The saucor, a new stereological tool for analysing the spatial distributions of cells, exemplified by human neocortical neurons and glial cells

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Summary

The 3D spatial arrangement of particles or cells, for example glial cells, with respect to other particles or cells, for example neurons, can be characterized by the radial number density function, which expresses the number density of so-called 'secondary' particles as a function of their distance to a 'primary' particle. The present paper introduces a new stereological method, the saucor, for estimating the radial number density using thick isotropic uniform random or vertical uniform random sections. In the first estimation step, primary particles are registered in a disector. Subsequently, smaller counting windows are drawn with random orientation around every primary particle, and the positions of all secondary particles within the windows are recorded. The shape of the counting windows is designed such that a large portion of the volume close to the primary particle is examined and a smaller portion of the volume as the distance to the primary object increases. The experimenter can determine the relation between these volumina as a function of the distance by adjusting the parameters of the window graph, and thus reach a good balance between workload and obtained information. Estimation formulae based on the Horvitz–Thompson theorem are derived for both isotropic uniform random and vertical uniform random designs. The method is illustrated with an example where the radial number density of neurons and glial cells around neurons in the human neocortex is estimated using thick vertical sections for light microscopy. The results indicate that the glial cells are clustered around the neurons and the neurons have a tendency towards repulsion from each other.

1. Introduction

Biological tissue is not fully described by first-order quantities like the mean number of cells per volume, but is also characterized by the spatial arrangement of cells. The necessity to quantify 3D spatial relationships has been stated in many medical fields such as embryology (Chandebois, 1976), oncology (Mattfeldt et al., 1993a,b) and diabetic nephropathology (Mayhew, 1999).

In the present paper, we analyse the spatial distribution of glial cells around neurons in different subregions of the human neocortex. According to the classical view of the nervous system, glial cells play an inferior role in that they simply provide an ideal environment for neuronal cell function. However, research has shown that glial cells are intimately involved in the active control of neuronal activity and synaptic transmission. Any change in the spatial arrangement of glial cells with respect to the neurons may reflect functional changes in the relationship between them. Because the functions of the glial cell subtypes are different (Berry & Butt, 1997; Araque et al., 1999; Ullian et al., 2001), the spatial arrangement of glial cell subtypes around neurons should be expected to differ as well.

Because the methods presented below are not restricted to glial cells and neurons but can be applied to any kind of cells or particles, we will refer to the neurons as 'primary' cells and to the glial cells as 'secondary' cells in what follows. This does
not mean that primary and secondary particles necessarily have to belong to different types. In fact, we have also analysed the distribution of neurons around other neurons in the practical part. The spatial distribution of secondary cells around primary cells is described by the so-called radial number density function \( N_{ij} \). The quantity \( N_{ij}(r) \) can be understood as the average number of secondary cells in shells limited by spheres of radii \( r - \delta \) and \( r + \delta \) around randomly picked primary cells, divided by the volume of the shell, with an infinitesimally small \( \delta \). The symbol \( N_i \) was adapted from the accepted stereological notation for number density. The indices \( i \) and \( j \) indicate the two types of cells, primary and secondary cells, respectively, as is common practice in the statistics of bivariate point processes.

\( N_{ij} \) is closely related to well known second-order functions from the theory of spatial point processes. In particular, it can be seen as an non-normalized version of the bivariate pair correlation function, also known as radial distribution function, see Section 2. Second-order functions are popular tools in the statistical analysis of spatial point patterns. Consequently, extensive writings exist on applications and estimation of these functions, for summaries see e.g. the books by Stoyan et al. (1995), Diggle (2003) or Illian et al. (2008). However, almost all applications concentrate on the 2D case. The few exceptions include the paper by Hanisch and Stoyan (1981), who propose a method for the stereological estimation of 3D second-order functions from planar sections under the additional assumption that the particles or cells are spherical. The studies by Baddeley et al. (1987, 1993) investigate the spatial arrangement of osteocytes in bone by registering the positions within thick ‘bricks’ with the help of a tandem scanning reflected light microscope. Jensen et al. (1990) provide a unified mathematical theory for the general \( n \)-dimensional case.

Schmitz et al. (2002) examine the effect of irradiation to the brain by characterizing the mutual spatial arrangement of neurons by means of nearest neighbour distributions. This is another popular statistic for spatial point patterns, capturing different aspects of the spatial arrangement than the second-order functions do. Again its estimation requires knowledge of cell positions in thick brick-shaped sections – the authors used 150-µm-thick cryostat sections. By contrast to the estimation of \( N_{ij} \) it does not allow for compensation of the missing information when only thinner sections are available.

The need for laborious registration of the positions of cells or particles in thick bricks has possibly been one of the main hindrances to applying second-order functions in the analysis of 3D structures. We therefore propose a new method that limits the sampling to thinner physical or optical sections, and moreover further reduces the data collection effort by restricting measurements to small subsets around primary cells. These subsets are called ‘saucers’ after their shape. The saucer sampling scheme extends earlier work of Evans and Gundersen (1989), who estimated \( N_{ij} \) from the positions of all secondary cells with given distance to a primary cell observed in a disector. The idea of using only a small subset around the primary cell was later introduced in Evans (2004). The shape of the saucer window appeared first in Stark et al. (2007), where the corresponding estimation method was called ‘saucer’. For details on the earlier estimators see Section 5. Unfortunately, none of them is unbiased. With the present paper, we therefore propose a corrected estimator and explain its mathematical background.

Saucer sampling can be used with isotropic uniformly random (IUR) sections as well as with vertical uniformly random (VUR) sections, thus enabling its application to tissue that is preferentially examined by vertical sections, such as the brain. We provide estimation formulae for the neighbour density function for both sectioning schemes. The estimators have been implemented in the CAST-GRID® software package (Visiopharm, Hrsholm, Denmark) for semi-automatic image analysis, which greatly facilitates the measurements and the computations.

2. The radial number density function \( N_{ij} \) and its relation to other second-order summary functions

In order to describe the spatial arrangement of secondary particles with respect to primary particles, we will reduce the particles to points in the following. Thus we deal with the description of the mutual arrangement of two point patterns, representing primary and secondary particles. The average radial number density \( N_{ij}(r_1, r_2) \) is defined as the expected number of secondary points with distance between \( r_1 \) and \( r_2 \) to a uniformly randomly sampled primary point, divided by the volume of the shell limited by spheres of radii \( r_1 \) and \( r_2 \), which is equal to \( 4/3 \pi (r_2^3 - r_1^3) \):

\[
N_{ij}(r_1, r_2) = \mathbb{E}\left[ \frac{\text{# secondary points within distance } r_1 \text{ and } r_2}{\text{to a uniformly sampled primary point}} \right] \frac{4/3 \pi (r_2^3 - r_1^3)}{N_{ij}(r_1, r_2)}
\]

(1)

If the pattern of secondary points is completely independent of the primaries, \( N_{ij} \) is constant and equal to the overall number density \( N_{ij} \) of secondary particles. High values of \( N_{ij} \) for small distances indicate clustering of secondary particles around primary particles. Conversely, repulsive behaviour results in small initial values of \( N_{ij} \). In general, we will observe that \( N_{ij} \) approaches \( N_{ij} \) for large distances. When dealing with real particles or cells, \( N_{ij} \) always vanishes for very small distances because cell or particle centres cannot come closer than the sum of the radii of the respective primary and secondary cells or particles. The different cases of spatial interaction between primary and secondary particles are illustrated in Fig. 1.

