

How to count cells: the advantages and disadvantages of the isotropic fractionator compared with stereology

Suzana Herculano-Houzel · Christopher S. von Bartheld · Daniel J. Miller · Jon H. Kaas

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Abstract The number of cells comprising biological structures represents fundamental information in basic anatomy, development, aging, drug tests, pathology and genetic manipulations. Obtaining unbiased estimates of cell numbers, however, was until recently possible only through stereological techniques, which require specific training, equipment, histological processing and appropriate sampling strategies applied to structures with a homogeneous distribution of cell bodies. An alternative, the isotropic fractionator (IF), became available in 2005 as a fast and inexpensive method that requires little training, no specific software and only a few materials

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S. Herculano-Houzel (✉)
Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
e-mail: suzanahh@gmail.com

S. Herculano-Houzel
Instituto Nacional de Neurociência Translacional, Ministério de Ciência e Tecnologia, São Paulo, Brazil

C. S. von Bartheld
Department of Physiology and Cell Biology, University of Nevada School of Medicine, Reno NV 89557, USA

D. J. Miller · J. H. Kaas
Department of Psychology, Vanderbilt University, Nashville TN 37240, USA

before it can be used to quantify total numbers of neuronal and non-neuronal cells in a whole organ such as the brain or any dissectible regions thereof. This method entails transforming a highly anisotropic tissue into a homogeneous suspension of free-floating nuclei that can then be counted under the microscope or by flow cytometry and identified morphologically and immunocytochemically as neuronal or non-neuronal. We compare the advantages and disadvantages of each method and provide researchers with guidelines for choosing the best method for their particular needs. IF is as accurate as unbiased stereology and faster than stereological techniques, as it requires no elaborate histological processing or sampling paradigms, providing reliable estimates in a few days rather than many weeks. Tissue shrinkage is also not an issue, since the estimates provided are independent of tissue volume. The main disadvantage of IF, however, is that it necessarily destroys the tissue analyzed and thus provides no spatial information on the cellular composition of biological regions of interest.

Keywords Numbers of neurons · Brain size · Isotropic fractionator · Stereology · NeuN

Introduction

Biological tissues are fundamentally composed of cells. Therefore, understanding the normal function of tissues of interest and when these have become abnormal, ultimately requires knowledge of the number of cells of various types that compose it. A major limitation to acquiring this knowledge is that most biological structures of interest have too many cells to be simply enumerated under a microscope, although this is, on occasion, painstakingly attempted and with

success (Ward et al. 1975; Hall and Russell 1991). However, this is a limitation that can fortunately be circumvented by techniques that allow cell numbers to be estimated from small fractions of tissue. Estimating cell numbers by sampling nevertheless has its own limitations, such as is presented by the heterogeneous distribution of cells, tissue shrinkage, or sectioning artifacts that occur during histological processing and the fact that estimates of numbers of cells obtained by multiplying cell density by structure volume are necessarily dependent on structure volume (which has the additional problem of being subject to shrinkage) and are thus not useful for allometric studies.

Unbiased stereology, by using tools such as the optical disector and optical fractionator, has been, for a couple of decades, the go-to approach (West and Gundersen 1990; West 1999). However, the sampling parameters have to be tailored to the size and heterogeneity of events of interest within each structure and multiple structures require multiple independent analyses. The application of stereology to an entire brain, including, for instance, structures organized into layers or arranged as clumps of cells, is not feasible, as it would require independently sampling each of these dramatically dissimilarly organized structures. Additionally, stereological methods often require an elaborate sequence of histological processing techniques that can be time-intensive, basic training in cellular and regional anatomy and laboratory and microscopy equipment that can be expensive to purchase and maintain.

It was to circumvent these limitations that a non-stereological technique, the isotropic fractionator (IF), was developed (Herculano-Houzel and Lent 2005; Herculano-Houzel 2012). This method estimates the total number of cells independently of tissue volume and anisotropy and can be applied to the whole brain or any dissectible structure therein. It does not require any specific software and although it requires a basic understanding of the mathematical principles that are employed in stereology, it is far more user-friendly and can yield reproducible estimates of the total number of cells and neurons within a single day. The essence of the IF consists in dissolving the tissue of interest into a soup of free-floating cell nuclei, whose density in the suspension is estimated directly and extrapolated to the whole suspension volume to yield an estimate of the total number of cells in the original structure.

In this review, we compare the two methods, namely stereology and IF, in a way that will be useful to the researcher who is interested in estimating numbers of cells in his or her tissues of interest but is not sufficiently familiar with either method to decide on the best approach to the particular need. A simple side-by-side comparison of the steps involved in each method is shown in Fig. 1.

Unbiased stereological methods for counting cells

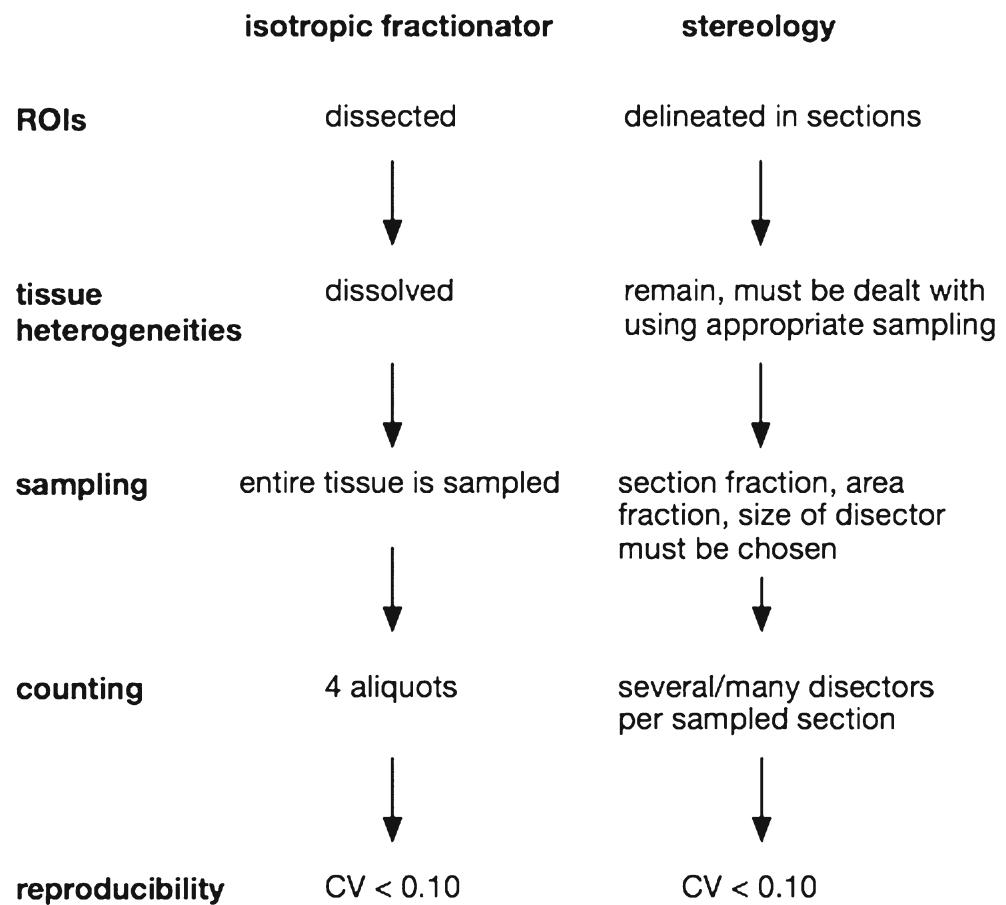
Stereology is a design-based modification of the traditional profile-counting approach for counting particles in histological sections. It consists in applying mathematical principles to the estimation of objects of interest within a definable three-dimensional (3D) volume based upon 2D data (e.g., planes of focus on a microscope). The crux of cell number estimation by using stereological methods is that a systematic and random sampling strategy is employed to evaluate the frequency of unambiguously identifiable objects. In other words, stereological probes need to be placed randomly throughout the entire region of interest. Stereology is described in detail in another contribution of this issue (Geuna and Herrera-Rincon 2015), so we will only briefly review the main features of one type of functionality (cell counting) in the interest of comparison with the IF.

