a/a'$ is the sampling period corresponding to a UR test system of quadrats, and $Q$ is the number of units directly sampled with the test system. An error variance predictor is proposed whose performance is checked by automatic Monte Carlo resampling.

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