A continuous version of the radial number density of secondary points as a function of the distance to the primary
is obtained by considering infinitesimally thin shells, limited by spheres of radii \( r_1 = r - \delta \) and \( r_2 = r + \delta \), namely
\[
N_{ij}(r) := \lim_{\delta \to 0} N_{ij}(r - \delta, r + \delta).
\] (2)

The functions \( N_{ij} \) defined by Eqs (1) and (2) are closely related to the bivariate \( K \)-function \( K_{ij} \) and the bivariate pair correlation function \( g_{ij} \). These functions have a long standing tradition in the statistical analysis of stationary spatial point processes consisting of several types of points, for summaries see e.g. the books by Cressie (1993), Stoyan et al. (1995), Diggle (2003) or Illian et al. (2008). The bivariate \( K \)-function \( K_{ij}(r) \) is defined as the expected number of points of type \( j \) in a sphere of radius \( r \) around a type \( i \) point, divided by the number density of type \( j \) points. In the setting of infinite stationary point processes, the \( K \)-function thus relates to \( N_{ij} \) via
\[
K_{ij}(r) = \frac{N_{ij}(0, r) \cdot \frac{4}{3} \pi r^3}{N_i}.
\] (3)

The derivative of the \( K \)-function, normalized by the surface area of a sphere of radius \( r \), is the popular bivariate pair correlation function \( g_{ij}(r) \), and is given by
\[
g_{ij}(r) = \frac{N_{ij}(r)}{N_i}.
\] (4)

In recent years, a 2D version of \( N_{ij} \), the so-called Wiegand–Moloney ring statistic (Wiegand & Moloney, 2004), has furthermore become popular in ecology to describe the spatial arrangement of plants.

The functions introduced above are in the literature referred to as ‘second-order summary functions’. Another popular way to characterize the spatial arrangement of points by means of their distances is the (bivariate) ‘nearest neighbour distance distribution function’ \( D_{ij}(r) \), which is the probability that at least one type \( j \) point can be found within distance \( r \) to a uniformly selected type \( i \) point. Although \( D_{ij} \) can also be used to detect clustering or repulsion, there is no one to one correspondence to second-order summary functions, that is \( D_{ij} \) and e.g. \( N_{ij} \) or \( K_{ij} \) capture different aspects of the spatial arrangement.

3. Estimation of radial number density using saucor sampling

3.1. The general estimation principle for \( N_{ij}(r_1, r_2) \)

The radial number density \( N_{ij} \) is defined as the expected number density of secondary particles around a uniformly sampled primary particle, which means that all primary particles contribute equally to \( N_{ij} \). Therefore we start by sampling primary particles with equal probability. This is achieved using a dissecter design (Sterio, 1984; Gundersen, 1986), which combines uniform random thick sections with a systematic uniform random positioned unbiased counting frame. In the second step, the number of secondary particles in the shell with radius between \( r_1 \) and \( r_2 \) around each sampled primary is separately estimated from the same thick section using an efficient unbiased estimator that is described in detail below. This second estimator requires that the thick sections be uniformly randomly orientated. Depending on the situation, the experimenter may choose either isotropic uniform random sections (IUR, randomly rotated around two axes, see Miles & Davy, 1976) or vertical uniform random sections (VUR, randomly rotated around an identifiable axis, see Baddeley,
Although generation of VUR sections is a relatively straightforward process as described in Baddeley et al. (1986), preparation of IUR sections requires special methods such as the ‘orientator’ (Mattfeldt et al., 1990) and the ‘isector’ (Nyengaard & Gundersen, 1992).

The estimator for \( N_{ij} \) is then simply obtained by averaging over the individual results. Formally, let \( n \) denote the number of primary particles sampled in the first step, and \( \hat{N}_{ij}(c_i; r_1, r_2) \) the estimated number of secondary particles with distance between \( r_1 \) and \( r_2 \) to the \( i \)th primary particle located in point \( c_i \), then the proposed estimator reads

\[
\hat{N}_{ij}(r_1, r_2) = \frac{1}{n} \sum_{i=1}^{n} \frac{\hat{N}_{ij}(c_i; r_1, r_2)}{4/3\pi (r_2^3 - r_1^3)}.
\]

Because the primary particles are sampled with equal probability and their number \( n \) is random, the estimator \( \hat{N}_{ij}(r_1, r_2) \) is ratio unbiased, which means that it is the ratio of two unbiased estimators. Many popular stereological estimators share this property. For a more detailed discussion and a formal proof, see the Appendix.

We now turn to the unbiased estimation of the number \( N_j(c_i; r_1, r_2) \) of secondary particles around the \( i \)th primary. In order to determine whether or not a secondary particle found in the thick section lies within a distance between \( r_1 \) and \( r_2 \) to the given primary, its 3D coordinates have to be recorded. As mentioned before, particles are reduced to associated points for distance measurement: we will refer to these points as the particle centres in the following sections.

If all observable secondary particles were evaluated, one would have to perform many more measurements in shells with large radii than in shells close to the primary centre \( c_i \). For radii \( r \) that exceed the section height \( h \), one would have to survey a volume that is proportional to \( r \); the volume of a shell limited by spheres with radii \( r - \delta \) and \( r + \delta \) around the primary which is enclosed in a section of height \( h < r \) is equal to \( 4\pi \delta \cdot \delta \cdot h \). Thus, the mean number of secondary cells to count would be proportional to \( r \), namely approximately \( r \cdot 4\pi \delta \cdot N_{ij}(r) \) for small \( \delta \). Moreover, the spatial distribution of secondary particles far away from the primary is expected to be less informative from a biological point of view. It is therefore desirable to reduce the measurement effort associated with the estimation of \( N_{ij}(r) \) for larger values of \( r \). This is achieved by restricting the counting of secondaries to a subset of the observation field, the saucor graph described in Section 3.3.

