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

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Abstract

How many cells compose biological structures is 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 fairly 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 few materials 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. It entails transforming the highly anisotropic tissue into a homogeneous suspension of free-floating nuclei which 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 multiple 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 different 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 has nevertheless 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, 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. Applying 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 which can be time-intensive, requires basic training in cellular and regional anatomy, as well as 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 total number of cells independently from 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 isotropic fractionator consists of 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 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 Figure 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 of applying mathematical principles to the estimation of objects of interest within a definable 3D volume based upon 2D data (e.g. planes of focus on a microscope). The crux of cell number estimation using stereological methods is that a systematic and random sampling strategy be 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 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, vibratome, paraffin or resin); sectioned into 10–100 μm thick slices; and stained with a dye, 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 be clearly identifiable, and that the particles be recognizable in an unambiguous fashion (Gundersen et al., 1988; Williams and Rakic, 1988; Schmitz and Hof, 2005; Geuna and Herrera-Rincon, 2015). Counting is done within an “optical disector,” a 3-dimensional polyhedral probe placed within tissue sections and analyzed through focal planes available with high-resolution microscopy, using an “unbiased counting scheme” that includes all particles inside the disector as well as those particles that touch any of the three “inclusion surfaces” of the probe, but 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 x 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 x Nv method reaches an 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 number of particles (cells) that is dependent on an accurate estimate of tissue volume. Although both methods are unbiased in theory, bias can arise due to tissue deformation and loss of particles during tissue processing and other errors (Guillery, 2002; von Bartheld, 2002; Baryshnikova et al.,

2006). For this reason, calibration against the ultimate gold standard, i.e. 3-dimensional serial section reconstructions of a tissue sample, is recommended (Coggeshall et al., 1990; von Bartheld, 2001; von Bartheld, 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 done 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 sufficiently sample the reference space with probes to ensure that probe counts are representative (i.e. capture >90% of the variability associated with heterogeneously distributed objects); to account for the differential shrinkage which 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; Guillery, 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 makes distinguishing glia from neurons difficult, which may also be compounded by poor antibody penetration when immunolabeling; and differential shrinkage in the z-axis that may 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 to accurate stereological estimation, and a short pilot study to determine the appropriate probe dimensions, area between probes and number of sections to be investigated are all critical to 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 counting 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 counting 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 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, while 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 very time-consuming because of the histological processing that may be necessary to unambiguously 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 which 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 to the isotropic fractionator in a separate section.

Isotropic fractionator

The isotropic fractionator 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 isotropic fractionator can be applied to any biological tissue composed of nucleated cells, even syncytial tissue such as muscle – the only caveat here is that the estimated numbers of nuclei no longer translate into estimated numbers of membrane-bound cells in the tissue.

The method consists of 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 Neuronal Nuclear antigen (NeuN), 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 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 different steps of the process are illustrated in Figure 2.

Because the tissue is necessarily destroyed, the isotropic fractionator 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 dissecting the tissue of interest into subregions according to anatomical and functional criteria (Herculano-Houzel et al., 2013; Miller et al., 2014; Young et al., 2013), into arbitrary smaller samples (Collins et al., 2010a), or into systematically spaced serial samples (Ribeiro et al., 2013).

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

Isotropic fractionator: manual counts on the microscope

When using the isotropic fractionator, estimating 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 next). The visual identification of DAPI-(4'6-diamidino-2-phenylindole) or immunolabeled nuclei has several advantages: it ensures a quality control of the samples, since broken nuclei (due to over-homogenization) and nuclei with dissolved membranes (due to insufficient fixation or over-exposure to detergents) are easily noticeable; stained debris (common in myelin-rich structures) are easily discernible from labeled nuclei; and clumps of nuclei (which indicate insufficient homogenization) are easily spotted 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 it is readily accessible to any laboratory equipped with an upright fluorescence microscope.

We routinely count 4 aliquots from each sample (that is, dissected tissue turned into a suspension of free nuclei). Each aliquot, of 10 μ l, 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 over 200 nuclei in the counting grid are considered too dense, and should be diluted into a larger final volume before counting.

