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An interviewer once asked Woody Allen whether you could see the human soul with a microscope. “Yes, you can,” he said, “but you need one of those good ones with two eyepieces.”

Beyond seeing the microstructures that comprise the nervous system, one possible location for the human soul, neuroscientists often face the task of quantifying what we see. We also share the responsibility to do our best to accurately convey our results to each other, which led to the idea for this book.

After he finished his first stereology study a few years ago, a postdoctoral student in my lab sent his coauthors a manuscript to review, along with a statement that read, to the effect, “like most stereology papers, the discussion section is short.”

When I asked him to elaborate, he said his reading of the literature showed that stereology papers generally have shorter discussion sections than nonstereology papers. With systematic-random sampling and unbiased estimators, the methodology avoids a wide range of potential pitfalls, problems, and assumptions associated with quantifying microstructures.

“When writing a paper,” he said, “the author simply includes the details of their stereology approach in the methodology. There is little to no need for further justification in the discussion.”

The situation is different for some areas of neuroscience, he explained, such as his field of experimental psychology, where the goal is to quantify animal behavior. Reviewers expect authors to detail all the possible confounds in the design and placement of the testing apparatus, motivational features of the task, and demands placed on the animal’s abilities, along with a full statistical analysis of variables and covariables.

For stereology, he concluded, “you just discuss whether changes such as fewer neurons were revealed by the study and the implications of those findings for the hypothesis in question.”

I reviewed his paper with this idea in mind. Well organized and written in the usual manner, the materials and methods section included descriptions of the procedures with correct citations for the stereological approaches such as the Cavalieri-point counting technique, disector principle, and the optical fractionator. As expected from a post doc in experimental psychology, the draft included a thorough statistical analysis of the findings with the total observed variability in the results partitioned into biological sources and method error. The concise discussion reviewed his findings relative to the original hypothesis and ended with a short conclusion. The study was submitted to a prominent peer-reviewed journal and was accepted with only minor revisions.

In the years since, my informal survey of stereology and nonstereology papers reveals that perhaps he was right. Because unbiased stereology has been carefully vetted for accuracy, with empirical comparisons to gold standards and rigorous reviews by theoretical experts, authors do
not spend much effort justifying, rationalizing, or analyzing the approaches, other than to explain any caveats that apply. Provided the investigators clearly state and properly apply the methods, the results should be reliable.

Yet Luis M. Cruz-Orive, the esteemed stereologist from Spain, reminds us that the situation is not always that simple.

“Stereology,” according to Professor Cruz-Orive, “is a committed task.” That is, generating reliable stereology results requires a commitment by each investigator to thoroughly identify and eliminate all known sources of systematic error (bias) from his or her study design.

Neurostereology: Unbiased Stereology of Neural Systems brings together theory and research from neuroscientists who explore the issues, pitfalls, and potential confounds associated with the applications of design-based stereology to their research. Contributions from neuroscientists in Turkey, Iran, Denmark, India, Japan, Australia, the United Kingdom, and the United States use unbiased stereology to quantify a diverse range of neural structures, from macroscopic studies of brain volumes on MRI images to detailed microstructures of unmyelinated nerve fibers. Because the authors were encouraged to explore the practical issues in their work, readers will find the length of discussion sections perhaps a bit longer, and hopefully with more valuable insights, than those found in the majority of published stereology papers. For neuroscientists with previous experience, as well as those just starting to ease into stereology, these papers exemplify applications to a wide range of tissues, from human brains to marine mammals and genetic, toxicological, and chemical models in rodents. In each case, the authors identify and address a number of important pitfalls, such as the reference trap, recognition bias from inadequate staining, profile versus object counting, and poorly defined reference spaces.

Readers will note that despite the major differences in these studies with regard to the types of tissues analyzed, wide range of hypotheses tested, and different microstructures analyzed, each study applies similar principles and practices of unbiased stereology. In each case, the investigators identify an anatomical reference space of interest, apply systematic random sampling, use unbiased estimators, and sample with sufficient stringency to generate reliable results. In some cases, particularly those involving autopsied human brains or less common species of animals, unavoidable conditions complicate a fully unbiased study design, that is, one free of all methodological bias. In those cases, the authors point out to the readers if and how these issues might influence their findings.

