Key questions in quantitative neuromorphology are why do we want to be able to generate numbers from microscope images and what questions can we ask and answer with numbers that we cannot ask and answer with photomicrographs? Histologic sections are used to define the normal or abnormal appearance of the brain tissue. In this regard histologic descriptions often use terms like “large,” “small,” “many,” “few,” “absent” or “present.” These terms are very helpful and often sufficient to describe features of sections, particularly when there are distinct differences between pathologic and physiologic conditions. However, they are not good enough to test for statistically significant changes in appearance resulting from a disease or an experimental treatment, particularly if differences between pathologic and physiologic conditions are discrete. In this case, one needs to attach numbers to the more or less subjective terms used in descriptions. Fortunately, almost all terms that are subjective can be associated with numbers, and design-based stereology is the state-of-the-art methodology for doing so.

Originally, the word “stereology” was coined to describe a set of methods that provide a three-dimensional interpretation of structures based on observations made by users for users, provides the reader with a description of all stereologic methods available. Rather, it is written by users for users, provides the reader with a guided tour through the relevant literature. It has been the experience of the authors that most neuroscientists potentially interested in design-based stereology need to analyze volumes of brain regions, numbers of cells (neurons, glial cells) within these brain regions, mean volumes (nuclear, perikaryal) of these cells, length densities of linear biological structures such as vessels and nerve fibers within brain regions, and the cytoarchitecture of brain regions (i.e. the spatial distribution of cells within a region of interest). Therefore, a comprehensive introduction to design-based stereologic methods for estimating these parameters is provided. It is demonstrated that results obtained with design-based stereology are representative for the entire brain region of interest, and are independent of the size, shape, spatial orientation, and spatial distribution of the cells to be investigated. Also, it is shown that bias (i.e. systematic error) in results obtained with design-based stereology can be limited to a minimum, and that it is possible to assess the variability of these results. These characteristics establish the advantages of design-based stereologic methods in quantitative neuromorphology.

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on two-dimensional sections (Weibel, 1979, 1980, 1992; for an overview on the history of neuromorphometry see Haug, 1986). However, in the current use of design-based stereology many methods (so-called probes) make use of three-dimensional sections. The term “design-based” indicates that the probes and the sampling schemes that define the newer methods in stereology are “designed,” that is, defined a priori, in such a manner that one need not take into consideration the size, shape, spatial orientation, and spatial distribution of the cells to be investigated (West, 2002). The elimination of the need for information about the geometry of the cells to be investigated results in more robust data because potential sources of systematic errors in the calculations are eliminated (Gundersen et al., 1988; West, 1993, 2002).

Design-based stereology can be divided into analyses of the global and local characteristics of tissues, the most important of which are volume, number, connectivity, spatial distribution, and length of linear biological structures. These characteristics can be expressed as absolute values (e.g. the volume of the rat cerebellar granule cell layer, the number of granule cells in the rat cerebellum, etc.) or as relative values (e.g. the volume fraction of the rat cerebellum occupied by the granule cell layer, the density of granule cells within the granule cell layer of the rat cerebellum, etc.). Both global and local characteristics can be analyzed by a variety of stereologic probes. For example, the volume of the granule cell layer is a global characteristic of the cerebellum that can be analyzed with Cavalieri’s principle and point counting techniques; the mean nuclear volume of the granule cells is a local characteristic of the cerebellum that can be analyzed with the so-called Nucleator, the Rotator, and the Point Sampled Intercepts method.

Design-based stereology can nowadays be viewed as a well-established methodology to reveal certain features of development, repair, natural aging, and normal anatomy of the brain which could not be detected otherwise. (A continuously updated list of currently more than 1100 papers describing stereologic investigations of the brain can be found in the Stereology Literature Database [Enterprise Biology Software Project; Medina, WA, USA].) Furthermore, the rigorous use of design-based stereologic methods allows one to directly compare results of different quantitative histologic studies on the same topic, as has been noted in the literature (Coggeshall and Lekan, 1996; Coleman, 1997; Harrison, 1999).

It is not the intention of this review to provide a full description of all design-based stereologic probes available. Rather, it aims to provide the reader with a guided tour through the relevant literature. It contains eight sections that can be read independently of each other. Among them, section 5 addresses the central question of what can be analyzed with design-based stereology, considering the experience of the authors that most neuroscientists potentially interested in design-based stereology need to analyze volumes of brain regions, numbers of cells (neurons, glial cells) within these brain regions, mean volumes (nuclear, perikaryal) of these cells, length densities of linear biological structures such as vessels and nerve fibers within brain regions, and the cytoarchitecture of brain regions (i.e. the spatial distribution of cells within a region of interest).

Section 1: Basic information about design-based stereology in the literature

More than 20 reviews and eight comprehensive books dedicated to design-based stereology and its applications in neurosciences are available. Among the reviews, some address more basic concepts of design-based stereology, whereas others deal with specific details of this methodology. Within the first category, the papers by Gundersen (1986, 1992), Cruz-Orive and Weibel (1990), Coggeshall and Lekan (1996), Mayhew and Gundersen (1996) and Royet (1991) provide introductions to the basic concepts of quantification, probes and sampling, whereas the papers by Gundersen et al. (1998), Mayhew (1992), Hyman et al. (1998) and particularly West (1993, 1999, 2002) are more application oriented. Comparisons between earlier “assumption-based” or “model based” stereologic methods (due to their reliance on a model of cell geometry for correction formulas) and the more recently developed “design-based” (assumption-free or model-free) stereologic methods are given in the papers by Coggeshall (1999), Geuna (2000) and West (2001).

Among the specialized reviews, the papers by Dorph-Petersen et al. (2001) and von Bartheld (2002) are dedicated to the strengths and limitations of profile- and dissector-based cell counting methods, with a particular focus on the problem of tissue deformation as a result of histologic preparations. The paper by Calhoun and Mouton (2001) addresses the history and problems of length estimations, and the paper by Glaser and Glaser (2000) the current advantages in computer-based stereology. The paper by Oorschot et al. (1991) describes the usability of design-based stereology in quantitative analyses of cultured explants of the nervous system. Finally, the papers by Mayhew (1996), Petersen (1999), Kubinova and Janacek (2001) and Kubinova et al. (2004) present strategies and illustrations of approaches to implementing design-based stereologic procedures in electron microscopy and confocal microscopy.