Estimating the cellular composition of tissues by using stereological techniques commonly takes advantage of the excellent cytological details apparent in formaldehyde-fixed tissues. Following fixation, tissues are often embedded in a suitable medium (e.g., frozen, paraffin, or resin) sectioned into 10–100 μm thick slices (microtome, vibrotome), and stained with a dye or processed for immunolabeling or DNA/RNA expression, so that all cells of interest are visible. Stereology analyzes sections with a systematic random sampling scheme, such that all particles (cells, nuclei, or nucleoli) across the entirety of the tissue of interest have the same chance of being sampled. This sampling strategy is important because it is the mechanism by which the heterogeneity of the distribution of items of interest (cells) is overcome. Probes must be representative of particle density and the same sampling strategy must apply to the entire reference space.

Stereology requires that the region of interest is clearly identifiable and that the particles are recognizable in an unambiguous fashion (Gundersen et al. 1988; Williams and Rakic 1988; Schmitz and Hof 2005; Geuna and Herrera-Rincon 2015). Counting is performed within an “optical disector,” a 3D polyhedral probe placed within tissue sections and analyzed through focal planes available with high-resolution microscopy by using an “unbiased counting scheme” that includes all particles inside the disector and those particles that touch any of the three “inclusion surfaces” of the probe but that ignores those that touch any of the three “exclusion surfaces” of the probe (Gundersen 1986; Williams and Rakic 1988; Schmitz and Hof 2005).

Modern design-based stereology for counting cells comes in two “flavors”: the “fractionator method” (Gundersen 1986) and the so-called VRef \times Nv method (West and Gundersen 1990). The fractionator method does not require a separate estimation of the volume of the structure of interest and therefore generates an estimate that is independent of structure volume. In contrast, the VRef \times Nv method reaches an

Fig. 1 Side-by-side comparison of the steps involved in estimating numbers of cells with the isotropic fractionator and with stereology (*ROIs* regions of interest, *CV* coefficient of variation)



estimate of cell number by multiplying an estimate of particle density (number of particles per volume counted) in the tissue and an estimate of the volume of the tissue, which must be obtained, for example, via the Cavalieri principle. This method thus yields an estimate of the number of particles (cells) that is dependent on an accurate estimate of the tissue volume. Although both methods are unbiased in theory, bias can arise because of tissue deformation and the loss of particles during tissue processing and other errors (Guillary 2002; von Bartheld 2002; Baryshnikova et al. 2006). For this reason, calibration against the ultimate gold standard, i.e., 3D serial section reconstructions of a tissue sample, is recommended (Coggeshall et al. 1990; von Bartheld 2001, 2002; Williams et al. 2003).

The stereological estimation of the number of cells in tissues of interest requires a minimum of a z-axis position encoder (microcator) on the microscope. Systematic random sampling and probe placement, however, is facilitated by a motorized stage that encodes position on the x-, y- and z-axes and can also be controlled by software in a computer next to the microscope. Typically, investigators employ a commercially available software package that automates the systematic random sampling strategy, the size and positioning of the optical disectors and available focal planes (video overlay

hardware and software). However, simple stereological counting can be performed by using a microcator and a drawing tube (Williams et al. 2003; Schmitz and Hof 2005).

Major challenges of the stereological approach to counting cells are to be able sufficiently to sample the reference space with probes to ensure that probe counts are representative (i.e., can capture >90 % of the variability associated with heterogeneously distributed objects); to account for the differential shrinkage that results from alternative tissue processing techniques (sectioning frozen tissue with a sledge microtome, dehydration steps for distinct staining procedures); to distinguish correctly between neurons and glia; to identify the true borders and dimensions of the region of interest; and to measure the actual height of tissue sections (von Bartheld 2001, 2002; Guillary 2002; Schmitz and Hof 2005; Miller et al. 2014). Accordingly, stereological estimation is more difficult in regions with a complex shape (e.g., mesencephalic nucleus of the trigeminal nerve); in tissues containing exceedingly high cell densities, which make distinguishing glia from neurons difficult and that might also be compounded by poor antibody penetration when immunolabeling; and following differential shrinkage in the z-axis that might preclude the use of guard zones, particularly across sections containing distinctly organized structures. However, the skilled investigator can easily

overcome each of these difficulties with a well-designed experimental approach that is rigorously quantitative and anatomically accurate (von Bartheld 2002).