My hope is that you find this book not only illuminating but helpful in your efforts to apply the methods of design-based stereology in an effective manner. Professor Cruz-Orive reminds us that stereology depends on a tacit agreement from each investigator to identify and eliminate all known sources of systematic error (bias) to the greatest extent possible. In line with this adage, Neurostereology: Unbiased Stereology of Neural Systems offers a multidisciplinary range of studies from an international group of neuroscientists who did their collective best to get it right.

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Neurostereology
Unbiased Stereology of Neural Systems
Background

Stereology combines mathematical and statistical approaches to estimate three-dimensional (3D) parameters of biological objects based on two-dimensional (2D) observations obtained from sections through arbitrary-shaped objects (for reviews of design-based stereology, see Howard and Reed, 1998; Mouton, 2002, 2011; Evans et al., 2004). Among the first-order parameters quantified using unbiased stereology are length using plane or sphere probes, surface area using lines, volume using points, and number using the 3D disector probe. These approaches estimate stereology parameters with known precision for any object regardless of its shape.

These criteria for stereological estimation of volume and surface area are met by standard magnetic resonance imaging (MRI) and computed tomography (CT) scans, as well as tissue sections separated by a known distance with systematic random sampling, that is, taking a random first section followed by systematic sampling through the entire reference space (Gundersen and Jensen, 1987; Regeur and Pakkenberg, 1989; Roberts et al., 2000; Mouton, 2002, 2011; García-Fiñana et al., 2003; Acer et al., 2008, 2010). Numerous studies have been reported using MRI to estimate brain and related volumes by stereologic and segmentation methods in adults (Gur et al., 2002; Allen et al., 2003; Acer et al., 2007, 2008; Jovicich et al., 2009), children (Knickmeyer et al., 2008), and newborns (Anbeek et al., 2008; Weisenfeld and Warfield, 2009; Nisari et al., 2012).

The Cavalieri Principle

Named after the Italian mathematician Bonaventura Cavalieri (1598–1647), the Cavalieri principle estimates the first-order parameter volume (V) from an equidistant and parallel set of 2D slices through the 3D object. As detailed later, the approach uses the area on the cut surfaces of sections through the reference space (region of interest) to estimate size (volume) of whole organs and subregions of interest. The point counting technique for area estimation uses a point-grid system superimposed with random placement onto each section through the reference space (Gundersen and Jensen, 1987). The number of points falling within the reference area is counted for each section (Figure 1.1). Total V of a 3D object, x, is estimated by Equation 1.1:
where $A(x)$ is the area of the section of the object passing through the point $x \in (a, b)$, and $b$ is the caliper diameter of the object perpendicular to section planes. The function $A(x)$ is bounded and integratable in a bounded domain $(a, b)$, which represents the orthogonal linear projection of the object on the sampling axis (García-Fiñana et al., 2003; Kubínová et al., 2005).

The Cavalieri estimator of volume is constructed from a sample of equidistant observations of $f$, with a distance $T$ apart, as follows (Eq. 1.2):

$$V = T \sum_{k \in \mathbb{Z}} f(x_0 + kT) = T(f_1 + f_2 + f_3 + \cdots + f_n),$$

where $x_0$ is a uniform random variable in the interval $(0, T)$ and $\{f_1, f_2, \ldots, f_n\}$ is the set of equidistant observations of $f$ at the sampling points which lie in $(a, b)$. In many applications, $Q$ represents the volume of a structure, and $f(x)$ is the area of the intersection between the structure and a plane that is perpendicular to a given sampling axis at the point of abscissa $x$ (García-Fiñana et al., 2003; García-Fiñana, 2006; García-Fiñana et al., 2009).

**Cavalieri Principle with Point Counting**

Unbiased and efficient volume estimates with known precision (Roberts et al., 2000; García-Fiñana et al., 2003) can be obtained from a set of parallel slices separated by a known distance ($T$), and sampled in a systematic random manner. These criteria are easily obtained from standard sets of MRI and CT scans (Roberts et al., 2000; Acer et al., 2008, 2010).
To apply the point-counting method, a square grid system is superimposed with random placement onto each Cavalieri section or slice and the number of points falling within the reference area (area of interest) counted on each section (Figure 1.1). Finally, an unbiased estimate of volume is calculated from Equation 1.3:

\[ V = T \times \left( \frac{a}{p} \right) \times (P_1 + P_2 + P_3 + \cdots + P_n), \]  

(1.3)

where \( n \) is the number of sections, \( P_1, P_2, \ldots, P_n \) show point counts, \( a/p \) represents the area associated with each test point, and \( T \) is the sectioning interval.