Dividing the standard deviation by the average of the 4 counts yields a coefficient of variation (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/ml by the total volume of the suspension (Herculano-Houzel, 2012). Typically, a total of 240–800 nuclei are counted across 4 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 to obtain an estimate of total cell number using the isotropic fractionator is the homogenization of the tissue to generate the suspension of free nuclei, which is typically 20 minutes per sample of up to 3g of tissue. Estimating numbers of cells in larger tissue requires breaking it down into a number of samples, which may or may not be combined as a single suspension before counting. Thereafter, counting 4 aliquots of the sample under the microscope to determine the total number of nuclei (cells) in the suspension requires as little as 10 minutes 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 using immunocytochemistry (Herculano-Houzel, 2012).

Isotropic fractionator: 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 isotropic fractionator reduces the amount of time necessary to obtain precise cell counts on dissected tissues to an absolute minimum (Collins et al., 2010; Miller et al., 2014; Young et al., 2012). In other words, the dissection, dissociation and immunohistochemical reactions necessary to count cells under the isotropic fractionator 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 seconds).

Automated counting is particularly advantageous when large numbers of samples from the same tissue are to be counted (for instance, when subdividing 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, for example when there are samples 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 nuclei in the sample. Manual counting nuclei under the microscope (e.g. 1 in 10 samples) is the best control measurement currently available to address the uncertainties brought up by poor tissue quality. Similarly, the expression of biomarkers (e.g. NeuN) may 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 hold great promise to further increase the precision of obtainable estimates. Aside from how rapidly the flow cytometer counts events of interest, its other major advantage over hand counts is the high repeatability of subsequent counts, in part due 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 seconds. Thus, estimates for a set of 20 aliquots can typically be obtained within 8–9 minutes. Accordingly, estimating the total number of cells as well as the percentage of neurons in a set of 20 aliquots can be performed in under 20 minutes.

Other alternatives

As mentioned above, cell-by-cell enumeration, the simplest counting method, is a useful alternative only in tiny biological structures. Even then, there is a concern that the same cell not be counted twice when working with serial sections, which can be avoided by counting smaller structures such as cell nuclei or even nucleoli (keeping in mind that some cells may 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). Yet, 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). It is however prohibitive to use in any routine or large-scale application due to the amount of time, cost and effort.

An alternative approach to histology and the isotropic fractionator is to extract and measure DNA content in the tissue and to calculate cell numbers based on knowledge of 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: complete recovery of DNA is required; there can be contamination with other nucleic acids; not all cells are euploid, and only total cell number, but not cell type is revealed. This method has never been used to estimate absolute cell numbers – it was used mostly in the 1950s–1970s to track relative numbers.

Comparing estimates and variability

Estimates of numbers of cells obtained with stereology and with the isotropic fractionator (both through manual and automated counts) can be compared directly, as each consists of 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 isotropic fractionator, CVs are computed across different aliquots of 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 isotropic fractionator. 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 make 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 to accurate stereological estimation and expending 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, variation may be too high (and sample number too low) to make meaningful comparisons. In this respect it is important to note that 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 to count?

When counting cells manually with the isotropic fractionator, 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, 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 isotropic fractionator 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 3,000–5,000 events in total [(2,000+ DAPI events) + (~1,000 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 1,000 or more) NeuN+ events relative to (often 5,000+) DAPI events.

How many cells 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 using computer simulations, however, revealed that a considerably larger number of cells should be counted (e.g. 700–1,000, Schmitz and Hof, 2000). It is possible that too small of a sample size may 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).

A direct comparison of results obtained with the isotropic fractionator and stereology

Some authors have raised concerns that estimates of cell numbers obtained with the isotropic fractionator had not been verified against stereology and might be inaccurate (Charvet et al., 2013; Carlo and Stevens, 2013; Verkhratsky and Butt, 2013). This concern no longer applies since two independent, direct comparisons of stereology and the isotropic fractionator carried out by two independent groups showed that similar estimates of cell numbers are obtained with the isotropic fractionator and with stereology. Notice 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, 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 of 0.031), and 69,000–79,000 nuclei/mg in the adjacent tissues with stereology (mean 69,624 nuclei/mg with a CE of 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 with a CE of 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 human cerebellum white matter, 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 macaque cerebellar white matter, the mean was 38,424 nuclei/mg with IF (CE: 0.025) and 37,294 nuclei/mg (CE: 0.022) with stereology. Thus, there was no consistent or statistically significant difference 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 isotropic fractionator as well as stereology) to chimpanzee visual cortex (V1), Miller et al. (2014) also found that the relationship between 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 using the isotropic fractionator 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 2,000–3,000 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 neuron number in V1 of 651.73 million and 664.72 million, respectively. Stereological estimation of neuron number in V1 produced an estimate of 695.47 million. The CV for estimates based upon the isotropic fractionator of the total number of cells in V1 was 0.035 (3,000–5,000 nuclei/events), and a CV for estimates of the percentage of nuclei that expressed NeuN of 0.036 (5,000–10,000 nuclei/events). The estimate of variability using stereological procedures was 0.032 to count the total cell number, and 0.028 to count neurons (Miller et al., 2014).