This graph is drawn individually with random orientation in the observation plane for every primary particle: we denote the graph belonging to the \( i \)th primary by \( W_i \). A secondary centre \( c_j \) is thus only sampled for the estimation of \( N_j(c_i; r_1, r_2) \) if it lies both in the randomly orientated thick section \( T \) and in the (randomly orientated) saucor graph \( W_i \). The probability that \( c_j \) is sampled as a ‘satellite’ of the primary \( c_i \) factorizes into the probability

\[
p_{\text{sect}}(c_j; c_i) := \text{Prob}(c_j \text{ lies in } T \ | \ T \text{ contains } c_i)
\]

that the \( j \)th secondary is observed in the thick section if the \( i \)th primary has been observed, and the probability

\[
p_{\text{sauc}}(c_j; c_i) := \text{Prob}(c_j \text{ lies in } W_i \ | \ T \text{ contains } c_i \text{ and } c_j)
\]

that \( c_j \) falls into the saucor graph around \( c_i \). The probability \( p_{\text{sect}} \) depends on the type of orientation graph (IUR or VUR) for the thick section sampling; we distinguish between \( p_{\text{beect}} \) and \( p_{\text{vsect}} \) correspondingly. Formulae are given for both cases in Subsection 3.2. Subsection 3.3 is devoted to the saucor graph and to the calculation of \( p_{\text{sauc}} \).

Following the Horvitz-Thompson principle (Horvitz & Thompson, 1952), we now obtain an unbiased estimator of the total number \( N_j(c_i; r_1, r_2) \) of secondary centres around the primary by weighting the individual counts with the inverse of the sampling probability \( p_{\text{sect}}(c_j; c_i) \cdot p_{\text{sauc}}(c_j; c_i) \), that is

\[
\hat{N}_j(c_i; r_1, r_2) = \sum_{c_j \in \text{saucor}(W_i)} \frac{1}{p_{\text{sect}}(c_j; c_i) \cdot p_{\text{sauc}}(c_j; c_i)}
\]

where dist \( (c_j; c_i) \) stands for the Euclidean distance between the secondary point \( c_j \) and the primary point \( c_i \), and the indicator function \( 1 \) is used to count the secondary points with distance between \( r_1 \) and \( r_2 \) to the primary \( c_i \).

By combining (5) with (6), the estimation procedure may be summarized in the following procedure:

1. Take a uniform random thick section (VUR or IUR) of the containing organ or reference space (here, a thick sections is to be understood as an optical section within the original physical section, see the remark about guard zones below).

2. Position a counting frame on the section, identify the primary particles therein and record the 3D positions of their centres (associated points). Let \( n \) denote their number and \( c_1, \ldots, c_n \) denote their centres.

3. For every centre \( c_i \), estimate the number of surrounding secondary particles as follows:

   a. According to the instructions in Subsection 3.3, make a randomly orientated saucor graph \( W_i \) around \( c_i \) on the observation window or screen.

   b. Identify the secondary particle centres within the thick section \( T \) that fall into \( W_i \) and record their positions.

   c. For every sampled secondary centre \( c_j \), calculate the probability \( p_{\text{sect}}(c_j; c_i) \) using formula (9) in the case of IUR sections or (11) in the case of VUR sections, and find the probability \( p_{\text{sauc}}(c_j; c_i) \) from (14).

   d. Apply formula (6) to get the estimate \( \hat{N}_j(c_i; r_1, r_2) \).

4. Obtain an estimate for the radial number density by averaging the results from step 3 according to formula (5).

Remark: Although secondary particles or cells are counted by means of an associated point such as the nucleolus centroid,
they do have a spatial dimension. This causes problems with counting particles close to the thick section boundaries – for example, particles may get lost due to the sectioning process, and for those that are hit by the section boundary, it is not clear whether or not their associated centre falls within the section. Similar problems also affect the dissector counting of primary particles. Therefore we equip the original section with an upper and a lower guard zone, as recommended by Andersen and Gundersen (1999). These guard zones actually reduce the original thick section of thickness \( h_{\text{original section}} \) to an optical thick section of thickness (or height)

\[
h := h_{\text{original section}} - (h_{\text{upper guard zone}} + h_{\text{lower guard zone}}).
\]  

Note that the guard zones used for sampling primary particles may be different from the guard zones used for sampling secondary particles. For example, primary particles could be subsampled using optical disectors that are considerably smaller in thickness than the original section, just to reduce the number of measurements. This does not affect the validity of the estimator, the height \( h \) in Eq. (7) (and later) then refers to the part of the section used to record secondary particles.

In the following text, we will always identify the section plane with the \( xy \)-plane and the focal depth with the \( z \)-coordinate. Thus the \( xy \)-plane is identical with what is seen on the microscopical screen. If the section is VUR, the direction of the \( y \)-axis is set to the vertical direction.

### 3.2. Uniform random section sampling

Saucor sampling starts with a uniform random thick section \( T \) of height \( h \) that is bounded by two parallel uniform random planes. Uniform random sectioning consists of a jointly uniform randomization of direction and position of the section plane. The direction is represented by the normal vector, and the position by the distance to the origin. For IUR sections, the normal vector takes values from the whole unit sphere (all directions in three dimensions). VUR sections are parallel to a predefined vertical direction that is often given by the situation Baddeley et al. (1986). VUR sections are necessary, e.g. when substructures in certain organs only can be identified if the organ is cut perpendicular to its surface. For a mathematical description of IUR and VUR sectioning see e.g. Baddeley and Jensen (2005).

In what follows, we will give heuristical derivations of the conditional probability \( p_{\text{sect}}(c_i; c_j) \) for observing a secondary point \( c_j \) in the section given the primary point \( c_i \) has fixed distance \( d_i \leq h/2 \) to the closer one of the two section boundary planes. One might object that this condition is somewhat stronger than the condition \( T \) contains \( c_i \) used in the previous definition of \( p_{\text{sect}}(c_i; c_j) \) in Subsection 3.1. However, this does not infringe the unbiasedness of the estimator \( \hat{N}_i(c_i; r_1, r_2) \) as given by (6). We will always let \( x_i, y_i, z_i \) denote the coordinates of a point \( c_i \) representing the primary particle centre and \( x_j, y_j, z_j \) the coordinates of the secondary particle centre \( c_j \) as measured under the digitally equipped microscope.

#### IUR sections

IUR sampling is designed such that all points in the reference space have equal probability to be contained in the IUR thick section. However, if we restrict randomization to sections \( T \) that contain a given point \( c_i \), we will sample points that are close to \( c_i \) with higher probability than points further away.

Due to the isotropic rotation of \( T \), all points on a sphere with fixed radius around \( c_i \) are sampled with the same conditional probability, given by the fraction of the sphere surface which is contained in the section, as illustrated in Fig. 2.