The books written by Howard and Reed (1998) and by Nurcombe et al. (1999) provide relatively short but comprehensive overviews of stereologic methodology and can be recommended in particular to newcomers in the field. Mouton’s (2002) book is a more in-depth description of the many stereologic probes; the books by Vedel Jensen (1998) and by Russ and Dephoff (2000) are more dedicated to the statistical and mathematical foundations of stereologic methods. Those particularly interested in stereology at the electron microscopy level may find useful information in the book by Reith and Mayhew (1988). Finally, the pioneering books by Weibel and Elias (1967), Elias and Hyde (1983) and Weibel (1979, 1980) must be mentioned as the original text dealing with these issues. However, these books do not cover the recent methodologic developments for measuring global and local volumes, for counting cells, and for analyzing the spatial distribution of cells within a given tissue or brain region.
Section 2: Considerations for specimen preparation for design-based stereologic analysis

Design-based stereologic methods have been developed to make statements about structures such as an identified organ, a definable brain region, a population of cells, or linear biological structures within a tissue. In the case of the brain, if such statements are to be valid for an entire brain region, then the analyzed sample of sections and microscopic fields has to be representative of it. This requires that one has access to the entire brain region, that all cells or linear biological structures within this region are recognizable by an appropriate (sensitive and specific) staining, and that all parts of the brain region have the same chance to contribute to the sample. Furthermore, if these statements are supposed to be unbiased (i.e. without systematic error), the estimates must be independent of the size, shape, spatial orientation, and spatial distribution of the cells or linear biological structures to be investigated (see section 4 for details).

It is an advantage of design-based stereology that all probes described in section 5 have been developed taking these prerequisites into account. Access to the entire region of interest is achieved by systematic-random sampling of sections from exhaustive section series encompassing the entire region (Gundersen, 1986; Gundersen and Jensen, 1987). Selection of microscopic fields in a systematic-random manner (explained in detail in section 5) guarantees that all parts of the region of interest have the same chance to contribute to the sample (Gundersen and Jensen, 1987). Independence of design-based stereologic estimates from the size, shape, spatial orientation, and spatial distribution of the neurons or linear biological structures under study is achieved by the three-dimensional design of almost all probes described in section 5, as well as by the development of special sections ("isotropic uniform random" [Miles and Davy, 1976] or "vertical" sections [Baddeley et al., 1986]), or the analysis of thin virtual sections within thick brain sections (Sterio, 1984; Williams and Rakic, 1988; West, 1993). In any case, the application of these new 3-dimensional (3D) stereologic probes requires the use of thick (i.e. 3D) sections instead of thin, 2-dimensional (2D) sections. In several studies, protocols have been provided and tested to appropriately prepare such thick (3D) sections (Hatton and von Bartheld, 1999; Heinsen et al., 2000; Messina et al., 2000; Perl et al., 2000; Schmitz et al., 2000; Dorph-Petersen et al., 2001; Gardella et al., 2003). Obviously, under certain conditions (such as stereologic analyses in electron microscopy) the use of thin (2D) sections cannot be circumvented. In this case, however, certain rules have to be considered such as the use of the so-called physical disector for counting neurons instead of the optical disector (see section 5.3 for details).

Thus, design-based stereologic analyses cannot be initiated right away on existing sections, if they have not been prepared adequately for design-based stereology. To circumvent this problem, one could argue that the analysis of only one or a few sections per investigated brain is sufficient, if they are “representative” sections and selected, for example, by clear anatomical landmarks. Furthermore, one could argue that the analysis of neuron densities (which does not require access to the entire brain region) is as useful as the analysis of the corresponding total numbers of neurons (requiring access to the entire brain region). However, alterations in neuron densities may considerably differ from alterations in the corresponding total numbers of neurons, and results obtained from systematically and randomly sampled sections may considerably differ from corresponding results obtained using single “representative” sections (for a particularly clear example of this issue see the companion study by Schmitz et al., 2004). Finally, one could argue that at least partial correction for the influence of size, shape, spatial orientation, and spatial distribution of the neurons to be investigated using approaches such as the Abercrombie’s correction for estimates of numbers of cells (based on the assumption that cells or cell nuclei are spheres; Abercrombie, 1946) may circumvent the need for using thick (3D) sections (Hedreen, 1998). In practice, however, it is rarely possible to figure out how well the application of such correction methods really adjusted for bias in quantitative analyses based on the size, shape, spatial orientation, and spatial distribution of the neurons to be investigated (for details see, e.g. Mouton, 2002). With the correct use of design-based stereology this is not any longer an issue.

Section 3: Laboratory equipment for design-based stereologic analyses

Recent developments in design-based stereology include techniques and analyses that have become possible only with the introduction of computer-interfaced microscopes and imaging instrumentation, in particular, estimates of local volumes with the Optical Rotator (section 5.5), estimates of the length of linear biological structures with Space Balls (section 5.7), and investigations on the spatial distribution of cells with Nearest-Neighbor analysis (section 5.8). Furthermore, the availability of semiautomated, computer-based stereology systems has substantially reduced both the observer’s effort and potential errors associated with the use of the other methods described in section 5. Moreover, these systems offer the combination of computer-based anatomical mapping and rigorous stereologic estimates.

Currently the commercially available stereology systems include (in alphabetical order): AIS stereology system (Imaging Research, St. Catharines, Ontario, Canada), CAST (Visiopharm, Hørsholm, Denmark), Digital Stereology (Kinetic Imaging, Bromborough, UK), Stereologer (Systems Planning and Analysis, Alexandria, VA, USA), Stereology Toolkit Plug-in for NOVA PRIME (Bioquant Image Analysis Corporation, Nashville, TN, USA) and Stereonvigator (MicroBrightField, Williston, VT, USA). These systems integrate a three-axis motor-driven specimen stage with a computer in order to acquire data from 3D structures, and implement (to various degrees) the stereologic probes described in section 5. The motorized stage is used to map brain regions and objects that are larger than a single microscopic field, to rapidly access specific locations throughout the entire region of interest.
regardless of optical magnification, and to perform systematic random sampling (for details see Glaser and Glaser, 2000). Furthermore, the microscope is usually equipped with a z axis position encoder (sometimes referred to as a microrator), that measures accurately the actual focal position of the microscope stage. This is particularly important when using 3D probes like the Optical Disector, the Optical Rotator, Space Balls and Nearest-Neighbor analysis (see section 5).