We used software that allowed the user to automatically sum the area of each slice and determine brain volumes by the Cavalieri principle. An unbiased estimate of volume was obtained as the sum of the estimated areas of the structure transects on consecutive systematic sections multiplied by the distance between sections, that is, \( V = \Sigma A \cdot T \). The program allowed the user to determine contrast, select true threshold value to estimate the point count automatically (Denby et al., 2009).

Coefficient of Error (CE) and Confidence Interval (CI) for Volume Estimation

The precision of volume estimation by the Cavalieri method was estimated by CE. Based on the original work of Matheron, the CE was adapted to the Cavalieri volume estimator by Gundersen and Jensen (1987) and more recently simplified by a number of stereologists (Gundersen et al., 1999; García-Fiñana et al., 2003; Cruz-Orive, 2006; Ertekin et al., 2010; Hall and Ziegel, 2011). The CE is useful for estimating the contribution of sampling error to the overall (total) variation for stereological estimates. A pilot study of the mean CE estimate allows the user to optimize sampling parameters, for example, mean CE less than one-half of total variation; to select the appropriate number of MRI sections through the reference space; and to set the optimal density of the point or cycloid grid.

García-Fiñana (2006) pointed out that the asymptotic distribution of the parameter volume as its variance is strongly connected with the smoothness properties of the measurement function. Using the Cavalieri method, we constructed both CE and a CI value for estimation of brain volumes. The first calculation involved the estimation of volume, variance of the volume estimate, and bounded intervals for the volume by Eq. (1.3).

Second, \( \text{Var} (Q_T) \) was estimated via Eq. (1.4) according to Kiêu (1997), which first requires calculation of \( \alpha(q), C_0, C_1, C_2, \) and \( C_4 \) (Table 1.1):

\[ \text{Var} \left( \hat{Q}_T \right) = \alpha(q) \times (3C_0 - 4C_1 + C_2)T^2, \quad q \in [0, 1]. \]  

(1.4)

Eq. (1.5) leads to

\[ C_k = \sum_{i=1}^{n-k} f_i f_{i+k}, \quad k = 1, 2, \ldots n - 1. \]  

(1.5)

The quantities \( C_0, C_1, \) and \( C_2 \) can be computed from the systematic data sample (García-Fiñana et al., 2003).

The smoothness constant \( (q) \) is then estimated from Eq. (1.6) as given in the following:

\[ q = \max \left\{ 0, \frac{1}{2 \log 2} \log \left[ \frac{(3C_0 - 4C_2 + C_4)}{(3C_0 - 4C_1 + C_2)} - \frac{1}{2} \right] \right\}. \]  

(1.6)
The coefficient $\alpha(q)$ has the following expression:

$$
\alpha(q) = \frac{\Gamma(2q + 2)\zeta(2q + 2)\cos(\tau q)}{2 \tau^{2q + 2} (1 - 2^{2q - 1})}, \quad q \in [0, 1],
$$

where $\Gamma$ and $\zeta$ denote the gamma function and the Riemann zeta function, respectively. For fairly regular, quasi-ellipsoidal objects, $q$ approaches 1, and for irregular objects, $q$ approaches 0. Under these circumstances, $\alpha(0) = 0.83$ and $\alpha(1) = 0.0041$ (García-Fiñana et al., 2003).

The bounded interval for the cerebral volume was obtained by Eq. (1.8):

$$
\hat{Q} = mT \lambda_\gamma \sqrt{\alpha(q)(3C_0 - 4C_1 + C_2)}.
$$

Note that Eq. (1.8) gives the approximate lower and upper bounds for $V_2 - V_1$.

### Example for Cerebral Volume, CE, and CI

Examples are provided for estimation of cerebral volume with upper and lower CI values and CE. To estimate brain volume, we used the total data set of 158 images with slice thickness 1 mm split into 15 Cavalieri planes, that is, every 10th magnetic resonance (MR) image with a different random starting point. Thus, each Cavalieri sample represents the area of cerebral cortex of a set of MR images at distance $T = 10 \cdot 1 \text{ mm} = 10 \text{ mm}$ apart (Table 1.1).