Consequently, the probability \( p_{\text{sect}}(c_i; c_j) \) is a function of the distance \( r_{ij} \) between \( c_i \) and \( c_j \),

\[
r_{ij} := \sqrt{(x_i - x_j)^2 + (y_i - y_j)^2 + (z_i - z_j)^2}. 
\]

The surface area of the spherical layer in \( T \) is equivalent to \( 2\pi r_{ij} h^* \), where \( h^* \) is the height of the spherical layer, which also depends on the distance \( d_i \) of \( c_i \) to the section boundary, see Fig. 3. The resulting probability is

\[
p_{\text{sect}}(c_i; c_j) = \frac{\text{Area(sphere around } c_i \text{ with radius } r_{ij} \cap \text{ thick section})}{\text{Area(sphere with radius } r_{ij})} \\
= \frac{2\pi r_{ij} h^*}{4\pi r_{ij}^2} \\
= \left\{ \begin{array}{ll}
1, & r_{ij} \leq d_i, \\
\frac{1}{2}(1 + d_i/r_{ij}), & d_i < r_{ij} < h - d_i, \\
h/(2r_{ij}), & r_{ij} \geq h - d_i.
\end{array} \right.
\]

#### VUR sections

Just as IUR sections, VUR sections also sample all points in the reference space with equal probability. Again,
we impose the condition that the point $c_i$ which represents the primary has a given distance $d_i$ to the closer one of the two boundary planes of the section. The VUR sections that fulfil this condition are obtained by uniform rotation around the vertical axis through $c_i$. This implies that all secondary points with the same given distance to that axis are sampled with the same probability $p_{V\text{sect}}(c_j; c_i)$. These points lie on a cylinder, as shown in Fig. 4. The sampling probability corresponds to the fraction of the cylinder surface which is contained in the thick section. Therefore, $p_{V\text{sect}}(c_j; c_i)$ depends on the distance

$$r_{ij}^{(xz)} := \sqrt{(x_j - x_i)^2 + (z_j - z_i)^2} \quad (10)$$

of $c_j$ to the vertical axis through $c_i$ (recall that the vertical direction is identified with the $y$-direction in the microscopical section, and the ‘horizontal plane’ perpendicular to this direction is the $(xz)$-plane).

A cylinder surface is obtained by multiplying the length of the profile on the horizontal plane with the cylinder height. The cylinder surface fraction within the section equals the length fraction of the circle with radius $r_{ij}^{(xz)}$ around the horizontal projection of $c_i$ that is covered by the horizontal profile of the thick section, see Fig. 5. Thus, the probability $p_{V\text{sect}}(c_j; c_i)$ is given by

$$p_{V\text{sect}}(c_j; c_i) = \frac{\text{Arc length(circle in } xz\text{-plane around } c_i \text{ with radius } r_{ij}^{(xz)} \cap \text{section})}{\text{Arc length(circle with radius } r_{ij}^{(xz)})}$$

$$= \begin{cases} 1, & r_{ij}^{(xz)} \leq d_i, \\ \frac{1}{2} + \frac{1}{\pi} \arcsin \left( d_i / r_{ij}^{(xz)} \right), & d_i < r_{ij}^{(xz)} < h - d_i, \\ \frac{1}{\pi} \left[ \arcsin \left( (h - d_i) / r_{ij}^{(xz)} \right) + \arcsin \left( d_i / r_{ij}^{(xz)} \right) \right], & r_{ij}^{(xz)} \geq h - d_i. \end{cases}$$

A rigorous mathematical derivation of this formula can be found in the Appendix.

### 3.3. The saucor

The idea of the saucor sampling method is to restrict coordinate measurement for secondary particles to independently randomly orientated sampling windows $W_i$ drawn on the microscopy screen around the individual primary particles $c_i$. The windows have a particular shape as depicted in Fig. 6 and explained later; Fig. 7 illustrates the rotation and for a practical example see Fig. 8 in Section 4.3. Once the random orientation of the window is established for a given primary
c_i, it remains fixed across focal depths. A secondary particle c_j is counted (or measured) with c_i if its x- and y-coordinates fall into W_i. The window is limited to points with distance less or equal to R_max to the centre c_i. This radius is chosen by the experimenter as the upper limit of biological interest for the determination of N_{ij}. On the other hand, the window contains all points with distance less or equal to a user determined R_mid. In the neuroanatomical study presented in Section 4 we chose R_mid = 12 µm and R_max = 48 µm.

The shape of W_i is defined by Eq. (12) in polar coordinates r and θ, where r denotes the distance in the focal plane to the centre point of W_i, and θ denotes the (planar) angle to the axis of W_i. A point with polar coordinates (r, θ) belongs to the W_i if and only if |θ| ≤ θ(r), and r ≤ R_max, where

\[
\theta(r) = \begin{cases} 
\pi, & r \leq R_{\text{mid}}, \\
\pi \left( \frac{R_{\text{max}}}{r} \right)^{1+\beta}, & r > R_{\text{mid}}.
\end{cases}
\]  

(12)

Fig. 5. VUR conditional sampling probability p_{Vsect(C_j|C_i)} depends on d_i and r_{ij}^{(x,y)}. The projections of the VUR thick section (with guard zones) and the cylinders as in Fig. 4 onto the horizontal plane are shown, that is, the vertical axis is perpendicular to the paper, and the black and white dots represent the projections of primary and secondary point c_i and c_j, i.e. their x- and z-coordinates. Points c_j with r_{ij}^{(x,y)} ≤ d_i are sampled with probability 1 (left part). The calculation of the sampling probability in the case r_{ij}^{(x,y)} > d_i requires determination of arc lengths – the hatched angle in the middle part is equal to \arcsin(d_i/r_{ij}^{(x,y)}).

Fig. 6. The saucor graph, here oriented along the x-axis. Left part: saucor graph for \beta = 1, right: saucor graphs for \beta = 0.5 (outer) and \beta = 1.5 (inner graph).

Fig. 7. Random rotation of the saucor graph around the primary point. Two realizations of the uniformly rotated saucor graph as drawn on the screen are shown, see also Fig. 8. Secondary cells are only sampled when they fall into the white area.
The parameter $\beta \geq 0$ determines how many of the secondary particles with distance larger than $R_{\text{mid}}$ to the primary have to be counted and how fast this number decreases. Figure 6 shows the graph of $W_i$ for various values of $\beta$ in the case where the axis of $W_i$ coincides with the x-axis. For the practical example, we chose $\beta = 1$. The name 'saucor' was inspired by the shape that is obtained if the graph in Fig. 6 were rotated around the $y$-axis.