The tissue specimen is usually viewed on a computer monitor via a high-resolution analog CCD video camera or a modern digital camera with more than 10 frames per second, allowing one to focus through the tissue in real time (see Glaser and Glaser, 2000, for details). In addition, as stereologic applications now extend to confocal microscopy (Petersen, 1999), electron microscopy (Mayhew, 1996), computed tomography (Pakkenberg et al., 1989) and magnetic resonance imaging (Roberts et al., 1993), some commercial stereology systems provide image file readers that are capable of accepting 3D confocal and magnetic resonance imaging image sets, as well as the file formats generated by a variety of electron microscopes and flatbed scanners.

When using any of these visualization methods, the user views the tissue specimen with the stereologic probe’s geometric overlay superimposed upon it. For example, when counting neurons with the Optical Fractionator, the system generates an unbiased counting frame (see section 5.3 for details). When investigating lengths of linear biological structures or Nearest-Neighbor analyses the software generates virtual spheres, whose intersections with the current focal plane are represented by circles of varying diameter, as the focal plane of the microscope lens is moved through the thick tissue sections (details in section 5.7). Then the user applies the counting rules of the probe by mouse-clicking on the sites of interaction of the specimen with the probe’s overlay. Depending on the system used, the resulting data may be presented and stored in different ways. Some systems save only the raw data obtained, such as the number of counted cells. Other systems save all of the acquired data in an object-oriented file containing the 3D locations of all points entered into the file and their relationship to one another, i.e. as a contour or a set of points of a particular type.

In summary, with the advent of advanced computer based systems, stereology became a practical laboratory method. Although some stereologic analyses can still be performed with simple microscopes and minimal computer assistance, the advantages provided by design-based stereology are best obtained when it is integrated into computer-based microscopy systems that optimize data collection, storage and analysis.

Section 4: Potential bias in results of design-based stereologic analyses

Let us assume a stereologic estimate of the total number of neurons within a certain brain region. If this estimate could be repeated ad infinitum and the mean of the estimates would equal the (unknown) true total number of neurons within this region, the estimates would be unbiased (i.e. without systematic error; for details see West, 2002). Importantly, the use of design-based stereology does not guarantee unbiasedness of the corresponding estimates (Guillery and Herrup, 1997). Rather, the use of design-based stereologic methods facilitates estimates of total numbers of neurons, cell volumes, object lengths etc., without having to take into consideration the size, shape, spatial orientation, and spatial distribution of the structures to be investigated (West, 2002). To minimize bias (i.e. systematic error) in stereologic estimates, a couple of additional requirements are necessary.

First, it is well known that different fixation and embedding protocols can result in very different shrinkage of the tissue (Bauchot, 1967; Kretschmann et al., 1982; Quester and Schröder, 1997; Dorph-Petersen et al., 2001). Therefore estimates of the volume of brain regions or cells (but also length estimates, investigations on neuron density and analyses of the spatial distribution of cells) will depend on the protocols used. In consequence, comparisons between groups should be restricted to material which has been processed under identical conditions. Note that estimates of total numbers of neurons (when carried out as described in sections 5.3 and 5.4) are not affected by tissue shrinkage during fixation and embedding. Second, one needs access to the entire brain region, the entire region must be recognizable, and all parts of the brain region under study must have the same chance to contribute to the stereologic sample. Third, the estimates must neither be affected by loss of structures at the upper or the lower surface of the sections when hit by the knife during cutting the tissue, nor by incomplete staining of the tissue, particularly in the middle of the section thickness (see section 5.3 for details). Fourth, particularly the use of methacrylate and paraffin as embedding media may cause inhomogeneous compression of tissue sections along the z axis during cutting of the tissue, resulting in differences in particle densities along this axis (Hatton and von Bartheld, 1999; von Bartheld, 2002; Gardella et al., 2003). This may be a potential source of bias, particularly in estimates of total numbers of neurons and analyses of the spatial distribution of cells. Cryostat sections have been proven to be unaffected by this problem (Hatton and von Bartheld, 1999; Gardella et al., 2003). However, the real impact of this problem might be much less than anticipated: in fact, estimates of the total number of pyramidal cells within the rat hippocampus obtained on methacrylate sections are very similar to corresponding estimates obtained on cryostat sections (for details see Schmitz et al., 2000). Fifth, irrespective of the embedding medium and the cutting procedure one has to consider shrinkage of the sections along the z axis (West et al., 1991; Messina et al., 2000; Dorph-Petersen et al., 2001). This shrinkage may be surprisingly high (Schmitz et al., 2000) and may vary from one part of a section to another. Measurements of the section thickness (preferably at each investigated microscopic field) with the z axis position encoder of a stereology system (see section 3 for details) can give reasonably accurate readings of the actual section thickness, provided...
that the entire thickness of the sections is stained, an oil objective is used for the measurements (see West and Slomianka, 1998), Köhler illumination is achieved, and the condenser is opened up appropriately. Note that correction for actual section thickness is crucial particularly for estimates of the volume of brain regions, investigations of total numbers of neurons and analyses of the spatial distribution of cells. It should be mentioned that problems of incomplete dye penetration cannot be excluded even with a simple Nissl stain (Cooper et al., 1988), although protocols are available to stain completely even 500 nm thick brain sections (Heinsen et al., 2000). Incomplete antibody penetration in immunohistochemistry may be prevented by free-floating staining procedures.

In summary, there are various potential sources of bias (systematic error) in design-based stereology. However, appropriate protocols have been developed to minimize this bias. The similarity of results reported by different authors on the same parameters (an example is provided in Table 1; see also Schmitz et al., 2000) represents a proof of the validity and effectiveness of these protocols.