Time elapsed 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, the cerebral cortex or hippocampus of multiple individuals (Bandeira et al., 2009; Young et al., 2013b); 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 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 isotropic fractionator, using manual counts at the microscope, the average processing time per sample is around 20 minutes to dissociate up to 3g of tissue (which is the amount of tissue that can be well homogenized at a time); 10 minutes to count 4 aliquots of the sample; 3 hours for the immunocytochemical reaction; and 15 minutes to determine the proportion of NeuN+ nuclei in the sample. Roughly, then, each sample under 3g requires 1 hour of processing and counting time, plus 3 hours for immunocytochemistry. However, as tissue can be processed in batches, the time to process multiple n samples is not $n \times 4\text{h}$, but rather $(n \times 1\text{h}) + 3\text{h}$. Thus, a whole mouse or rat brain, dissected into 5 structures (cortex, cerebellum, hippocampus, olfactory bulb, rest of brain, as in Bandeira et al., 2009), can be counted in 8h of work; 20 mouse cerebella can be processed in 23 hours of work by a single person; an entire human cerebral cortical hemisphere, with ca. 400g, will require 140 hours of dedicated work by a single person.

The amount of time needed to estimate a single sample/aliquot of under 3g with automated counting is identical to that described for manual counting, except to decrease the time spent counting, and to add time to include controls for areas or species that require calibration. Therefore, the timeline for a single aliquot includes: 20 minutes to dissociate the tissue; approximately 3 hours for the immunocytochemical reaction; and 25 seconds to count and analyze events of interest for both stains (e.g. DAPI, NeuN). As previously mentioned, the time to process n samples is thus $(n \times 20.42 \text{ minutes}) + 3 \text{ hours}$. Thus, a whole mouse brain ($N = 5 \text{ aliquots} + 3 \text{ controls [e.g. cortex]} + 3 \text{ controls [e.g. cerebellum]}$) can be counted in ~7 hours; 20 mouse cerebella ($N = 20 \text{ aliquots} + 3 \text{ controls}$) can be counted in ~11 hours; 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 hours.

Stereological procedures to estimate 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 in the preparation of the tissue, including the sectioning, staining and mounting of multiple series to facilitate anatomical accuracy. Our experience using stereology as well as manual and automated counting with the isotropic fractionator on equivalent samples (the primary visual cortex of the two hemispheres) of a chimpanzee brain helps illustrate how the two methods compare.

The chimpanzee primary visual cortex processed with the isotropic fractionator was first flattened (6 hours) and then divided into 61 samples of <0.2 grams each and, as calculated above, obtaining estimates using manual and automated procedures required a total of about 70 and 30 hours, respectively. In comparison, processing the opposing hemisphere for stereological counts required the sectioning of approximately 500 slices beginning at the occipital pole, requiring approximately 6 hours. Next, two sets of 1-in-6 series of brain slices were processed (each set containing ~84 slices) for either Nissl substance (required 5 hours in 1 day) or staining against a NeuN antibody (required approximately 8 hours over 2–3 days). Following staining, sections were mounted on glass slides (~7 minutes per section), coverslipped and permitted to dry for 48 hours. After processing, counts were obtained from approximately 300 sampling probes (Nissl, 316; NeuN, 324); we estimate that each probe required 1 minute to count a single cell type (counting and categorizing two cells types requires not more than 2 minutes per probe). Thus, a general formula to calculate 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 hours sectioning) + [(6 hours Nissl) + (8 hours NeuN) staining] + [(10 hours Nissl) + (10 hours NeuN) mounting] + [(5–10 hours Nissl) + (5 hours NeuN) counting] = 50–55 hours. It should be noted that V1 is easily distinguishable based upon cytoarchitecture alone, and thus we did not need additional histological stains to unambiguously 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 hours of work (when using our study as an example, although the investigator should tailor these calculations to their own needs: 6–8 hour staining + 10 hours mounting + 8 hours counting). It should also be noted that more recently developed stains, such as to reveal RNA expression using *in situ* hybridization, may require longer than our example of immunohistochemical staining for NeuN. Furthermore, it is necessary to consider the time requirements outlined above 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, it should be noted that stereological procedures must adequately sample each region independently, such that collecting data on multiple regions of interest requires time spent counting each region on its own (8 hours counting V1 + 8 hours counting V2 etc.) whereas the isotropic fractionator provides independent estimates for each aliquot across a given structure and can therefore more rapidly address hypotheses involving multiple brain areas – provided these subdivisions can be dissected with confidence. In summary then, our work on the primary visual cortex in the chimpanzee, which should be seen as representing the minimal time requirement to collect data on cell and neuron number in a single neurobiological structure of interest using stereology, required 50–55 hours of work spread over about 2 weeks. In addition, based upon this example, each subsequent stain might require 24 hours 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 isotropic fractionator 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 it is worth noting that new technologies may be applicable to both approaches, as was immunolabeling in our research). In summary, providing 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 hours over 8–9 days or two weeks (mouse), 88 hours over 14 days or three weeks (macaque) or ~200 hours over about 26 days or 5–6 weeks.