We illustrate the calculation steps involved in the estimation of a lower and upper bound for the true cerebral cortex volume by applying Eq. (1.8) to one set of Cavalieri planes (i.e., 26, 66, 98, 132, 156, 163, 158, 150, 115, 85, 22). This data sample represents the area of cerebral cortex in square centimeters on $n = 11$ MR sections a distance $T = 1 \text{ cm}$ apart. The Cavalieri volume for cerebrum was obtained using Eq. (1.3) as follows:

$$
V = 1 \times 1^2 \times (26 + 66 + \cdots + 85 + 22) = 1171.0 \text{ cm}^3.
$$

### Table 1.1 Calculation of the constants $C_0$, $C_1$, $C_2$, and $C_4$ for brain volume

<table>
<thead>
<tr>
<th>Section(i)</th>
<th>$P_1$</th>
<th>$P_1^2$</th>
<th>$P_1P_{i+1}$</th>
<th>$P_1P_{i+2}$</th>
<th>$P_1P_{i+4}$</th>
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<td>130,612</td>
<td>109,433</td>
</tr>
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</table>
The smoothness constant \( (q) \) is estimated from Eq. (1.6) as follows:

\[
q = \left\{0, \frac{1}{2\log 2} \log \left[\frac{3 \times 151363 - 4 \times 130612 + 109433}{3 \times 151363 - 4 \times 145489 + 130612}\right] - \frac{1}{2}\right\} = 0.815.
\]

(1.11)

Applying Eq. (1.7) with \( q = 0.815 \) leads to \( \alpha(q) \) as follows:

\[
\alpha(0.815) = \frac{\Gamma(2 \times 0.815 + 2) \zeta(2 \times 0.815 + 2) \times \cos(3.14 \times 0.815)}{(2\pi)^{2 \times 0.815^2} (1 - 2^{2 \times 0.815 - 1})} = 0.008.
\]

(1.12)

Therefore, the estimate of \( \text{Var}(\hat{Q}_T) \) obtained via Eq. (1.4) is

\[
\text{Var}(\hat{Q}_T) = \alpha(q)(3C_0 - 4C_1 + C_2) \times T^2
\]

\[
\text{Var}(\hat{Q}_T) = 0.008 \times (3 \times 151363 - 4 \times 145489 + 130612) \times (1)^2
\]

\[
\text{Var}(\hat{Q}_T) = 22.65.
\]

The CE for this estimate is calculated as shown in Eq. (1.13):

\[
CE(Q_T) = \sqrt{22.65 / 1171} = 4\%.
\]

(1.13)

Values for CI were calculated using Eq. (1.8):

\[
\left(1171 - 3.38 \times \sqrt{22.65}, 1171 + 3.38 \times \sqrt{22.65}\right) = (1154 - 1187) \text{ cm}^3
\]

\[
V_2 - V_1 = 1154 \text{ cm}^3 - 1187 \text{ cm}^3.
\]

(1.14)

This example allowed us to identify the \( \lambda \) value as 3.38 according to García-Fiñana (2006). Predictive CE values were calculated using the R program using developed R codes to calculate the contribution to the predictive CE. After the initial setup and preparation of the formula, the point counts and other data were entered for each scan, and the final data were obtained automatically using the R program (Appendix A).

### Volume Estimation Using Spatial Grid of Points

The method of volume estimation using a spatial grid of points (Gundersen and Jensen, 1987; Cruz-Orive, 1997; Kubínová and Janáček, 2001) is an efficient modification of the Cavalieri principle. If a cubic spatial grid of points is applied, the object volume can be estimated by the formula (Eq. 1.15):

\[
V = u^3 \times P,
\]

(1.15)
where \( u \) is the grid constant (distance between two neighboring points of the grid), and \( P \) is the number of grid points falling into the object.

Using slice thickness and distance between two test points of the grid for the same value such as 1 cm leads to simple estimates of brain volume. A 1 cm \( \times \) 1 cm \( \times \) 1 cm grid was chosen, indicating a grid point spacing of 1 cm\(^3\) in the plane of the image and through the depth of the volume.