The choice of sampling strategy and size of disector are of fundamental importance for accurate stereological estimation and a short pilot study to determine the appropriate probe dimensions, the area between the probes and the number of sections to be investigated are all critical for obtaining a precise and accurate estimate. The size of the probe depends upon the size of the cells of interest and the number of probes to be counted depends on the homogeneity of cell distribution and the required precision of the project. Initially, it was claimed that the counting of about 100 particles with a coefficient of error (CE) of ~10 % was sufficient (Gundersen 1986; Pakkenberg and Gundersen 1988; Coggeshall and Lekan 1996) but more recent work, based on calibrations and computer simulations, recommends the counting of a considerably larger number of particles (700–1000) per individual with a CE of 3 % (Schmitz and Hof 2000). Some investigators err on the side of caution and risk “wasting” time by counting a multiple of this guideline (see Miller et al. 2012, 2013, 2014). However, given that the majority of time spent producing accurate estimates by using stereological procedures is taken up by tissue processing (see direct comparison below), prospective investigators may adopt a similar approach depending on the needs of the individual project.

Determining whether a sufficient number of particles have been scored can be done by referring to the coefficient of error (CE), a parameter for within-sample variation. The CE is defined as the standard deviation (SD)/mean of the number of events per probe. Accordingly, the larger the number of probes, the lower the CE tends to be (for an example, see Miller et al. 2014). A CE of 0.01–0.15 is considered adequate, whereas a CE of more than 0.20 indicates an insufficient number of probes. The coefficient of variation (CV) is a parameter for total observed variation, usually at the population level, between groups or individuals.

Stereology can be highly time-consuming because of the histological processing that may be necessary unambiguously to identify a region or particles of interest (e.g., accompanying stains to reveal characteristic chemo- or cytoarchitectural features), in addition to the fact that independent counting procedures are necessary to determine the cellular composition of multiple regions of interest. This contrasts with the manual or automated counting of cells after tissue dissolution, a method that obtains independent estimates for each subdivision of tissue (i.e., region of interest). The average time elapsed from tissue collection to final estimates will be considered in direct comparison with the IF in a separate section.

Isotropic fractionator

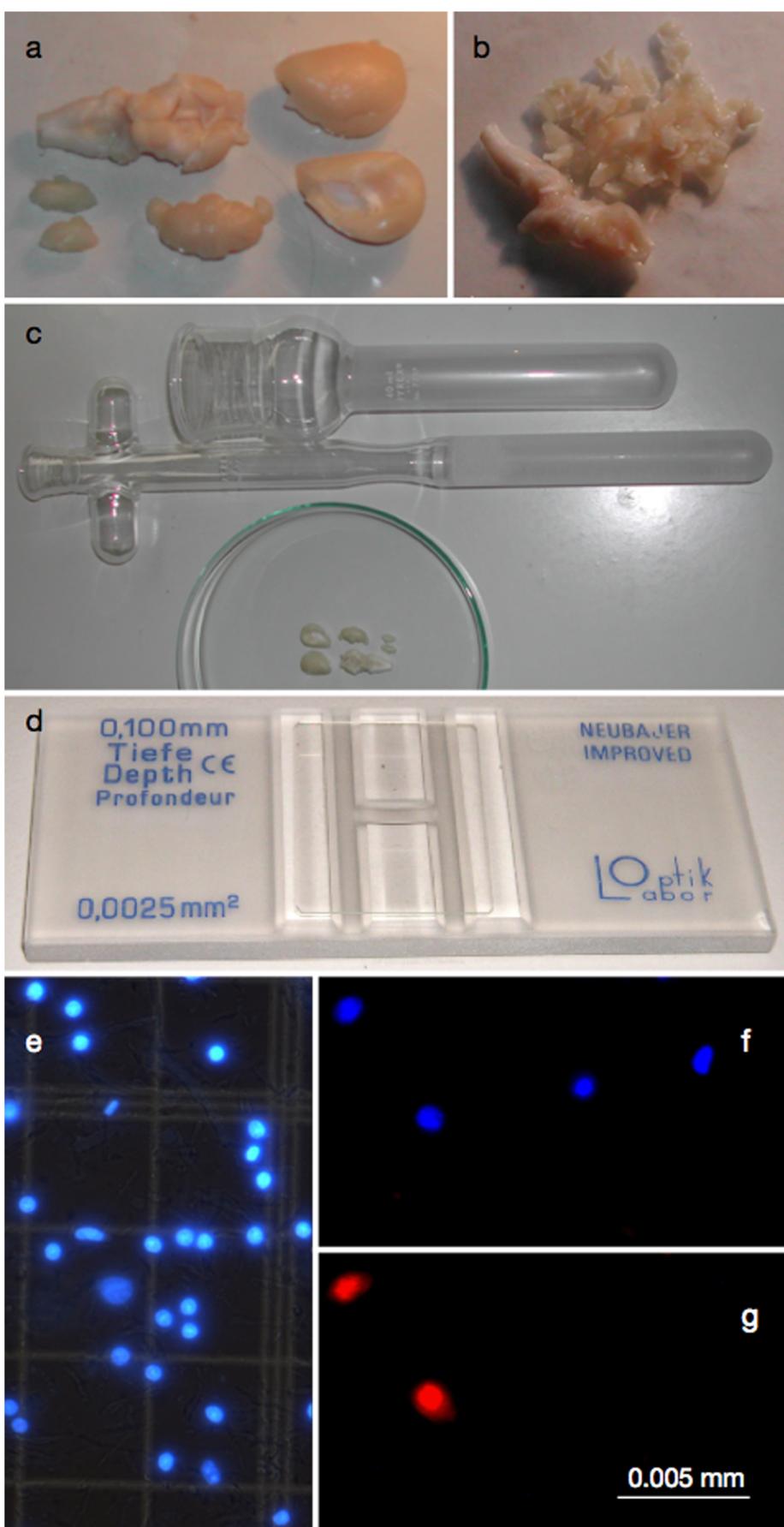
The IF method entails counting free-floating cell nuclei that once composed a biological tissue that has been dissolved with detergent and turned into a soup or suspension (Herculano-Houzel and Lent 2005; Herculano-Houzel 2012). As long as every cell in the tissue of interest contains one and only one nucleus, the total number of nuclei estimated in the suspension corresponds to the total number of cells in the original tissue of interest. Although developed for brains, the IF can be applied to any biological tissue composed of nucleated cells, even syncytial tissue such as muscle; the only caveat with regard to muscle is that the estimated numbers of nuclei no longer translate into estimated numbers of membrane-bound cells in the tissue.

The method consists in processing fixed brains (or any other fixed tissue), as a whole or dissected into subregions, into an isotropic suspension of isolated nuclei in which cytoarchitectural heterogeneities have been literally dissolved. Since this suspension has a known defined volume and can be made isotropic (homogeneous) by agitation, the total number of nuclei therein and therefore the total number of cells in the original tissue, can be estimated by determining the density of nuclei in small aliquots of the suspension. The density of nuclei in each aliquot is determined by counting unambiguously identifiable particles (stained nuclei) within a counting chamber employing a similar set of “inclusion” and “exclusion” boundaries as described above for stereology (Herculano-Houzel and Lent 2005).