**Section 5: Parameters that can be assessed by design-based stereology**

**5.1: Considerations about delineation of brain regions.** Each stereologic investigation starts with the identification of the boundaries of the brain region of interest (thereafter abbreviated as BROI) on a systematic-random series of sections throughout this BROI (Fig. 1A). In many cases (such as the cerebellar granule cell layer) the boundaries of the BROI can easily be identified on Nissl-stained sections, and one can trace the boundaries on video images displayed on a computer (Glaser and Glaser, 2000). Alternatively, one can perform only a rough delineation of the BROI at low magnification so that the entire BROI is included in the traced area, then to switch to a higher magnification, to scan the traced area systematically, and to investigate only those microscopic fields which belong to the BROI (as has been proposed for the vocal control nucleus of songbirds; Vogels, 1997). Boundaries of cortical areas in the primate brain can be established by standardized, observer-independent methods such as the "gray level index (GLI)" method (Schleicher and Zilles, 1990; Schleicher et al., 1999) or with a chemoarchitectural approach, i.e. making use of differences in the regional distribution of certain subsets of neurons (for examples see Carmichael and Price, 1994; Hof and Morrison, 1995; Ongür et al., 2003; Bussière 2003a,b). Anyway, each stereologic study should provide a description of the method selected to identify the boundaries of the BROI.

5.2: Volume of a brain region. Estimates of global volumes (such as the volume of a brain region) can be performed with Cavalieri's principle (Cavalieri, 1635). In brief, the volume of any BROI can be estimated without bias (i.e. without systematic error) from the profile areas of the cut sections of the BROI (Fig. 2A, B). An initial random cut through the BROI is required, with subsequent cuts at consistent intervals (systematic–random sampling). Provided the sections through the BROI are systematic–random, i.e. all sections through the region have an equal probability of being sampled, Cavalieri’s principle gives an unbiased estimate of the volume of this BROI by multiplying the sum of the profile areas of the BROI on all sections with the distance between the sections (Gundersen and Jensen, 1987).

The profile areas of the sections through the BROI can be measured by tracing the boundaries of the BROI on video images displayed on a computer, and let the software calculate the profile area. Alternatively one can use point counting, which can be achieved by randomly placing a rectangular lattice of known side lengths on the surface of a section, and counting the intersections of the lattice and the BROI (Fig. 1B). Provided the position of the lattice on the BROI is random, i.e. all parts of the BROI have an equal probability of being hit by the lattice, this method gives an unbiased estimate of the profile area of this BROI by multiplying the sum of the counted intersections (or points) with the uniform area represented by each intersection (or point, respectively; Gundersen and Jensen, 1987). This area is determined by the side lengths of the lattice.

One has to consider potential overestimation of global volumes by overprojection when investigating sections under the microscope (Fig. 2C; Uylings et al., 1986). Such overestimation can be accounted for by disregarding the section with the largest projection area (Gundersen and Jensen, 1987). Alternatively, overestimation of global vol-

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**Table 1. Estimates of the total number of hippocampal granule and pyramidal cells in the rat brain with design-based stereologic methods (unilateral values)**

<table>
<thead>
<tr>
<th>First author</th>
<th>Strain</th>
<th>Sex</th>
<th>Age (months)</th>
<th>GC (DG)</th>
<th>PC (CA1-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lukoyanov¹</td>
<td>Wistar</td>
<td>Male</td>
<td>14</td>
<td>1,310,000</td>
<td>687,000</td>
</tr>
<tr>
<td>Lukoyanov²</td>
<td>Wistar</td>
<td>Male</td>
<td>9</td>
<td>1,300,000</td>
<td>650,000</td>
</tr>
<tr>
<td>Rapp³</td>
<td>Long-Evans</td>
<td>Male</td>
<td>6</td>
<td>1,200,000</td>
<td>615,000</td>
</tr>
<tr>
<td>Rasmussen⁴</td>
<td>Wistar</td>
<td>Male</td>
<td>2</td>
<td>1,550,000</td>
<td>930,000</td>
</tr>
<tr>
<td>Schmitz⁵</td>
<td>Long Evans</td>
<td>Male</td>
<td>3</td>
<td>1,307,000</td>
<td>694,000</td>
</tr>
<tr>
<td>Sousa⁶</td>
<td>Wistar</td>
<td>Male</td>
<td>7</td>
<td>1,200,000</td>
<td>660,000</td>
</tr>
<tr>
<td>West⁷</td>
<td>Wistar</td>
<td>?</td>
<td>1</td>
<td>1,200,000</td>
<td>632,000</td>
</tr>
</tbody>
</table>

¹ DG, dentate gyrus; GC, granule cell layer; PC, pyramidal cell layer. The data were taken from the following publications: ¹ Lukoyanov et al. (1999); ² Lukoyanov et al. (2000); ³ Rapp and Gallagher (1996); ⁴ Rasmussen et al. (1996); ⁵ Schmitz et al. (2002b); ⁶ Sousa et al. (1999); ⁷ West et al. (1991).
umes can almost be eliminated by cutting the BROI into an exhaustive series of sections of uniform thickness, selecting a systematic-random series of these sections with a random start for analysis (e.g. every 10th section: sections no. 7, 17, . . .), and performing the analysis using objectives with short depth of focus, with Köhler illumination, and open condenser.

5.3: Number of neurons within a given tissue volume.
Cutting brain tissue into sections also results in cutting the neurons (or glial cells, respectively) within this tissue. However, the number of neuron fragments in the sections differs from the number of neurons within the tissue. As a consequence estimates of neuron numbers based solely on counts of neuron fragments in sections are biased (Fig. 3). Numerous correction procedures have been developed for solving this problem such as Abercrombie’s formula, based on the assumption that cells or cell nuclei are spheres (Abercrombie, 1946). In practice, however, it is rarely possible to figure out how well the application of such correction methods really adjusted for bias in estimates of numbers of neurons (for details see, e.g. Mouton, 2002).