Randomization of the saucor orientation is performed by uniform rotation of the saucor axis around the primary particle. The probability $p_{\text{sau}}(c_j; c_i)$ that a secondary point $c_j$ with coordinates $x_j, y_j$ as seen on the microscopy screen lies inside the graph therefore depends only on the $xy$- (or screen-) distance $r_{ij}^{(xy)}$ to the primary with coordinates $x_i, y_i$.

$$r_{ij}^{(xy)} = \sqrt{(x_j - x_i)^2 + (y_j - y_i)^2}. \quad (13)$$

This probability is given by the length fraction of the arc through $c_j$ in the saucor window, see Fig. 7.

$$\frac{\text{Arc length(circle around } c_i \text{ with radius } r_{ij}^{(xy) \cap \text{saucor graph})}}{\text{Arc length(circle with radius } r_{ij}^{(xy)})} = \frac{2\theta r_{ij}^{(xy)}}{2\pi r_{ij}^{(xy)}}.$$

that is,

$$p_{\text{sau}}(c_j; c_i) = \begin{cases} 
1, & r_{ij}^{(xy)} \leq R_{\text{mid}}, \\
\left(\frac{R_{\text{mid}}}{r_{ij}^{(xy)}}\right)^{1+\beta}, & R_{\text{mid}} < r_{ij}^{(xy)} \leq R_{\\max}. 
\end{cases} \quad (14)$$

Note that the saucor estimator holds only for $N_{ij}(r)$ with $r < R_{\max}$. Very few secondary particles may be observed on the outer edge of the saucor graph with 3D distance $r_{ij}$ larger than $R_{\max}$, because the screen distance $r_{ij}^{(xy)}$ is smaller than the true 3D distance (unless $z_i = z_j$), namely

$$r_{ij}^{(xy)} = \sqrt{r_{ij}^2 - (z_i - z_j)^2}. \quad (15)$$

These particles normally constitute less than 1% of all measured particles and are excluded from the computation.

3.4. Number of measurements associated with saucor sampling

The workload associated with measuring particle coordinates in a thick section is approximately proportional to the expected number of particles in the evaluated volume. Brick counting methods as proposed by Baddeley et al. (1987, 1993) and Schmitz et al. (2002) capture all secondary particles that can be seen by focusing up and down in a given counting frame. The
expected number $E_Q(\text{disector})$ of secondary particles depends on the area of the frame, the height $h$ of the section and the number density $N_{ij}$, namely

$$E_Q(\text{disector}) = N_{ij} \cdot \text{Area(frame)} \cdot h.$$  \hspace{1cm} (16)

When sampling secondaries individually for every primary, the effort increases with the number of primary particles in the disector. For the saucor method, one has to evaluate cylinder volumes that equal the area of the saucor graph multiplied by the height $h$. This yields an expected number $E_Q(\text{saucor})$ of secondary counts given by

$$E_Q(\text{saucor}) = N_{ij} \cdot \text{#primaries} \cdot \text{Area(saucor)} \cdot h.$$  \hspace{1cm} (17)

Thus, saucor sampling will require less measurement effort than disector sampling if and only if the number of primaries per disector is less than the ratio $\text{Area(frame)}/\text{Area(saucor)}$.

Using the standard formula for the area swept out by a radius-vector function, we find

$$\text{Area(saucor)} = \int_0^{R_{\text{max}}} 2\theta(r)r\,dr \, r$$

$$= \pi R_{\text{mid}}^2 + \int_{R_{\text{mid}}}^{R_{\text{max}}} 2\pi r^{-\beta} R_{\text{mid}}^1 \, dr$$

$$= \pi R_{\text{mid}}^2 \begin{cases} 1 + 2 \ln \frac{R_{\text{max}}}{R_{\text{mid}}}, & \text{if } \beta = 1, \\ 1 + \frac{1}{1-\beta} \left( \frac{R_{\text{max}}}{R_{\text{mid}}} \right)^{1-\beta}, & \text{otherwise.} \end{cases}$$  \hspace{1cm} (18)

In the following neuroanatomical study, a saucor graph was used with $\beta = 1$. $R_{\text{mid}} = 12 \mu m$ and $R_{\text{max}} = 48 \mu m$, having area $1707 \mu m^2$.

### 4. Practical application: estimating radial number density around neurons in the human neocortex in VUR sections

#### 4.1. Tissue and tissue preparation

To illustrate the saucor method we analysed vertically uniformly random neocortical sections. The post-mortem tissue consisted of one hemisphere from a 61-year-old woman who had no history of psychiatric or neurological disorders and died of cardiac arrest. The brain was removed 48 h post-mortem and fixed in 0.1 M sodium phosphate buffered (pH 7.2) 4% formaldehyde for 5 months. The meninges were removed, and the cerebellum and brainstem detached at the level of the third cranial nerve. The frontal, temporal, parietal and occipital regions were delineated and indicated by applying different colours to the pial surface of the right hemisphere Pakkenberg and Gundersen (1997). The hemisphere was embedded in 6% agar, and sliced coronally at 7 mm intervals, and the neocortical volume of the sliced hemisphere was estimated by Cavalieri’s principle. From every second slice, using a special sampling plate, transcortical wedges were sampled uniformly and systematically random from each neocortical region. Each wedge was cut into 2-mm-wide parallel bars. The bars were subsampled so each region was represented by approximately 8 to 10 bars. All sampled bars were rotated around their vertical (long) axis and embedded in Historesin®. One 35-μm-thick vertical section was cut from each block, and stained with a modified Wolbach’s Giemsa stain. This resulted in 9 frontal, 10 temporal, 8 parietal and 6 occipital bars with an average section thickness of 28.5 μm. The bars were individually mounted on glass slides for microscopic examination.

#### 4.2. Equipment

The slides were placed on the stage of a BH-2 Olympus (Olympus, Tokyo, Japan) microscope. The slide holder could be freely rotated to enable sampling along the direction of the vertical axis of the section. The image of the section was captured by a digital video camera and transmitted to a computer screen. A high image resolution and a thin focal plane was obtained using a high numerical aperture (=1.4), 100 X oil-immersion objective for cell counting and cell volume estimation. A Heidenhain microcator with digital readout was used for measuring movements to the nearest 0.5 μm kept track of the z-direction. The stage of the microscope and hence the specimen could be moved precisely in $x$-, $y$- and $z$-directions and its movements were tracked and recorded. The position of the cursor was also tracked and the 3D coordinates of the points marked with the cursor were recorded automatically. The final magnification at the computer screen was 3040 X.

The volume of each sampled neuron was estimated by the vertical planar rotator method (Jensen & Gundersen, 1993) using the CAST-GRID computer program (Visiopharm), which also controlled all data acquisition from the saucor.

#### 4.3. Data acquisition

The primary neurons were identified in an optical disector (Gundersen, 1986; Gundersen et al., 1988) with a disector area of $3500 \mu m^2$ and a disector height of 10 μm, leaving guard zones of 8 to 10 μm on the top and 10 μm on the bottom. Then the saucor was applied (see details below). Positions of secondary cells were recorded covering a larger focal depth of 20 μm, with guard zones of a few micrometres in the top and 5 μm in the bottom. The dimensions of the saucor were defined by $\beta = 1$, $R_{\text{mid}} = 12 \mu m$ and $R_{\text{max}} = 48 \mu m$. An example of the saucor on the screen is shown in Fig. 8.