Once the total cell number is known, the proportion of neurons is typically determined by immunocytochemical detection of the neuronal nuclear antigen (NeuN), which is expressed in all nuclei of most neuronal cell types (notable exceptions are Purkinje cells, inferior olive neurons, mitral cells and photoreceptors) and not in non-neuronal cells (Mullen et al. 1992) and the number of non-neuronal cells is derived by subtraction. Other nuclear markers, such as cell-specific transcription factors, can be applied equally well and combined in multiple-antibody reactions. Alternatively, morphological criteria can be used to determine numbers of readily identifiable nuclear types, such as those of Purkinje cells. The various steps of the process are illustrated in Fig. 2.

Because the tissue is necessarily destroyed, the IF method does not allow one to obtain any information regarding the spatial distribution of cells within counted samples. However, spatial information can be obtained by using adequate sampling strategies such as the dissection of the tissue of interest into subregions according to anatomical and functional criteria (Herculano-Houzel et al. 2013; Miller et al. 2014; Young et al. 2013a, 2013b), into arbitrary smaller samples (Collins et al. 2010a), or into systematically spaced serial samples (Ribeiro et al. 2013).

Fig. 2 Outline of the use of the IF.
a Example of rat brain dissected into regions of interest, namely two olfactory bulbs, two cortical hemispheres, whole cerebellum and rest of brain, in this case. **b** Cerebral cortex cut into smaller pieces for processing; rest of brain still intact. **c** Tenbroeck glass homogenizer used for homogenizing the tissue in a solution of 1 % Triton X-100 in 40 mM sodium citrate. **d** Neubauer chamber (improved) used for determining the density of nuclei in the suspension. **e** Appearance of a suspension of free nuclei (blue) from a marmoset cerebellum, stained with DAPI (4,6-diamidino-2-phenylindole), ready for counting in the Neubauer chamber for the determination of the total number of cell nuclei in the suspension. **f, g** Double-labeling of nuclei with DAPI (blue; **f**) and NeuN (neuronal nuclear antigen; red; **g**) allows the determination of the percentage of all nuclei that express NeuN and are therefore scored as neuronal



Importantly, the tissue processed with the IF is destroyed but not lost: the suspension, or aliquots of it, can be stored in an anti-freeze solution and kept frozen for years for later re-analysis, for instance, when new antibodies become available, with little loss of immunoreactivity (Herculano-Houzel 2012).

IF: manual counts on the microscope

When using the IF, an estimation of the density of free nuclei in the prepared suspension always starts under a fluorescence microscope, even if automated counting methods are to be used (see below). The visual identification of DAPI-(4'6-diamidino-2-phenylindole) or immunolabeled nuclei has several advantages: it ensures quality control of the samples, since broken nuclei (the result of over-homogenization) and nuclei with dissolved membranes (because of insufficient fixation or over-exposure to detergents) are easily noticeable; stained debris (common in myelin-rich structures) is readily discernible from labeled nuclei; and clumps of nuclei (which indicate insufficient homogenization) are conspicuous under the microscope at low magnification (in which case the suspension needs to undergo further homogenization). Counting manually under the microscope also allows the identification of specific cell types by their nuclear morphology and can be readily carried out in any laboratory equipped with an upright fluorescence microscope.

We routinely count four aliquots from each sample (that is, dissected tissue turned into a suspension of free nuclei). Each aliquot of 10 µl is placed in a Neubauer chamber, which allows the determination of the density of nuclei in the suspension (details of the procedure can be found in Herculano-Houzel 2012). Aliquots should contain a minimum of 60 nuclei in the counting grid; when too few nuclei are present, the suspension should be centrifuged and re-suspended in a smaller volume. Conversely, suspensions that give more than 200 nuclei in the counting grid are considered too dense and should be diluted into a larger final volume before being counted.

Dividing the standard deviation by the average of the four counts yields a CV that corresponds to the CE in stereology (that is, variation across probes, within the sample). Four aliquots are typically sufficient to yield a CV below 0.15 and usually below 0.10, which indicates that the suspension from which the aliquots were taken was indeed isotropic. Larger CVs indicate that the suspension was not isotropic and should be agitated again before new aliquots are taken to start over the counting procedure. Conversely, when more precision is needed, smaller CVs can be obtained by increasing the number of aliquots sampled. Once a suitably low CV is obtained, the total number of cells (nuclei) in the original tissue sample is calculated by multiplying the density of nuclei per milliliter by the total volume of the suspension (Herculano-Houzel 2012). Typically, a total of 240–800 nuclei are counted across

four aliquots; because their homogeneous (isotropic) distribution in the suspension can be ascertained by the low CV values, they can be considered representative of the entire suspension and thus of the original tissue.

The most time-consuming step in obtaining an estimate of total cell number by using the IF is the homogenization of the tissue to generate the suspension of free nuclei, which is typically 20 min per sample of up to 3 g tissue. The estimation of numbers of cells in larger tissues requires breaking the tissue down into a number of samples, which may or may not be combined as a single suspension before counting. Thereafter, the counting of four aliquots of the sample under the microscope to determine the total number of nuclei (cells) in the suspension requires as little as 10 min for a trained counter. A more detailed comparison with the time required to estimate numbers of cells with stereology will be given below.

Once total numbers of cells are obtained, subtypes can be estimated by determining the proportion of nuclei that express a particular morphology or particular nuclear markers by using immunocytochemistry (Herculano-Houzel 2012).

IF: automated counts using flow cytometry

Overall, the use of flow cytometry or fluorescence-activated cell sorting (FACS) equipment to automate the counting of samples prepared with the IF reduces the amount of time necessary to obtain precise cell counts from dissected tissues to an absolute minimum (Collins et al. 2010b; Miller et al. 2014; Young et al. 2012). In other words, the dissection, dissociation and immunohistochemical reactions necessary to count cells under the IF are still employed and the main advantage lies in the theoretical ability of the flow cytometer to count nuclei more rapidly and with greater reproducibility than a human observer. Specifically, the flow cytometer counts hundreds of fluorescent events (nuclei) in a single second, essentially pushing the time to obtain estimates as close to zero as is physically possible (a single estimate based upon approximately 1000 events is typically obtainable within 8–12 s).