In design-based stereology this problem is solved by counting neurons with so-called unbiased virtual counting spaces (Schmitz and Hof, 2000; often also referred to as “optical dissector” [for example see West, 1993] or “counting box” [Williams and Rakic, 1988]), consisting of a virtual space within the section thickness and a so-called unbiased counting frame (Gundersen, 1977; Fig. 1D, E and Fig. 4). Alternatively, neurons can be counted with the so-called dissector (Sterio, 1984) or physical dissector (analysis of two focal planes within a thick [3D] section, or of two thin [2D] adjacent sections; Fig. 5).

A potential source of bias in neuron counts with unbiased virtual counting spaces are so-called lost caps, i.e. loss of nucleoli or neurons at the upper or the lower surface of sections when hit by the knife during sectioning of the tissue (Andersen and Gundersen, 1999). Lost caps can be prevented using adequate histologic techniques (Schmitz et al., 2000) and by introducing a “guard zone” at both the upper and the lower surface of the sections (Fig. 1D). This also prevents potential bias in neuron number estimates produced by uneven or wavy surfaces of the sections. Another potential source of bias is incomplete staining of the tissue, particularly in the middle of the section thick-
ness. Thus, the height of the unbiased virtual counting spaces should always be adjusted appropriately to the thickness of the sections showing adequate staining, figured out in a small pilot study on the sections prior to stereologic analysis (for example see Jinno et al., 1998).

5.4: Number of neurons within a given brain region. In most experimental settings it is not possible to count all neurons within the BROI. Rather, one has to select a proper sample of microscopic fields to be investigated, and to derive an estimated total number of neurons within the region of interest from the number of neurons in the sample and the sampling probability. The solution to this problem in design-based stereology is shown (for a single section) in Fig. 1C. Unbiased virtual counting spaces are placed in a systematic (spacing) and random (position of grid on section) manner within a series of systematically and randomly sampled sections throughout the BROI, and neurons are counted according to the criteria discussed above at the selected microscopic fields. Note that a unique identifier needs to be defined for each neuron (i.e., profile, nucleus, nucleolus, top of the nucleus, etc.), preferably a punctate one (i.e., the so-called characteristic point; König et al., 1991; see also Fig. 4).

Estimated total numbers of neurons within a BROI can be obtained by the "V_{\text{Ref}}/H_{11003}N_{V}" method (West and Gundersen, 1990), i.e. by calculating the product of the mean neuron density within the unbiased virtual counting spaces \(N_v\) and the global volume of the BROI \(V_{\text{Ref}}\); estimated with Cavalieri’s principle; see section 5.2). Alternatively, one can multiply the number of neurons counted within all unbiased virtual counting spaces with the reciprocal value of the sampling probability ("Fractionator" method; West et al., 1991). The sampling probability depends on the number of investigated sections compared with the total number of sections (the "section sampling fraction"), the base area of the unbiased virtual counting spaces compared with the product of the side lengths of the rectangular lattice used for placing the counting frames within the sections (the "area sampling fraction"), and the height of the unbiased virtual counting spaces (h in Fig. 1D) compared with the average section thickness after histologic processing (the "thickness sampling fraction").

Estimating the number of neurons within a given BROI with the Fractionator method is from an economical point of view more efficient than doing the same with the \(V_{\text{Ref}}\times N_v\) method. This is due to the fact that the Fractionator method does not require estimates of the global volume of the BROI. Interestingly, estimated numbers of neurons obtained with the Fractionator method are also more precise (i.e. statistically more efficient) than corresponding estimates obtained with the \(V_{\text{Ref}}\times N_v\) method (Schmitz and Hof, 2000).

5.5: Mean cellular/nuclear volume. Alterations in perikaryal or nuclear volumes of neurons or glial cells (so-called local volumes) under pathologic or experimental conditions can be regarded as, non-specific in situ indicators of alterations in cell metabolism (Arnold et al., 1995; Insausti et al., 1997; Bussière et al., 2003b). Among other

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**Fig. 2.** Procedure to estimate the volume of a brain region with Cavalieri’s principle. (A) Schematic drawing of an oblate brain region. (B) The brain region is cut into an exhaustive series of sections of uniform thickness, and the top profile area of each (or every nth) section is measured (arrows). An unbiased estimate of the volume of this brain region is obtained by multiplying the sum of the profile areas of the brain region on all sections with the uniform section thickness. Note that the first section of this brain region, shown on top of (B), does not show a top profile area and is therefore not taken into account for the analysis. (C) A potential bias in estimates of global volumes with Cavalieri’s principle can be caused by overprojection when investigating sections at the microscope (arrows). Methods to prevent this are described in the text.
approaches, the "Nucleator" method (Gundersen, 1988), the "Rotator" method (Vedel Jensen and Gundersen, 1993) and the "Optical Rotator" method (Tandrup et al., 1997) have been developed to obtain unbiased estimates of number-weighted mean local volumes (i.e. each neuron has the same probability of being selected for investigation; see summary in Fig. 6). Alternatively, volume-weighted mean local volumes can be estimated with the "Point Sampled Intercepts" method (Gundersen and Jensen, 1985). Here the probability of a neuron being selected for investigation depends on its individual size.

Importantly, these methods require the use of certain types of sections, the so-called isotropic uniform random (Miles and Davy, 1976) or vertical sections (Baddeley et al., 1986). It is quite possible to prepare such sections from a given BROI (Baddeley et al., 1986; Mattfeldt et al., 1990; Nyengaard and Gundersen, 1992; Schmitz et al., 1999). However, in isotropic uniform random or vertical sections the plane of section is unknown, resulting in potential loss of orientation within the sections. To circumvent this problem, the Optical Rotator method was developed (Tandrup et al., 1997), which works well on sections showing only a minimum shrinkage in the z axis (e.g. sections embedded in methacrylate; Sousa et al., 1999; Lukoyanov et al., 1999; Messina et al., 2000). Unfortunately, embedding in methacrylate is not possible in most applications in neuroscience since it makes immunohistochemistry impossible. Frozen or vibratome sections, however, can show considerable shrinkage in the z axis (Messina et al., 2000; Schmitz et al., 2000), preventing the use of the Optical Rotator. This problem has to be individually solved in each application (see Schmitz et al., 1999, for an example in the mouse hippocampus and cerebellum).