The secondary cells were classified on morphological characteristics into neurons, oligodendrocytes, astrocytes, microglial or endothelial cells. Cell classification was based solely on morphology because immunohistochemical staining cannot penetrate plastic sections. The cells were identified as neurons if they had a combination of a single large nucleolus free of any surrounding heterochromatin, a typical
Fig. 9. Neurons, glial and endothelial cells. Two typical fields of view from the 61-year-old female brain used in this pilot study, with post-mortem interval of 48 h. The bar indicates 10 \( \mu m \). N: neuron, O: oligodendrocyte, A: astrocyte, M: microglial cell, E: endothelial cell.

pale chromatin pattern in a triangularly rounded nucleus, and were surrounded by a visible cytoplasm. Astrocytes were defined as cells with a round and pale nucleus having the heterochromatin concentrated in granules in a rim below the nuclear membrane and a relatively translucent cytoplasm. A small nucleolus was not always identified, but when present, it was most often situated eccentrically. The nuclear membrane of astrocytes has a sharp profile, and the cells are often located singularly. Oligodendrocytes are often situated in groups and in close proximity to neurons or blood vessels. They are characterized by a small rounded or oval nucleus with dense chromatin structure and a perinuclear halo. Microglial cells are defined by a small elongated or comma-shaped nucleus with dense peripheral chromatin. Endothelial cells are polygonal and flat, and frequently found clustered in a vessel structure; however, the vessel structure is often only visible when focusing through the height of the section. Figure 9 shows two typical examples with neurons, astrocytes, oligodendrocytes, microglial and endothelial cells.

A total of 151 primary neurons were sampled for the estimation of radial number density, 32 in the frontal region, 49 in the temporal, 37 in the parietal and 33 in the occipital lobe. This means that an aggregate saucor area of 151 \( \mu m \times 1707 \mu m = 257757 \mu m^2 \) had to be scanned for secondary cells. The traditional disector box counting methods would have required inspection of all dissectors containing primary neurons. Because 100 of the 120 dissectors that were screened did contain neurons, this would correspond to an area of 100 \( \times 3500 \mu m^2 \), which is about 36% larger than the aggregate saucor area.

4.4. The practical use of the Saucor and the final computations

First the direction of the vertical axis was indicated, in this case the longitudinal axis of the sections. With a random start, the sampling of primary cells was performed with a constant sampling period of 1500 \( \mu m \) between the dissectors along the centre of the section. All primary neurons in a disector must be sampled to ensure uniform sampling.

When the centre of a primary cell is indicated with the mouse in the relevant frame, the system moves the stage so that the primary is at \((0, 0)\), and a saucor probe is drawn, rotated uniformly random, cf. Fig. 8. The user indicates all secondary cell centres that in any way touch the saucor graph. Coordinates \((x, y, z)\) are automatically recorded for primary and secondary cells by clicking on the cell midpoint, which is determined visually. When sampling in a saucor probe is exhausted, the investigator activates the rotator probe to estimate the volume of the primary neuron. This procedure is done for all primary neurons within a disector.

For simplification we have described the primary cells as neurons and secondary cells as glial cells. One can of course pick any type of cell or other objects to be defined as primary or secondary, the same type of object can also be defined as both primary and secondary. For this practical application we chose primary cells to be neurons and both neurons and glial cells as secondary cells.

4.5. Data analysis and results

We present the radial number density of neocortical cells around primary neurons as a sequence sorted according to radial distance. The distance is classified or binned based on the observed radii, \( r_g \). We used wide classes at the perimeter to obtain enough observations in the decreasing observed shell volume fractions and narrow classes in the centre and its vicinity to ensure precision and details of the most interesting parts of the distribution.

Unless many observations are made (>200), there is no extra information in more than roughly 10 informative classes. Because there will often be some empty and thereby uninformative classes in the centre (the beginning of the distribution will often start some distance from the primary
The factor of geometric progression, \( f \approx 1.21 \), means that each class is 21% wider than the previous one, providing an essentially constant sampling volume per bin outside of \( R_{\text{mid}} \), as shown in Fig. 10.

Figure 11 illustrates the difference in the spatial arrangement of glial cells and neurons with respect to (primary) neurons by comparing the radial glial and neuron number density averaged over all four regions. The graph for glial cells shows a pattern with high densities close to the neurons and a gap with lower density before the background density is reached, thus indicating clustering of glial cells around neurons. On the contrary, the radial density of neurons around neurons is small for small distances and approaches the final density from below. This suggests a tendency towards repulsion from the primary neuron.

We have chosen to illustrate the distribution of all cells around neurons with the four subdivisions (frontal, temporal, parietal and occipital cortex) with the distribution in neocortex in the same graphs to make the regional differences more visible (see Fig. 12). For estimating total radial number density of neocortical cells, each region is weighted with the fraction of the total number of neurons in that specific region. It is a very

---

Fig. 10. Binning of the saucor. Left panel: Profile of the binned saucor. In the central black circle secondary particle centres are excluded due to the presence of the primary cell. Right panel: The volume per bin of a saucor with a thickness of 10 \( \mu m \).

object centre) a few extra classes are needed to end up with about 10 informative classes; we have chosen \( n = 14 \).

Finally, secondary particle centres cannot be arbitrarily close to that of the primary, so we have fixed the beginning of the sequence of bins at \( R_1 = 2.4 \mu m \). The binning is a refined geometric progressing or quasi-logarithmic sequence of classes, which provides freedom to make it fit the three constants \( R_1, R_{\text{mid}}, \) and \( R_{\text{max}} \) (in a logarithmic sense it is symmetric around \( R_{\text{mid}} \)). The generating equation for the complete sequence of lower bin limits is

\[
R_i = c \cdot f^{i-1} - \text{off} = 3.490 - 1.20783^{3i-1} - 1.09,
\]

where

\[
c = \frac{(R_{\text{mid}} - R_1)^2}{R_{\text{max}} - 2R_{\text{mid}} + R_1},
\]

\[
f = \left[ \frac{R_{\text{mid}} - R_1}{c} + 1 \right]^{2/m}\]

and

\[
\text{off} = c - R_1.
\]
limited sample but there is a tendency towards glial attraction in all four regions, the glial cells are distributed in the same pattern over the four neocortical regions. The distribution of secondary neurons on the other hand presents a difference in the density when comparing the pattern of the frontal region to the other regions, probably due to the simple fact that the neurons in the frontal cortex have a larger volume than the neurons in the other regions.

For this study the embedding material was historesin, which has low shrinkage potential, unlike for example paraffin or frozen sections. For studying spatial distribution of cells it is important to choose an embedding material with as little shrinkage as possible. Frozen and vibratome sections are special in that it is usually possible to restrict the shrinkage to the z-axis, and that is easily monitored locally Dorph-Petersen et al. (2001). If the sections are cut using a calibrated microtome one may then correct for the local shrinkage of the z-coordinate.