Automated counting is particularly advantageous when large numbers of samples from the same tissue are to be counted (for instance, when subdividing the cerebral cortex into many functional or anatomical areas; Young et al. 2013a, 2013b). On the other hand, a major disadvantage of the automated counting of dissociated nuclei is the need for on-site access to a flow cytometer and either a technician or the time required to master the applicable software. Additional disadvantages include the need for increased technical precision (e.g., accurate pipetting) and the need to calibrate the flow cytometer when tissue quality is variable (e.g., when samples have been obtained from differentially processed tissues or distinct species). Furthermore, when tissue quality is poor (in particular, with large amounts of white matter debris), the need to calibrate the flow cytometer becomes paramount as

the presence of debris can mask the signal generated by the nuclei in the sample. The manual counting of nuclei under the microscope (e.g., 1 in 10 samples) is the best control measurement currently available to address the uncertainties brought about by poor tissue quality. Similarly, the expression of biomarkers (e.g., NeuN) might vary across species and areas, requiring further calibration. Despite some difficulty in these calibration steps, particularly in non-perfused tissues, the use of more effective antibodies and steps to remove debris holds great promise to increase further the precision of obtainable estimates. Aside from the rapidity with which the flow cytometer counts events of interest, its other major advantage over hand counts is the high repeatability of subsequent counts; this, in part, is attributable to the internal control afforded by the use of Countbright beads (Invitrogen), as nuclei are counted in relation to the known density of beads in the aliquot (Miller et al. 2014; Young et al. 2012).

In summary, the automated counting of fluorescently tagged nuclei reduces the variability of and time required to obtain estimates for a given aliquot of tissue. For example, running an aliquot in duplicate, to obtain a measure of variability and including the time needed to confirm or calibrate the flow cytometer gating schema requires approximately 25 s. Thus, estimates for a set of 20 aliquots can typically be obtained within 8–9 min. Accordingly, the estimation of the total number of cells and the percentage of neurons in a set of 20 aliquots can be performed in under 20 min.

Alternatives

As mentioned above, cell-by-cell enumeration, the simplest counting method, is a useful alternative only in tiny biological structures. Even then, the concern that the same cell not be counted twice is present when working with serial sections; this can be avoided by counting smaller structures such as cell nuclei or even nucleoli (keeping in mind that some cells might contain more than one nucleolus; e.g., Coggeshall et al. 1984).

Serial section 3D reconstruction is an extremely time-consuming endeavor that can be applied to very small collections of particles (Coggeshall et al. 1990; Hatton and von Bartheld 1999). Nevertheless, it is still considered the “gold standard” and can be used to calibrate a small defined region to determine bias (von Bartheld 2002; Williams et al. 2003). The use of this method is however prohibitive in any routine or large-scale application because of the amount of time, cost and effort needed.

An alternative approach to histology and the IF is to extract and measure DNA content in the tissue and to calculate cell numbers based on the knowledge of the DNA content per cell nucleus (Heller and Elliott 1954; Zamenhof et al. 1964; Hess and Thalheimer 1971; Dobbing and Sands 1973; Bahney and von Bartheld 2014). However, this technique has severe

drawbacks: the complete recovery of DNA is required; contamination with other nucleic acids can occur; not all cells are euploid; and only the total cell number but not the cell type, is revealed. This method has never been used to estimate absolute cell numbers; it was used mostly in the 1950s to 1970s to track relative cell numbers.

Comparison of estimates and variability

Estimates of numbers of cells obtained with stereology and with the IF (both through manual and automated counts) can be compared directly, as each consists in counting events in a known volume (i.e., the counting probe, the counting chamber, or the calibrated number of beads per volume, respectively). Estimates of variability are therefore also comparable across the methods.

For the IF, CVs are computed across different aliquots from the same sample (tissue) and thus indicate the variability across aliquots. Typically, four aliquots yield CVs between 0.05 and 0.10; CVs above 0.15 are considered an indication that the suspension of nuclei was not homogeneous (isotropic) when the aliquots were collected and the counting must start over with new aliquots from a well-mixed suspension. A larger number of aliquots can decrease the CV, usually to below 0.05.

The CV is calculated in a similar way when using automated counting to estimate the number of cells in samples prepared with the IF. The only difference between the two is that in automated counting, the CV reflects fewer estimates ($n=2$), each of which is based upon a much larger set of observations or events (thousands), whereas when counting by hand, the CV reflects a larger number of estimates ($n=4$ to 8), each of which is based upon a smaller set of events (typically 60–200). However, the number of observations to be made can be increased in accordance with the needs of the project.

In stereology, the CV is calculated for each of the measurements of interest: for example, the cell density or volume of a region of interest, when applicable. The determination of an appropriate sampling strategy (e.g., area between probes or probe dimensions), often the result of a short pilot study, is absolutely critical for accurate stereological estimation and the expenditure of more effort to reduce the CE is recommended. At a subsequent step (and similar to the IF), different samples from different individuals need to be compared and at this population level, the variation might be too high (and sample number too low) to make meaningful comparisons. Notably, in this respect, when additional samples are needed, it is much less time-consuming to process further samples with the IF than with stereology (see the direct comparison below).

How many cells should be counted?

When counting cells manually with the IF, one typically counts 60–200 nuclei per aliquot on the Neubauer chamber and therefore 240–800 nuclei (cells) per sample in the first step, to establish the total number of cells in the original tissue. Each individual nucleus counted thus corresponds to only 0.1 to 0.4 % of the estimate, so that errors on the part of the counter, namely counting one nucleus too many or too few, have an impact on the final estimate of that small range. Similarly, when determining the percentage of all nuclei that belong to a particular cell type, at least 500 nuclei are counted, so that each nucleus scored as positive or negative contributes only 0.2 % of the final estimate.

The automated estimation of total cell number in DAPI-stained aliquots of tissue samples prepared with the IF is based upon the number of DAPI events per Countbright bead events and, thus, is the result of the flow cytometer counting somewhere in the range of 3000–5000 events in total [(2000+ DAPI events) + (~1000 bead events)], per aliquot. In a second step, the automated estimation of the percentage of neurons in a given aliquot is the result of investigating the number of (typically 1000 or more) NeuN⁺ events relative to (often 5000+) DAPI events.