**Surface Area Estimation**

Surface area can be estimated on vertical uniform random (VUR) and isotropic uniform random (IUR) tissue sections (Baddeley et al., 1986). Estimations of surfaces require randomness of slice direction, also known as isotropy, as well as slice position (Henery and Mayhew, 1989; Mayhew et al., 1996; Roberts et al., 2000).

**Estimation of Surface Area Using Vertical Sections**

With this approach, surface area is sampled at systematic random positions in 3D and combines the Cavalieri principle with vertical sectioning (Baddeley et al., 1986), thus offering major advantages over earlier methods for estimation of surface area (Mayhew et al., 1990, 1996).

The vertical section technique for surface area estimation uses cycloid test probes (Baddeley et al., 1986). A cycloid probe is a line for which the length of arc oriented in a particular direction is proportional to the \( \sin \) of the angle to vertical. The bias produced by taking VUR as opposed to IUR sections is exactly canceled by the inherent \( \sin \) weighting of the orientation of the cycloid test lines; thus, IUR and VUR sections with cycloids are equivalent (Baddeley et al., 1986; Gual-Arnau and Cruz-Orive, 1998; Gokhale et al., 2004).

Vertical sections are planar sections longitudinal to either a fixed (but arbitrary) axial direction or perpendicular to a given horizontal plane. After rotating the object of interest by \( \phi \), the user cuts sections with uniform random position perpendicular to the vertical axis, thereby generating planes of fixed distance \( T \) apart, all vertical with respect to the horizontal reference plane (Baddeley et al., 1986; Howard and Reed, 2005). For example, if the horizontal plane is an axial section, coronal and sagittal sections will be vertical sections (Michel and Cruz-Orive, 1988; Pachê et al., 1993). The 3D of objects is divided with uniform random position along each generating orientation to form \( n \) Cavalieri series of vertical sections (Figure 1.2a,b).

Both CT and MRI allow for sampling 3D objects into an exhaustive series of vertical sections in several systematic random orientations, each randomly offset with respect to a fixed vertical axis (Roberts et al., 2000). Thus, these data sets meet the requirement for isotropic and thus random orientations may be obtained for estimation of surface area as described previously (Roberts et al., 2000; Kubínová and Janáček, 2001).

A random direction of the uniform random line in the first orientation was selected as a random angle \( a \) from the interval \((0^\circ, 180^\circ)\), then VUR sections generated a fixed distance \( T \) apart, \( T = 1 \) cm. Starting with a \( 5^\circ \) random angle, the direction of vertical sections in the \( j \)th segment is given by the angle \( a_j = a_0 + (j - 1) \times (180^\circ/m) \) \( (j = 1, \ldots, m) \) (Figure 1.3a–c). For example, if \( m = 4 \) and \( a_0 = 5^\circ \), then \( a_2 = 5^\circ + 1 \times (180^\circ/4) = 50^\circ \), \( a_3 = 95^\circ \), \( a_4 = 140^\circ \) (see Figure 1.3).

The relevant formula for estimating surface area from an exhaustive series of vertical sections was calculated from Equation 1.16 (Roberts et al., 2000; Ronan et al., 2006; Acer et al., 2010):

\[
S = 2 \times T \times a / l \times I.
\] (1.16)
Figure 1.2  (a) The object is fixed horizontal plane (H), and is given isotropic rotation on the horizontal plane. (b) The object is divided with uniform random position along each generating orientation \( n = 3 \) to form nine Cavalieri sections of vertical section. \( \Theta_1 \) = random angle (obtained in the interval \( \pi/n \), \( n \) = number of orientations, \( T \) is the interval of the sections. For example, if \( n = 3 \) and \( \Theta_1 = 20^\circ \), then \( \Theta_2 = 20^\circ + 1 \times (180^\circ/3) = 80^\circ \), \( \Theta_3 = 140^\circ \).

Figure 1.3  (a) An example of four systematic orientations (if \( n = 4 \) and \( a_1 = 5^\circ \), then \( a_2 = 5^\circ + 1 \times (180^\circ/4) = 50^\circ \), \( a_3 = 95^\circ \), \( a_4 = 140^\circ \). (b) A total of seven Cavalieri sections with slice separation (T) of 2 cm. (c) Illustration of cycloid probe overlaid on one subject all brain sections.