5. Discussion of the saucor in relation to other methods for characterizing 3D spatial arrangement

As mentioned in the ‘Introduction’ and in Section 2, a variety of statistical characteristics have been proposed for the quantification of spatial arrangement of particles or cells in a material or tissue. Both second-order statistics, including $K_{ij}$ and $N_{ij}$, and the nearest neighbour distribution $D_{ij}$ reduce the information about positions of particles or cells to information about distances. Estimation of $D_{ij}(r)$ requires that all type $j$ (secondary) particles within distance $r$ to a type $i$ (primary) particle are observable, and therefore can only be achieved when positions are recorded in thick brick-shaped sampling windows as reported by Schmitz et al. (2002). By contrast, estimation of second-order statistics can also be done on thinner sections – here, missing information about the positions of secondary particles can be compensated for with a Horvitz–Thompson estimator.

The traditional second-order statistics $K_{ij}$ and $g_{ij}$ are normalized by the overall number density $N_{ij}$ of secondary particles, compare Eqs (3) and (4). Methods for the estimation of these statistics therefore include counting of secondary particles in windows that are independent of the position of primary particles, namely in the same window that is used for sampling the primary particles. This window has therefore to be relatively large. An example is the work by Baddeley et al. (1987, 1993), who estimate second-order statistics for the pattern of osteocyte lacunae in the skull bones of Macaque monkeys. Lacunae positions are recorded by confocal microscopy in brick-shaped sampling windows that have a fixed orientation determined by the surface of the specimen. Second-order characteristics are evaluated for distances up to $r = 50 \mu m$, which is in the order of magnitude of the brick side lengths ($82 \mu m, 100 \mu m$, and $60 \mu m$). An edge-corrected estimator for $K(r)$ is applied, that is a Horvitz–Thompson estimator that compensates for unobserved point pairs. The estimator is model based, assuming stationarity and isotropy of an underlying point process.
In the present paper however, we follow a design based approach, which entails that the sections have to be randomized both in position and direction, in particular because we use sections that are thinner than the maximum distance $R_{\text{max}}$ at which $N_{ij}$ is evaluated. If the direction of the thick sections were fixed, secondary particles lying above or below the boundary planes but still within distance $R_{\text{max}}$ to a recorded primary would be completely unobservable, whilst the Horvitz–Thompson method requires that all such secondary particles are sampled with positive probability.

A Horvitz–Thompson approach to correct for edge effects is also taken by Evans and Gundersen (1989) for estimating $N_{ij}$ from measurements in optical disectors on VUR sections. The estimation formula given there (without rigorous mathematical explanation) yields biased results, in particular for small radii $r$. As a consequence, a very strong clustering of glial cells around neurons is reported with a number density reaching up to $N_{ij}(r) = 600$ million glial cells per cm$^3$ at $r = 6 \, \mu m$. This is about four times as much as observed in the present study, where we found a peak value of 140 million glial cells per cm$^3$ at $r = 9 \, \mu m$, whilst the values of $N_{ij}(r)$ for large $r$ were of comparable magnitude in both studies. In a later version of that estimator (Evans, 2004), the idea of sampling secondary particles using a randomly rotated window drawn around the primary is introduced. This window, called a ‘spatial distribution grid’, is composed of a circle, surrounded by a half circular shell which is followed by a quarter circle and one-eighth of a circle. It can be seen as a stepwise version of the saucor graph.

The final smooth saucor graph was first published in Stark et al. (2007). It is more flexible in choosing the sampling probability of secondary particles as a function of the distance to the primary and probably also more user friendly in the actual practical measurement because it has a smooth shape as opposed to the rather jagged appearance of the spatial distribution grid. The ‘saucer’ estimation method in Stark et al. (2007) is restricted to VUR sections and differs from the present ‘saucor’ method in that it requires sine weighted randomization of the sampling window graph orientation. There, estimates for $N_{ij}$ are obtained by dividing the number of secondary particles with a given distance $r \in [r_1, r_2]$ by the corresponding sampling volume (the intersection of the thick section and sampling window with the shell bounded by spheres of radius $r_1$ and $r_2$), thus following the idea of fractionator sampling. This does in general not lead to unbiased estimates because particles at different positions within the sampling volume are sampled with different probability.

With the present new version, the randomization of the sampling window orientation was simplified, and the estimation formula has been completely changed, now being based on individual positions of secondary particles because these are recorded anyway. The main idea – rotating a sampling graph around observed primary particles – as well as the form of the sampling window however remain unaltered. We therefore tried to find a name that is different from but still reminiscent of the old name.

6. Conclusions

The spatial distribution of glial cells is of interest because the distribution of cells within a given region of the brain may have important implications for the function of that region. The ability of a group of neurons to work in a coordinated fashion depends on the intensity of its synaptic connectivity, which in turn may be reflected in the physical proximity or arrangement of the cells. Spatial arrangement of cells or particles with respect to each other is suitably characterized by the radial number density which expresses the local mean number per volume of cells of one type as a function of their distance to cells of another (or the same) type. With this paper, we introduce a stereological probe, the saucor method that allows efficient sampling and estimation of the radial number density from vertical or IUR sections.

Application of the saucor is of course not restricted to neuroscience, but in this field of science it is a particularly valuable tool because it enables the researcher to obtain data on the cellular spatial relations with a reasonable workload and sensitivity which previously were considered difficult to acquire. Even though the saucor is a manual method and the data collection cannot be done by a computer, the labour burden is manageable; the data collection from the brain studied in Section 4 took 4 to 6 h per region.

Our results indicate that glial cells are clustered around neurons, whereas neurons showed a tendency towards repulsion from each other. This could be explained with the role of neurons as the morphological, ontogenetical and functional units of the central nervous system, whereas glial cells primarily are the metabolically supportive cells, which assist neurons in their function and development. Although these outcomes are plausible, they are difficult to verify because they were obtained on only one neocortex and there are only few previous studies concerning spatial distribution of cells in healthy human neocortex they could be compared against. Still, the densities found in the present study are within the same ranges as reported earlier for both neurons (Pakkenberg & Gundersen, 1997) and glial cells (Pelvig et al., 2008).

The saucor method allows analysing the spatial distributions with respect to the size of the primary neurons. Neuron size crudely indicates function (small local interneurons, larger neurons communicating with distant regions in the brain and very large neurons with axons going far outside the brain). The spatial distributions around neurons of very different sizes are therefore likely to be quite different. This may or may not be interesting in itself, but it represents a known source of variation which may be effectively handled by analysing with respect to a number of volume classes of neurons. Although data on neuron volume were collected...
in the present investigation, we did not include any results because the sample size was too small.

When dealing with tissues like the human neocortex, it would also be interesting to study the layer specific spatial distribution of cells with respect to each other. However, due to the random rotation of the tissue during preparation and the much folded cortical surface it is impossible to identify all six layers in all sections. Knowledge of the 3D spatial relationship between neurons and glial cells may give new insight to the organization and function of the central nervous system, in brains with and without disease. In an earlier pilot study of the human cell distribution in neocortex (Stark et al., 2007), only few statistically significant differences were found between healthy male and female brains, and between young and old brains. This apparent uniformity could be interpreted as an indicator for the importance of spatial arrangement to healthy brain function. Non-healthy brains might show a deviation from that spatial distribution pattern. Thus, the radial number density data might provide important additional information when trying to understand the complexity of diseases such as Alzheimer’s dementia and schizophrenia, where histological data so far have provided valuable results, but do not lead us to fully understand the pathology behind the diseases.