The number of cells that need to be counted in order to obtain an estimate with stereology varies between investigators and projects. It also depends on the precision required by the study. Initially, stereologists thought that counting 100–200 particles was sufficient to obtain meaningful estimates (Gundersen 1986; Coggeshall and Lekan 1996). Subsequent work with computer simulations, however, revealed that a considerably larger number of cells should be counted (e.g., 700–1000, Schmitz and Hof 2000). Indeed, too small a sample size might explain some of the discrepant results seen in stereology studies, e.g., those discussed in previous work (Guillery and Herrup 1997; Peters et al. 1998; Schmitz et al. 1999; von Bartheld 2001; Dorph-Petersen et al. 2009).

Direct comparison of results obtained with the IF and stereology

Some authors have raised concerns that estimates of cell numbers obtained with the IF had not been verified against stereology and might be inaccurate (Charvet et al. 2015; Carlo and Stevens 2013; Verkhratsky and Butt 2013). This concern no longer applies, since two independent direct comparisons of stereology and the IF carried out by two independent groups have shown that similar estimates of cell numbers are obtained with the IF and with stereology. Notice should however be taken that, because the IF destroys the tissue, the two estimates compared were necessarily obtained not from the same tissue but either from neighboring tissue (Bahney and von Bartheld

Fig. 3 Decision flowchart to determine whether the IF or stereology is better suited for a research project aimed at quantifying the cell composition of brains, other organs, or parts thereof (*CV* coefficient of variation, *FACS* fluorescence-activated cell sorting)

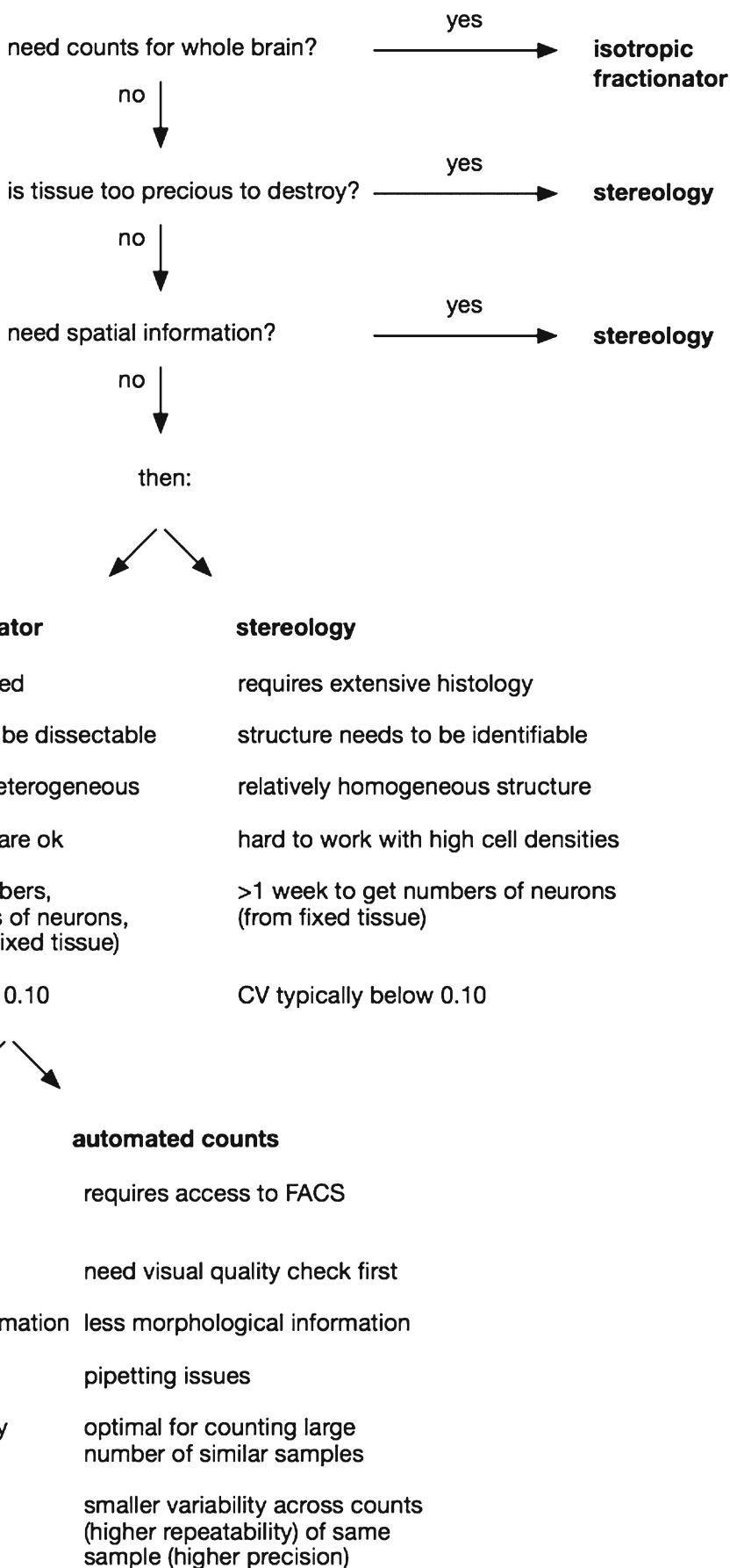
2014) or from the contralateral cortical hemisphere (Miller et al. 2014).

Using macaque and human cortical tissue, Bahney and von Bartheld (2014) found 70,000–92,000 nuclei/mg in the white matter of the human forebrain corpus callosum with the IF (mean: 72,276 nuclei/mg, CE: 0.031) and 69,000–79,000 nuclei/mg in the adjacent tissues with stereology (mean: 69,624 nuclei/mg, CE: 0.016). The same study showed 87,000–92,000 nuclei/mg in the corpus callosum of the macaque forebrain with the IF (mean: 87,211 nuclei/mg, CE: 0.026) and 84,000–110,000 nuclei/mg in the adjacent tissues with stereology (mean: 110,604 nuclei/mg, CE: 0.020). In the white matter of the human cerebellum, the mean was 32,520 nuclei/mg with the IF (CE: 0.063) and 39,497 nuclei/mg for stereology (CE: 0.031) and in the white matter of the macaque cerebellum, the mean was 38,424 nuclei/mg with the IF (CE: 0.025) and 37,294 nuclei/mg (CE: 0.022) with stereology. Thus, no consistent or statistically significant difference was present between the results obtained by IF and by stereology and the CEs were also comparable.