We modified the formula for surface area estimations of radiological images as shown in Equation 1.17:

\[
S = \frac{2}{n} \times T \times \left( \frac{a/I \times SU}{SL} \right) \times I,
\]  

(1.17)
where \( n \) is the number of random systematic orientation, \( a/l \) is the ratio of the test area to the cycloid length, \( T \) is the distance between serial sections, “SU” is the scale unit of the printed film, “SL” is the measured length of the scale printed on the film, and \( I \) is the number of intersections on all sections.

Eqs. (1.18, 1.19) that were used to estimate the surface area of an object by vertical sections are given in the following two formulas:

\[
\hat{S}_{n1} = \frac{\pi}{n} \left( \hat{f}_1 + \hat{f}_2 + \ldots + \hat{f}_n \right) \quad \text{(1.18)}
\]

\[
\hat{f}_i = \frac{2}{\pi} \cdot \frac{a}{l} \cdot T \cdot \sum_{j=1}^{k_i} I_{ij}, \quad i = 1, 2, \ldots, n \quad \text{(1.19)}
\]

\[
\hat{f}_1 = \frac{2}{\pi} \times 1 \times 1 \times 457 = 291.109
\]

\[
\hat{f}_2 = \frac{2}{\pi} \times 1 \times 1 \times 426 = 271.362
\]

\[
\hat{f}_3 = \frac{2}{\pi} \times 1 \times 1 \times 389 = 247.793
\]

\[
\hat{f}_4 = \frac{2}{\pi} \times 1 \times 1 \times 382 = 243.334
\]

\[
\hat{S}_{n1} = \frac{\pi}{4} (291.109 + 271.362 + 247.793 + 243.334) = 827.0749 \text{ cm}^2,
\]

where \( n \) represents the number of random systematic orientation about a central vertical axis through the object, \( a/l \) represents the ratio of test area to cycloid test length, \( T \) represents distance between Cavalieri vertical sections, \( I_{ij} \) represent the number of intersections between cycloids and the \( j \)th vertical trace of the \( i \)th series; \( k_i \) is the number of nonempty vertical sections in that series \((n = 4, T = 1 \text{ cm}, a/l = 1 \text{ cm}, \sum_{i=1}^{n} I_i = 1654)\):

\[
\hat{S}_{n2} = \frac{2}{n} \cdot \frac{a}{l} \cdot T \cdot \sum_{i=1}^{n} I_i, \quad I_i = \sum_{i=1}^{k_i} I_{ij} \quad \text{(1.19)}
\]

\[
\hat{S}_{n2} = \frac{2 \times 1 \times 1 \times 1654}{4} = 827.074 \text{ cm}^2.
\]

Four orientations were used to calculate the \( a/l \) using \( d = 0.637 \text{ cm} \). In our experiment, cycloid test lines had a 1-cm ratio of area associated with each cycloid for \( a/l \) according to Eq. 1.22 (Figure 1.4). In each orientation, the number of intersections on each of the MRI sections was counted on Cavalieri slices:

\[
a/l = \pi \times d / 2 \quad \text{(1.22)}
\]

\[
a/l = 3.14 \times 0.637 / 2 = 1 \text{ cm}. \quad \text{(1.23)}
\]

After transferring MR images to a personal computer, surface area estimation was done using different softwares. The first approach required conversion of the images to analyze format using
ImageJ, which is available free from the NIH (http://rsb.info.nih.gov/ij). After formatting, hdr or img file was opened using MRIcro (http://www.mccauslandcenter.sc.edu/mricro/mricro/index.html). MRIcro allows for rotation of each image for selection of random angles (Figure 1.3). Finally, the stereological parameters were estimated using EasyMeasure software.

Error Prediction for Surface Area for Vertical Section Method

Error prediction was divided into three processes: systematic orientations; systematic parallel sections for each orientation; and intersection counting with a cycloid test system on each section (Cruz-Orive and Gual-Arnau, 2002):

1. $\text{Var}_0(\tilde{S}_n)$, the variance due to $n$ systematic orientations
2. $\text{Var}_{\text{cav}}(\tilde{S}_n)$, the variance due to Cavalieri vertical sections
3. $\text{Var}_{\text{cyc}}(\tilde{S}_n)$, the variance due to application of cycloid probe,

where $n$ represents the number of orientations.