5.6: Surface area. Gender-related differences in cortical surface area (Luders et al., 2004) or age-related alterations in the surface area of brain capillaries (that can be interpreted as alterations in the exchange surface between blood and nerve tissue; Villena et al., 2003) are examples of parameters that can be estimated with design-based stereologic methods. The methodological approach is based on counting intersections between the surface of interest and a spatial grid, consisting of systematic probes of parallel test lines (resembling nails of a fakir bed piercing the surface, hence termed "Fakir probes"; Cruz-Orive, 1993). Fakir probes are available for the use of

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**Fig. 3.** Occurrence of bias (systematic error) in counting neurons by inspecting sections of tissue. In all examples shown there is the same number and density of neurons (gray elements), as well as the same spatial distribution of these neurons when focusing on their midpoints. In (A) and (B) the neurons differ in size, whereas in (C) and (D) they differ in their spatial orientation. The gray bar at the bottom of each example represents a section through the tissue. Asterisks indicate neuron fragments which are detected at the upper surface of the section, whereas fragments detected at the lower surface of the section are marked by arrows. Counting all neuron fragments would result in five counted fractions in (A), three in (B), four in (C), and one in (D). In contrast, counting only those neuron fractions which can be detected at the upper surface of the section would result in three counted fractions in (A), one in (B), two in (C), and zero in (D). Furthermore, counting only those neuron fractions which can be detected at the lower surface of the section would result in three counted fractions in (A), two in (B), two in (C), and one in (D). This illustrates the problems resulting from estimates of neuron numbers within a given tissue volume based solely on counts of neuron fragments in the sections.
Fig. 4. Procedure to investigate the number of neurons within a given tissue volume with unbiased virtual counting spaces. The procedure is shown for pyramidal neurons in layer III of an adult mouse neocortex as an example. After perfusion fixation, the mouse brain was frozen and cut into an exhaustive series of 30 μm-thick frontal sections. Immunohistochemical detection of NeuN was carried out according to standard protocols using a monoclonal mouse anti-neuronal nuclei antibody (MAB377; Chemicon, Temecula, CA, USA; 1:100) and a secondary donkey anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA; 1:100). The figure shows the upper surface of a section as well as the same microscopic field at 17 consecutive focal planes below the upper surface, with a distance of 1 μm between the focal planes. Between −3 μm and −14 μm an unbiased counting frame is shown at each focal plane (exclusion lines in red, inclusion lines in yellow), representing an unbiased virtual counting space with a height of 12 μm. The midpoint of the nucleolus was used as the sampling unit (i.e. the “characteristic point”; König et al., 1991) of the neurons. At several focal planes neurons were found where the midpoint of the nucleolus came into focus (arrows). However, only the neurons found at −9 μm, −11 μm and −14 μm were counted. The neuron found at −2 μm was positioned above the unbiased virtual counting space, and the neuron found at −15 μm was positioned below the counting space. The midpoint of the nucleolus of the neuron found at −3 μm was outside the unbiased counting frame. Note that if one is counting nucleoli (or cell tops, etc.) and the nucleoli are outside the counting frame, the neuron should not be counted even if the cell body profile is partly inside the counting frame (as being the case for the neuron found at −3 μm). Vice versa, if one is counting nucleoli (or cell tops, etc.) and the nucleoli are inside the counting frame and not touching the exclusion lines, the neuron should be counted even if the cell body profile does touch the exclusion lines (as being the case for the neurons found at −9 μm and at −14 μm). Photomicrographs were produced by digital photography using a Nikon Dxm 1200F digital camera (Nikon, Tokyo, Japan) and ACT-1 software (Nikon); the final figures were constructed using Corel Photo-Paint v. 11 and Corel Draw v. 11 (Corel, Ottawa, Canada). Only minor adjustments of contrast and brightness were made, which in no case altered the appearance of the original materials. Scale bar=20 μm. Section contributed by B. P. F. Rutten, University of Maastricht, Netherlands.
“isotropic uniform random” sections (see section 5.5, Spatial Grid; Sandau, 1987), the use of “vertical” sections (see section 5.5, Vertical Spatial Grid; Cruz-Orive and Howard, 1995) and the use of (thick) sections with any plane of section (Isotopic Fakir method; Künbonia and Janacek, 1998). As shown in Fig. 7 for the Isotropic Fakir method, orthogonal triplets of perpendicular test lines are systematically and randomly placed within thick sections containing the surface of interest. Then, the intersections between the surface and the test lines are counted, and the surface area is calculated from the total number of intersections.

For the sake of completeness it should be mentioned that surface areas of local entities (such as single cells) can also be investigated with the Optical Rotator method (Kiêu and Jensen, 1993; Tandrup et al., 1997; see also section 5.5). However, the Fakir methods can be universally applied so that the surface areas of capillaries, ventricles and other hollow bodies can also be estimated.

5.7: Fiber length. Quantification of linear biological structures (LBS) has important applications in neuroscience. For example, it may be relevant to know the ratio of the diameter of the space balls and the diameter of the fibers investigated, and the z axis shrinkage of the tissue due to histologic processing (details can be found in Gundersen, 2002). It is actually not possible to correct for this shrinkage, unlike with 3D reconstructions. Thus, if the LBS are not isotropic (i.e. if they have a preferential direction), the length estimates will be different if sections are cut in different planes (see Gundersen, 2002, for practical solutions to minimize bias in investigations of LBS).

Another possibility to quantify LBS with design-based stereology is the use of “isotropic virtual planes” (Larsen et al., 1998). In this case, the LBS under study contained in thick (3D) sections are investigated with software-randomized isotropic virtual planes in volume probes in systematically sampled microscope fields. A disadvantage of this technique may be the fact that the analysis has to be carried out on virtual planes within the thick sections (which can be tedious and cumbersome), whereas by using the space balls the plane of analysis is always the focal plane of the microscopic fields.

5.8: Three-dimensional cytoarchitecture. Discrete alterations in the normal cytoarchitecture of the adult cerebral cortex attributable to disturbed neuronal migration during development have usually been investigated by labeling precursors of neurons in the brain in utero with bromodeoxyuridine (BrdU). Then, the distribution patterns of BrdU-labeled cells have been analyzed postnatally by immunohistochemistry (for example see Fushiki et al., 1997). However, this approach suffers from several methodological limitations (see discussion in Schmitz et al., 2002a). Most importantly, it cannot be applied to the human brain, as it is impossible to label cells in the developing human cerebral cortex with BrdU.