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References


Appendix

Formal proof of ratio unbiasedness

An estimator \( \hat{Q} = \hat{A}/\hat{B} \) for a quantity \( Q = A/B \) is called ratio unbiased if \( E(\hat{Q}) = Q \). Clearly, this does not entail unbiasedness of \( \hat{Q} \), but often such estimators still have desirable properties such as consistency. A thorough discussion of ratio estimators for particle populations can be found in the excellent book by Baddeley and Jensen (2005).

In the following text, we give a formal proof of ratio unbiasedness for the saucor estimator. Consider a finite population of \( N_1 \) primary particles or cells – this could consist of all cells in an organ, or in a set of organs. In this setting, the radial number density is defined as

\[
N_{r/\phi}(r_1, r_2) = \frac{\sum_{i=1}^{N_1} N_j(c_i; r_1, r_2)}{N_1},
\]

where

\[
N_j(c_i; r_1, r_2) = \sum_{\text{all secondary cells } c_j} 1(r_1 < \text{dist}(c_j, c_i) \leq r_2)
\]

denotes the number of secondary cells with distance between \( r_1 \) and \( r_2 \) to the primary cell \( c_i \).

In the first sampling step, primary particles are sampled using a disector design (Sterio, 1984). This ensures that all particles are sampled with the same probability which is equal to the volume sampling fraction \( sf \). Writing the saucor estimator Eq. (5) as

\[
\hat{N}_{r/\phi}(r_1, r_2) = \frac{1/sf \cdot \sum_{i=1}^{N_1} \hat{N}_j(c_i; r_1, r_2)}{n/sf}.
\]

the denominator can be read as an unbiased estimator for the total number \( N_1 \) of primaries, i.e. for the denominator of (23). The numerator is an unbiased estimator for the numerator of (23), because

\[
\hat{N}_j(c_i; r_1, r_2) = \sum_{\text{all sampled secondary cells } c_j} 1(r_1 < \text{dist}(c_j, c_i) \leq r_2) \cdot p_{\text{sau}}(c_j; c_i)
\]

is an unbiased estimator for \( N_j(c_i; r_1, r_2) \), for every sampled primary \( c_i \). This assertion is verified in the following text.

Let \( D_i \) denote the (random) distance of a given primary particle \( c_i \) to the lower boundary plane of the (random) thick section \( T \). The primary is sampled if and only if \( D_i \in [0, \ h] \). We will show that

\[
E(\hat{N}_j(c_i; r_1, r_2) | D_i = d) = N_j(c_i; r_1, r_2)
\]

for all \( d \in [0, \ h] \) – actually a stronger statement than unconditional unbiasedness of \( \hat{N}_j \). A secondary point \( c_j \) with distance between \( r_1 \) and \( r_2 \) to \( c_i \) is counted by \( \hat{N}_j \) if and only if it lies within both the thick section \( T \) and the saucor window \( W_i \) around the primary. Because

\[
E(\hat{N}_j(c_i; r_1, r_2) | D_i = d) = \frac{E_T \left[ \sum_{c_j \in T \cap W_i} \frac{1}{p_{\text{sau}}(c_j; c_i) \cdot p_{\text{sect}}(c_j; c_i)} \right]}{p_{\text{sect}}(c_i; c_i)}
\]

it suffices to show that

\[
E_T(1(c_j \in T) | D_i = d) = p_{\text{sect}}(c_j; c_i)
\]

and

\[
E_W(1(c_j \in W_i) | c_j \in T, D_i = d) = p_{\text{sau}}(c_j; c_i)
\]

holds with the probabilities \( p_{\text{sect}} \) given by Eqs (9) and (11) for IUR and VUR sections and with \( p_{\text{sau}} \) as in Eq. (14). We will only demonstrate that, for VUR thick sections,

\[
E_T(1(c_j \in T) | D_i = d) = p_{\text{sect}}(c_j; c_i).
\]
focus- (z-) direction is for sake of simplicity identified with the normal direction of the sectioning plane. A vertical section of thickness \( h \) consists of all points \((x, y, z)\) that are included between two parallel vertical planes, namely

\[
T(\phi, p) = \{(x, y, z) : x \cos \phi + z \sin \phi - p \in [0, h]\}.
\]

VUR thick sections are defined as uniformly random drawn from all vertical thick sections that intersect a given reference space. The uniform measure for vertical thick sections factorizes into the uniform measures for \( p \) and for \( \phi \), viz \( dT = dpd\phi \). Probabilities or expectations for stereological quantities obtained from VUR (thick) sections are obtained by integration with respect to that measure, normalized by the measure of the support.

For sake of readability, we will without loss of generality set \( c_i \) equal to origo. To verify (24), the conditioning event \( D_i = d \) is written as

\[
(\phi, p) : D_i = d = (\phi, p) : \phi \in [0, 2\pi], p = -d).
\]

Thus, randomization is restricted to the direction \( \phi \) of the VUR thick planes whilst the distance \( p \) to the origin is expressed by \( \phi \) and \( d \). Hence

\[
E(1(c_j \in T) | D_i = d) = \int_{[0, 2\pi)} 1(c_j \in T(\phi, -d))d\phi / \int_{[0, 2\pi)} d\phi.
\]

With \( c_j = (x, y, z) \), \( r := r_{ij}^{(x,y,z)} = \sqrt{x^2 + z^2} \) and \( \alpha \) such that \( r \sin \alpha = x \) and \( r \cos \alpha = z \), we have

\[
E(1(c_j \in T(\phi, -d)) | D_i = d) = \begin{cases} 1, & r \leq d^*, \\ \frac{1}{2} + \frac{1}{2} \arcsin \frac{d^*}{r}, & d^* < r < h - d^*, \\ \frac{1}{2} \left( \arcsin \frac{b - d^*}{r} + \arcsin \frac{d^*}{r} \right), & r \geq h - d^*. \end{cases}
\]

with the conditions \( A : r \leq \min (d, h - d) \), \( B : d < r < h - d \), \( C : h - d < r < h - d^* \), and \( D : r > \max (d, h - d) \). Writing \( d^* = \min (d, h - d) \) and integrating, one gets

\[
E(1(c_j \in T) | D_i = d) = \begin{cases} 1, & \frac{1}{2} \arcsin \frac{d^*}{r}, & d^* < r < h - d^*, \\ \frac{1}{2} \left( \arcsin \frac{b - d^*}{r} + \arcsin \frac{d^*}{r} \right), & r \geq h - d^*. \end{cases}
\]

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