The CE is directly related to the variance of the surface area estimator:

$$CE(\tilde{S}_n) = \frac{\sqrt{\text{Var}(\tilde{S}_n)}}{S_n} \times 100$$  \hspace{1cm} (1.24)

$$CE^2(\tilde{S}_n) = CE_0^2(\tilde{S}_n) + CE_{\text{cav}}^2(\tilde{S}_n) + CE_{\text{cyc}}^2(\tilde{S}_n)$$  \hspace{1cm} (1.25)

$$ce(\tilde{S}_n) = \sqrt{ce_0^2(\tilde{S}) + ce_{\text{cav}}^2(\tilde{S}) + ce_{\text{cyc}}^2(\tilde{S})},$$  \hspace{1cm} (1.26)

where $CE_0$, $CE_{\text{cav}}$, and $CE_{\text{cyc}}$ are orientation, Cavalieri, and cycloid CE values, respectively.

Although error prediction for surface area on systematic sampled images has not been previously reported in the literature, Cruz-Orive and Gual-Arnau (2002) examined precision of circular systematic sampling and discussed error prediction formulae for the surface area estimator on vertical sections. For this approach based on Cruz-Orive and Gual-Arnau (2002), the variance from each level sampling in vertical section provides useful information for optimization of sample size to design at different stages for estimation of cerebral surface. The error variance $\hat{\text{var}}_{m}(\tilde{S}_n)$ based on a global model was calculated from systematic sampling using a semicircle:

$$\hat{\text{var}}_{m}(\tilde{S}_n) = \frac{r^2 B_{2m+2} \left( \hat{C}_0 - \hat{C}_1 - \hat{v}_n \right)}{(B_{2m+2} - B_{2m+2} (1/n)) \cdot n^{2m+3} + T^2 \hat{v}_n}, \hspace{0.5cm} n \geq 2, m = 0, 1, \ldots$$  \hspace{1cm} (1.27)
The right side of Eq. (1.27) $T^2\hat{\nu}_n$ estimates the local error. The constants $\hat{C}_0$ and $\hat{C}_1$ (Table 1.3) are determined by

$$C_k = \sum_{i=1}^{n-k} f_i f_{i+k}, \quad i = 1, 2, \ldots, n.$$  \hspace{1cm} (1.28)

$B_2(x)$ and $B_2(0) \equiv B_2$ are a Bernoulli polynomial and number, respectively, as shown in Eq. (1.29):

$$B_2(x) = x^2 - x + 1/6, \quad B_4(x) = x^4 - 2x^3 + x^2 - 1/30.$$  \hspace{1cm} (1.29)

Choosing between the two values $m = 0, 1$ will typically suffice:

$$\hat{D}_m = \frac{\hat{C}_0 - \hat{C}_2 - \hat{\nu}_n}{\hat{C}_0 - \hat{C}_1 - \hat{\nu}_n} - \frac{B_{2m+2} - B_{2m+2}(2/n)}{B_{2m+2} - B_{2m+2}(1/n)}, \quad n \geq 4.$$  \hspace{1cm} (1.30)

For $m = 0$,

$$\frac{B_{2m+2}}{B_{2m+2} - B_{2m+2}(1/n)} = \frac{2(n-2)}{n-1}.$$  \hspace{1cm} (1.31)

For $m = 1$,

$$\frac{B_{2m+2} - B_{2m+2}(2/n)}{B_{2m+2} - B_{2m+2}(1/n)} = \frac{4(n-2)^2}{(n-1)^2}.$$  \hspace{1cm} (1.32)

We must compute $\hat{\nu}_n$:

$$\hat{\nu}_n = \sum_{i=1}^{n} \sigma_i^2.$$  \hspace{1cm} (1.36)

After computing $\hat{\nu}_n$, we have to calculate $\sigma_i^2$ following Equation (1.37). $\sigma_i^2$ is the variance due to Cavalieri sampling in each orientation $k$:

$$\sigma_i^2 = \left(\frac{2}{\pi} \times \frac{a_{ij}}{f} \right)^2 \times h^2 \times \left\{ \alpha(q_i) \times \left[ 3(\hat{C}_{0i} - \hat{\nu}_n) - 4\hat{C}_{ii} + 4\hat{C}_{2i} \right] + \hat{\nu}_{ai} \right\}, \quad n_i \geq 3.$$  \hspace{1cm} (1.37)