To circumvent these limitations, a novel 3D design-based stereologic method was recently developed, named Nearest Neighbor analysis (Schmitz et al., 2002a). This method, which essential steps are shown in Fig. 9, is based on the “nearest-neighbor distance distribution function” analysis founded in theoretical statistics (Diggle, 1983). It makes it possible to determine whether the neurons within a given BROI exhibit spatial randomness, a clustered distribution, or a more dispersed distribution. The application of Nearest Neighbor analysis requires the use of thick sections encompassing the entire BROI (approximately 100–150 μm thick sections for the mouse brain, and up to 750 μm thick sections for the human brain; any
plane of section), as well as a staining method that guarantees staining throughout the section thickness (e.g. gallo cyanin). Nearest Neighbor analysis comprises estimates of both the volume of the BROI using Cavalieri's principle (see section 5.2) and of the total number of neurons within this BROI using the optical fractionator method (see section 5.4).

Another approach to study the spatial distribution of neurons within a BROI is the so-called Voronoi tessellation (Duyckaerts et al., 1994; Duyckaerts and Godefroy, 2000; Hof et al., 2003). This approach focuses on the region of space that any cell within a BROI occupies, i.e. the region of space that is closer to that cell than to any other. Unfortunately, it has not been possible so far to adapt algorithms that have been published for 3D tessellations to histologic specimens.

Section 6: Variability of estimates obtained with design-based stereology

The results of quantitative histologic investigations performed with design-based stereologic methods are estimates rather than exact measurements. Thus, the results will vary if the same stereologic estimate were to be independently repeated. This implies that information about the variability of stereologic estimates is an important topic.

If the same stereologic estimate would be repeated ad infinitum, one could calculate the coefficient of variation of these estimates (note that the mean of such ad infinitum estimates must equal the true value under study; otherwise, the estimates would not be unbiased). In practical applications of design-based stereology, however, this coefficient of variation is unknown. It is one of the most desirable advantages of design-based stereology over other quantitative histologic techniques that predictions about this coefficient of variation (usually presented as “coefficient of error,” CE) are possible.
For estimates of the total number of neurons within a given brain region obtained with the Fractionator method (thereafter Fractionator estimates), 16 different procedures to predict the CE have been described (summarized in Table 1 in Schmitz and Hof, 2000). Extensive computer simulations have shown that several of these methods can be applied to predict precisely the CE of Fractionator estimates (for details see Schmitz, 1998; Glaser and Wilson, 1998, 1999; Schmitz and Hof, 2000). As a rule of thumb, the CE of a Fractionator estimate approximately equals the reciprocal value of the square root of the number of counted neurons (Schmitz, 1998; Schmitz and Hof, 2000). For example, if a Fractionator estimate results in counting 400 neurons, the CE is predicted as $1/\sqrt{400}=0.05$.

Three different procedures have been proposed to predict the CE of estimated total numbers of neurons obtained with the $V_{Ref}/N_V$ method (summarized in Table 1 in Schmitz and Hof, 2000). However, computer simulations have shown that these procedures do not result in adequate predictions of CE (Schmitz and Hof, 2000). Moreover, four different procedures have been described to predict the CE of estimates of global volumes obtained with Cavalieri’s principle (thereafter Cavalieri estimates; Gundersen and Jensen, 1987; Roberts et al., 1994; Geinisman et al., 1996; Garcia-Fiñana et al., 2003). Pilot computer simulations have shown that the procedure described in Garcia-Fiñana et al. (2003) is likely the best choice for predicting the CE of Cavalieri estimates based on point counting (C. Schmitz and P. R. Hof, unpublished observations). For the remaining methods described in section 5, proven procedures to predict precisely the CE are currently not available.

An unsolved problem in design-based stereology is the establishment of criteria for sampling requirements to achieve a specific variability in the estimates. In this respect it has been a long-standing rule in design-based stereology that sufficient precision of estimates is obtained when in analyses of a sample of cases, the mean of the squared CEs of the individual estimates is smaller than the squared coefficient of variation of the estimates (Gundersen and Østerby, 1981). Recent computer simulations, however, have shown the limitation of this approach (Schmitz and Hof, 2000). In fact, there is currently no general answer to the question how to design stereologic sampling protocols so that both sufficient precision of the estimates is achieved while the work is efficient by performing as few measurements as possible to achieve this precision. This is essentially due to the fact that different scientific questions to be addressed by design-based stereology require different levels of precision in the estimates. For example, a correlation analysis usually requires more precise estimates than the comparison of two group means with a Student’s $t$-test. It is always advisable to design an individual sampling scheme for each new study, taking into account all potential factors that will likely affect the precision of estimates such as potential biologic variability, the number of animals or brains to be investigated, the number of sections to be analyzed, the number of neurons to be counted, etc.

Fig. 7. Procedure to estimate the surface area of biological objects. (A) A schematic drawing of an object (e.g. a brain region) is shown which is hit by perpendicular test lines. For the sake of clarity, only two test lines each in directions X, Y and Z are shown. For a real estimate of the surface area of this object, a systematically and randomly placed array of test lines in directions X, Y and Z each would have to be used, as shown in (B) for direction Z. (C) Schematic drawing of two thick sections from the object shown in (A). In this case each section contains only a part of the surface of the object, and only a few intersections of the test lines and the surface area of the object are found in a given section. However, the total number of intersections between the test lines and the surface area of the object provides an estimate of the surface area (as explained in detail in Kubinova and Janacek, 1998), as is the case when the test lines hit the object not cut into sections (as shown in A).
Section 7: Presentation of results obtained with design-based stereology

Investigators and readers, as well as editorial boards and reviewers have all been concerned by the issue of what information and discussion are essential in a stereologic paper. Whereas there is no general answer to this question, the following recommendations might be useful.