Applying all three techniques (manual and automated counting with the IF and stereology) to the chimpanzee visual cortex (V1), Miller et al. (2014) also found that the relationship between the average estimates and the variance of estimates for a given tissue sample was comparable across techniques and all gave CVs below 0.05. Specifically, manual and automated counts of nuclei produced by using the IF gave an estimate of the total number of cells in V1 of 998.48 million and 1.01 billion, respectively. Alternatively, counting 300+ optical fractionator probes (typically including 2000–3000 cells/events) resulted in an estimate of the total number of cells in V1 of 961.08 million. Manual and automated counts of the number of nuclei expressing NeuN produced an estimate of the neuron number in V1 of 651.73 million and 664.72 million, respectively. Stereological estimation of the neuron number in V1 produced an estimate of 695.47 million. The CV for estimates based upon the IF of the total number of cells in V1 was 0.035 (3000–5000 nuclei/events) and the CV for estimates of the percentage of nuclei that expressed NeuN was 0.036 (5000–10,000 nuclei/events). The estimate of variability by using stereological procedures was 0.032 for counts of the total cell number and 0.028 for counts of neurons (Miller et al. 2014).

Time elapsing from tissue to final estimate

When trying to decide on a method to estimate numbers of cells in a tissue, the time required to obtain each estimate can



be a crucial factor, particularly when large numbers of samples are to be analyzed, for instance, from the cerebral cortex or hippocampus of multiple individuals (Bandeira et al. 2009; Young et al. 2013b); from many different areas of a single large cerebral cortex (Collins et al. 2010a; Ribeiro et al. 2013; Herculano-Houzel et al. 2013; Young et al. 2013a); or from one very large structure, such as the cerebral cortex of a human or elephant brain (Azevedo et al. 2009; Herculano-Houzel et al. 2014).

With the IF and by using manual counts at the microscope, the average processing time per sample is as follows: around 20 min to dissociate up to 3 g tissue, which is the amount of tissue that can be well homogenized at a time; 10 min to count four aliquots of the sample; 3 h for the immunocytochemical reaction; and 15 min to determine the proportion of NeuN+ nuclei in the sample. Roughly, then, each sample under 3 g requires 1 h of processing and counting time, plus 3 h for immunocytochemistry. However, as tissue can be processed in batches, the time to process multiple n samples is not $n \times 4$ h but rather $(n \times 1\text{ h}) + 3$ h. Thus, a whole mouse or rat brain, dissected into five structures (cortex, cerebellum, hippocampus, olfactory bulb, rest of brain, as in Bandeira et al. 2009) can be counted in 8 h of work; 20 mouse cerebella can be processed in 23 h of work by a single person; an entire human cerebral cortical hemisphere, with ca. 400 g, will require 140 h of dedicated work by a single person.

The amount of time needed to estimate a single sample/aliquot of under 3 g with automated counting is identical to that described for manual counting, except that the time spent counting is decreased and the time for the inclusion of controls for areas or species that require calibration has to be added. Therefore, the timeline for a single aliquot includes: 20 min to dissociate the tissue; approximately 3 h for the immunocytochemical reaction; and 25 s to count and analyze events of interest for two stains (e.g., DAPI, NeuN). As previously mentioned, the time to process n samples is thus $(n \times 20.42\text{ min}) + 3$ h. Thus, a whole mouse brain ($n=5$ aliquots+3 controls [e.g., cortex]+3 controls [e.g., cerebellum]) can be counted in ~7 h; 20 mouse cerebella ($n=20$ aliquots+3 controls) can be counted in ~11 h; and an entire human cortical hemisphere ($n=134$ aliquots+3 controls [e.g., cortex]+3 controls [e.g., cerebellum]) can be investigated within approximately 51 h.

Stereological procedures for estimating biological features of interest, as mentioned above, are critically dependent upon unambiguously identifying the events of interest across the entirety of the reference space, e.g., cells or neurons in a neurobiological structure. Although recent technology has expanded the repertoire of techniques to accomplish this identification process, the time required to produce these results has concomitantly increased. Thus, the most time-consuming steps in producing stereological estimates of biological features of interest do not have to do with the counting procedures per se (although, as we will see, this will add a

significant amount of time to the process, as each region of interest requires its own counting procedure) but with the preparation of the tissue, including the sectioning, staining and mounting of multiple series to facilitate anatomical accuracy. Our experience of using stereology and of manual and automated counting with the IF on equivalent samples (the primary visual cortex of the two hemispheres) of a chimpanzee brain helps illustrate a comparison of the two methods.

The chimpanzee primary visual cortex processed with the IF was flattened (6 h) and then divided into 61 samples of <0.2 g each and as calculated above, the obtaining estimates by using manual and automated procedures required a total of about 70 and 30 h, respectively. In comparison, processing the opposing hemisphere for stereological counts required the sectioning of approximately 500 slices beginning at the occipital pole, a procedure taking approximately 6 h. Next, two sets of 1-in-6 series of brain slices were processed (each set containing ~84 slices) for either Nissl substance (required 5 h in 1 day) or staining against a NeuN antibody (required approximately 8 h over 2–3 days). Following staining, sections were mounted on glass slides (~7 min per section), coverslipped and permitted to dry for 48 h. After processing, counts were obtained from approximately 300 sampling probes (Nissl, 316; NeuN, 324); we estimate that each probe required 1 min to count a single cell type (counting and categorizing two cell types requires not more than 2 min per probe). A general formula for calculating the requisite time is: (time sectioning)+(time staining)+(time mounting)+(time counting). Thus, the absolute minimal amount of time needed to obtain stereological estimates of cell and neuron number in the chimpanzee V1 was: (6 h sectioning)+[(6 h Nissl)+(8 h NeuN) staining]+[(10 h Nissl)+(10 h NeuN) mounting]+[(5–10 h Nissl)+(5 h NeuN) counting]=50–55 h. Notably, V1 is easily distinguishable based upon cytoarchitecture alone and thus we did not need additional histological stains unambiguously to identify the region of interest. However, the vast majority of studies will require additional stains (such as for cytochrome oxidase or myelinated fibers) to identify the event(s) or region(s) of interest and this necessitates an additional 24–26 h of work (when using our study as an example, although the investigator should tailor these calculations to their own needs: 6–8 h staining + 10 h mounting + 8 h counting). Of additional note, more recently developed stains, such as those used to reveal RNA expression by *in situ* hybridization, can require longer than our example of immunohistochemical staining for NeuN. Furthermore, the time requirements outlined above must be considered in terms of the logistics involved: the workload necessary for our research on chimpanzee V1 was spread over about 8–11 days: 1 day to cut, 3–5 days to complete the minimal staining procedures and mount (i.e., only Nissl and NeuN), 2 days to allow the mounting medium to solidify and 2 days to quantify cell and neuron numbers for a single region of interest. Finally, stereological