Decision flowchart

When deciding what method is best to estimate numbers of cells in experimental tissue, some key issues should be kept in mind. The main ones are outlined in Figure 3, and they start with whether estimates of numbers of cells in the whole brain are desired – which can only be obtained with the isotropic fractionator.

A second issue is whether the tissue can be destroyed for analysis. Both stereology and the isotropic fractionator require irreversibly processing 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 remaining intact for future analysis), while the isotropic fractionator should be performed on the entirety of the tissue of interest. On the other hand, as pointed out above, samples processed with the isotropic fractionator can be frozen for later reanalysis, provided they are stored in an appropriate antifreeze solution (Herculano-Houzel, 2012). Additionally, because only a very small fraction of the processed sample is used for immunocytochemistry, it is possible to use the same sample for many different types of analysis.

Additional advantages of the IF

In our experience with both methods, we find that the isotropic fractionator 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, as well as 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.

While there is good correspondence between estimates of numbers of cells in the literature obtained with stereology and with the IF (Azevedo et al., 2009; Tsai et al., 2009; Brautigam et al., 2012; Bahney and von Bartheld, 2014; Miller et al., 2014), there is much larger variation across studies using stereology than across studies using the IF (Table 1). While estimates obtained with the IF vary by around 15% across groups for the same species, which is in the range of biological variation, different investigators using the same stereological method on the same species and brain structures report estimates that often are more than 100% different (Table 1). In one study where the same data set of original slides were sent to 3 different teams of investigators for stereological analysis, the authors found that the estimates varied by 20% across investigators due to the different sampling schemes used (Kaplan et al., 2011). While it might be too early to evaluate the consistency of the IF across investigators, since it has been available for less 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 a now 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 to calibrate or confirm stereological or profile-based estimates, provided sufficient anatomical detail is available during dissection.

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Fig. 1.

Side-by-side comparison of the steps involved in estimating numbers of cells with the isotropic fractionator and with stereology. Abbreviations: ROIs, regions of interest; CV, coefficient of variation.

Fig. 2.

Outline of the isotropic fractionator. A, example of rat brain dissected into regions of interest (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 dissolving 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 from a marmoset cerebellum, stained with Dapi, ready for counting in the Neubauer chamber to determine the total number of cell nuclei in the suspension. F, G, double labeling of nuclei with Dapi (F) and NeuN (G) allows the determination of the percentage of all nuclei that express NeuN and are therefore scored as neuronal.

Fig. 3.

Decision flowchart to determine whether the isotropic fractionator or stereology is better suited for a research project aiming to quantify the cell composition of brains, other organs or parts thereof. Abbreviations: CV, coefficient of variation; FACS, fluorescence-activated cell sorting.

TABLE 1

Variability in numerical estimates obtained with stereology or isotropic fractionator in different studies.

Region or Population	Estimates of Neuron Number	Max. Difference*	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 & 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 & Begovic, 2007
	1.99×10^6		Dorph-Petersen et al., 2009
Isotropic Fractionator (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

Data from stereological estimates were compiled in the following references: Guillery and Herrup, 1997; Schmitz et al., 1999; Schmitz and Hof, 2005; von Bartheld et al., 2015 (in preparation).

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