The materials and methods of stereologic studies should provide a description of the histologic processing, with consideration of the specific requirements...
to be met (outlined in section 2) and the potential sources of bias (summarized in section 4). The stereologic probes which are used should briefly be mentioned, together with a reference to the corresponding...
Table 2. Methodological details to be given in papers describing design-based stereologic studies*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Stereologic estimate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$V_{G1}$</td>
</tr>
<tr>
<td>Mean number of investigated sections</td>
<td>x</td>
</tr>
<tr>
<td>Mean number of investigated microscopic fields</td>
<td>(1)</td>
</tr>
<tr>
<td>Mean actual section thickness after histologic processing</td>
<td>x</td>
</tr>
<tr>
<td>Settings</td>
<td>(3)</td>
</tr>
<tr>
<td>Mean number of counted points</td>
<td>(1)</td>
</tr>
<tr>
<td>Mean number of counted cells</td>
<td>x</td>
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<tr>
<td>Mean number of investigated cells</td>
<td>x</td>
</tr>
<tr>
<td>Mean number of counted intersections between space balls and linear biological structures</td>
<td>x</td>
</tr>
<tr>
<td>CE</td>
<td>x</td>
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* $L$, length estimates with Space Balls; $N$, estimates of total numbers of neurons with virtual unbiased counting spaces or physical dissectors; $NN$, investigations on the spatial distribution of neurons with nearest-neighbor analysis; $V_{G1}$, estimates of global volumes with Cavalieri’s principle; $V_{Lo}$, estimates of local volumes with the Nucleator, the Rotator, the Optical Rotator, or the Point Sampled Intercepts method. (1) When point counting is performed. (2) When the Optical Rotator is used. (3) When point counting is performed: distance between points. (4) When unbiased virtual counting spaces are used: base area, height and guard zones of the counting spaces, and distance between the counting spaces; when the physical dissector is used: area of the unbiased counting frame, distance between look-up frame and reference frame, and guard zones. (5) When the Nucleator is used: number of rays, and which kind of sections is investigated (isotropic uniform random sections or vertical sections); when the Rotator is used: number of lines or grid line separation, and which kind of sections is investigated; when the Optical Rotator is used: focal plane separation, guide line separation, optical slab thickness, and number of grid lines. (6) Radius of Space Balls. (7) Base area, height and guard zones of the counting spaces, and distance between the counting spaces. Descriptions of these methodological details can be found in the corresponding literature summarized in section 5.

Section 8: The future of design-based stereology in neuroscience

Over the recent years design-based stereology has become the state-of-the-art methodology in quantitative histologic analyses, and the application of design-based stereologic methods to the analysis of the CNS has considerably contributed to our understanding of the functional and pathological morphology of the brain. Nevertheless, design-based stereology is still an evolving field and it can be expected that its applications in neuroscience will be subject to many improvements and extensions in the near future. Some of them are already predictable and are briefly outlined in the following.

First, research is under way to develop tools for predicting precisely the variability of estimated mean cellular (neuronal and perikaryal) volumes (section 5.5), of estimated length densities of LBS (section 5.7), and of stereologic investigations on the spatial distribution of neurons within a region of interest (section 5.8). Another improvement will be the development of new strategies to design stereologic sampling protocols so that both sufficient precision of the estimates is achieved and the work is efficient by performing as few measurements as possible to achieve this precision (section 6).

A second domain of development is the advanced integration of anatomical mapping with stereologic analyses. As briefly outlined in section 5.1, each stereologic investigation starts with the identification of the boundaries of the region of interest, and there are many possibilities for doing so. The easiest situation is that the identification of the boundaries of the region of interest can be achieved on the same sections and with the same microscope which are used for performing the stereologic analyses. How-
ever, in many situations this is not the case. For example, if boundaries of the region of interest are established with a chemoarchitectural approach (i.e. making use of differences in the regional distribution of certain subsets of neurons) it can become necessary to identify the boundaries of the region of interest and to perform the stereologic analyses on adjacent, differently stained sections. Furthermore, identification of the boundaries of cortical and subcortical areas in the human brain can require the use of special equipment to apply the GLI method (Schleicher et al., 2000; see section 5.1 for details) or a stereomicroscope (Heinsen et al., 1999). Once appropriate software interfaces will have been developed, the boundaries of the brain regions of interest identified with the GLI method can directly be used by the commercially available stereology systems (see section 3). Another advance will be to fit stereomicroscopes with transparent graphic tablets and transfer the delineations directly to the software of the stereology systems.

Third, quantitative neuromorphology will greatly benefit from further integration of design-based stereology with confocal microscopy and electron microscopy. The optical resolution of confocal microscopy and electron microscopy as well as the ability to collect registered series of focal planes is ideally suited for the three-dimensional sampling of design-based stereology (see section 5). First attempts to integrate the collection of confocal and electron microscopic images with the implementation of design-based stereology has been undertaken (Mayhew, 1996; Petersen, 1999; Glaser and Glaser, 2000; Kubinova and Janacek, 2001; Howell et al., 2002). As for light microscopy, it is to be expected that this field will considerably expand with the recognition that the integration of design-based stereology with confocal microscopy and electron microscopy is fundamental in revealing certain features of development, repair, aging, pathology and normal anatomy of the brain that would not have been detected otherwise.

Concluding remarks

Many important questions in neuroscience cannot simply be answered with a simple qualitative analysis. Whenever quantitative morphologic approaches are used, the following issues should be considered: are the results representative for the entire BROI? Are the results independent from the size, shape, spatial orientation, and spatial distribution of the cells to be investigated? Is bias (i.e. systematic error) in the results kept to a minimum? Is it possible to assess the variability of the results? In numerous published studies reporting quantitative data not obtained with design-based stereologic methods, these questions cannot be adequately answered. In some cases, such quantitative data can even be more misleading than purely qualitative statements, because they are based on inadequate methods and are prone to overinterpretation. The use of design-based stereologic methods permits one to answer all these questions positively. Furthermore, design-based stereology comprises rigorous emphasis on prospective experimental design and the requirement for proper sampling, presumably the most important contributions of this methodology to neuroscience. Hopefully the same principles will be increasingly applied to other fields such as 3D neuronal reconstruction, quantitative (immuno) electron microscopy, and neurophysiology.

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