procedures must adequately sample each region independently, so that the collection of data from multiple regions of interest requires time spent counting each region on its own (8 h counting V1+8 h counting V2 etc.), whereas the IF provides independent estimates for each aliquot across a given structure and can therefore more rapidly address hypotheses involving multiple brain areas, provided that these subdivisions can be dissected with confidence. In summary, then, our work on the primary visual cortex in the chimpanzee can be seen as representing the minimal time requirement to collect data on cell and neuron number in a single neurobiological structure of interest by using stereology and required 50–55 h of work spread over about 2 weeks. In addition, based upon this example, each subsequent stain might require 24 h of work spread over about 3–5 days and each event type within each region of interest requires its own counting time. These calculations permit the following generalization: if the project only needs data on the cellular composition of dissectible areas, the IF is fast and reliable; however, if the project requires information on the spatial distribution of events of interest or requires data on the molecular or biochemical organization of a specific region (whether that is to identify the region itself or is of direct interest to the investigation), then stereology provides a flexible framework capable of delivering data relevant to a variety of hypotheses with quantitative and computational

needs (although new technologies might be applicable to both approaches, as was immunolabeling in our research). In brief, the provision of stereological data on the number of cells and neurons in one hemisphere of the cerebral cortex in a mouse (~1 cm long), macaque monkey (~6 cm long) or human (~14 cm long) requires approximately 28 h over 8–9 days or 2 weeks (mouse), and 88 h over 14 days or 3 weeks (macaque). For estimating the numbers of cells in experimental tissue, some key issues should be kept in mind. The main ones are outlined in Fig. 3 and start with whether estimates of the numbers of cells in the whole brain are desired; these can only be obtained with the IF.

A second issue is whether the tissue can be destroyed for analysis. Both stereology and the IF require the irreversible processing of the tissue: although spatial information is maintained, for example, in Nissl-stained tissue sections, only in specific situations can these sections be reprocessed for other stains or antibodies. However, stereology only consumes a fraction of the tissue sections (the other sections remain intact for future analysis), whereas the IF should be performed on the entirety of the tissue of interest. On the other hand, as pointed out above, samples processed with the IF can be frozen for later re-analysis, provided that they are stored in an appropriate antifreeze solution (Herculano-Houzel 2012). Additionally, because only a very small

Table 1 Variability in numerical estimates obtained with stereology or the isotropic fractionator (IF) in various studies (data from stereological estimates were compiled in the following references: Guillory and Herrup 1997; Schmitz et al. 1999; Schmitz and Hof 2005; C.S. von Bartheld et al., in preparation)

Region or cell population	Estimates of neuron number	Maximum difference ^a	Authors and year
Stereology			
Purkinje cells, rat cerebellum	2.1×10^5		Mayhew 1991
	6.1×10^5	+200 %	Korbo et al. 1993
Purkinje cells, human cerebellum	1.6×10^7		Mayhew 1991
	3.5×10^7	+100 %	Andersen et al. 1992
Pyramidal cells, mouse CA1, unilateral	80×10^3		Insausti et al. 1998
	205×10^3	+160 %	Calhoun et al. 1998
Pyramidal cells, rat CA1-3, unilateral	615×10^3		Rapp and Gallagher 1996
	930×10^3	+50 %	Rasmussen et al. 1996
Dorsomedial thalamic nucleus, human	1.8×10^6		Pakkenberg and Gundersen 1988
	3.5×10^6	+100 %	Popken et al. 2000
	7.3×10^6	+300 %	Dorph-Petersen et al. 2004
Lateral geniculate nucleus, human	3.48×10^6	+80 %	Selemon and Begovic 2007
	1.99×10^6		Dorph-Petersen et al. 2009
IF			
Neocortex, human	16.3×10^9	+25 %	Azevedo et al. 2009
	12.7×10^9		Andrade-Moraes et al. 2013
Cerebellum, human	65×10^9	+20 %	Azevedo et al. 2009
	54×10^9		Andrade-Moraes et al. 2013
Whole brain, human	86×10^9	+25 %	Azevedo et al. 2009
	67.3×10^9		Andrade-Moraes et al. 2013

^a Note that biological variation may account for 25 % or more, especially in humans and with small sample sizes

fraction of the processed sample is used for immunocytochemistry, the same sample can be used for many different types of analysis.

Additional advantages of IF

In our experience with both methods, we find that the IF is more robust than methods requiring histology in two important ways: first, it is less sensitive to fixation parameters; and second, it is more fool-proof (user-friendly) than stereology, which requires training in anatomical and morphological details relevant to the planned research and careful pilot studies to decide on a sampling strategy.

Bahney and von Bartheld (2014) found that samples from human tissues fixed with embalming fluid could not be used for histology/stereology but they could be processed with no problems for the IF and rendered comparable numerical estimates. Similarly, Herculano-Houzel and Kaas (2011) were able to obtain reliable estimates of numbers of cells in great ape cerebella that had been fixed for over 10 years, because cell nuclei could still be visualized with DAPI. Thus, data can still be extracted from archived tissues by using the IF, even when the tissues are no longer suitable for histology and stereology.

Although the correspondence between estimates of numbers of cells in the literature obtained with stereology and with the IF is good (Azevedo et al. 2009; Tsai et al. 2009; Brautigam et al. 2012; Bahney and von Bartheld 2014; Miller et al. 2014), much larger variation occurs across studies involving the use of stereology than across studies with the IF (Table 1). Whereas estimates obtained with the IF vary by around 15 % across groups for the same species and are thus within the range of biological variation, different investigators using the same stereological method on the same species and brain structures reported estimates that often are more than 100 % different (Table 1). In one study in which the same data set of original slides were sent to three different teams of investigators for stereological analysis, the authors found that the estimates varied by 20 % across investigators because of the different sampling schemes used (Kaplan et al. 2010). Despite it probably being too early to evaluate the consistency of the IF across investigators, since it has been available for fewer than 10 years and its number of users is still small, the IF has the clear advantage over stereology of not depending on sampling schemes, since the entirety of the tissue of interest is processed and analyzed as an isotropic suspension of nuclei. Another advantage is that the IF is more user-friendly, requiring less specific training. Given that the IF has a straightforward workflow requiring no adjustment of sampling strategies, we suggest that the IF is sufficiently accurate and robust to the point of being a suitable method for calibrating or

confirming stereological or profile-based estimates, provided sufficient anatomical detail is available during dissection.

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