The Isotropic Fractionator:
A Fast, Reliable Method to Determine Numbers of Cells in the Brain

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Introduction

Stereological techniques that estimate cell numbers require specific training and elaborate sampling strategies to infer total numbers of cells in well-defined structures of measurable volume. The isotropic fractionator is a fast and inexpensive method that requires little specific training and few materials before it can be used to quantify total numbers of neuronal and nonneuronal cells in the whole brain or any dissectable regions thereof. It transforms highly anisotropic (paraformaldehyde-fixed and dissected) brain structures into homogeneous, isotropic suspensions of cell nuclei, which can be counted and identified morphologically and immunocytochemically as neuronal or nonneuronal. Estimates of total cell, neuronal, and nonneuronal numbers can be obtained within 24 h and vary by less than 10% among samples of the same structure. And, because the estimates obtained are independent of brain volume, they can be used in comparative studies of brain volume variation among species and in studies of phylogenesis, development, adult neurogenesis, and pathology.

Traditionally, stereological methods such as the optical disector and fractionator have been the gold standard for estimating numbers of cells in discrete brain regions and determining how they compare across structures, species, ages, conditions, or experimental manipulations (West, 1999). These methods, however, are very time-consuming. They require familiarity with stereological techniques, depend on accurate measurement of structure volume, and either must be restricted to well-defined structures of isotropic architecture and measurable volume, or require elaborate sampling strategies to ensure that the counted samples are representative.

To circumvent these drawbacks, we created an alternative method: the isotropic fractionator (Herculano-Houzel and Lent, 2005), which estimates the total number of neuronal and nonneuronal cells independently from tissue volume and anisotropy, and can be applied to the whole brain or any dissectable structure. It does not require familiarity with stereological techniques and yields reproducible estimates of total numbers of cells and neurons within a single day. The isotropic fractionator has recently been compared with stereology by two independent groups and was found to yield similar results in far less time (Bahney and von Bartheld, 2014; Miller et al., 2014). We have already used this method in comparative studies of the cellular composition of the brain of different mammalian species (Herculano-Houzel et al., 2006, 2007, 2011, 2014; Sarko et al., 2009; Gabi et al., 2010; Neves et al., 2014). These include studies of the entire human brain (Azevedo et al., 2009), of the distribution of neurons across functional areas of the mouse cerebral cortex (Herculano-Houzel et al., 2013), across the human cerebral cortex (Ribeiro et al., 2013), and of the changes in the cellular composition of the developing rat brain (Bandeira et al., 2009). Other possible uses of the isotropic fractionator include the analysis of pathological alterations in the cellular composition of the brain and of experimental manipulations expected to affect it.

The isotropic fractionator relies on the single assumption that every cell in the brain contains one and only one nucleus. This method consists of processing fixed brains, either as a whole or dissected into subregions, into an isotropic suspension of isolated nuclei in which cytoarchitectural heterogeneities have been literally dissolved. Because this suspension has a known, defined volume and can be made 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. Once the total cell number is known, the proportion of neurons is determined by immunocytochemical detection of 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) but not in nonneuronal cells (Mullen et al., 1992; Gittins and Harrison, 2004). Then, the number of nonneuronal cells can be derived by subtraction. Alternatively, morphological criteria can be used to determine the numbers of readily identifiable nuclear types, such as those of Purkinje cells.

The single most important limitation of the isotropic fractionator is that it relies on nuclear labels to identify specific subpopulations of cell nuclei. This eliminates several useful neuronal and glial cell markers, which are restricted to the cytoplasm, such as MAP2 (microtubule-associated protein-2) and GFAP (glial fibrillary acidic protein). On the other hand, more and more cell-type-specific transcription factors are being discovered, thereby expanding the applications of the method.

Isotropic Fractionator Steps

The isotropic fractionator is a simple, fast, and reliable method for counting cells in brain (and other) tissue that consists of only a few steps.

1. Tissue fixation

The tissue to be analyzed is ideally perfusion-fixed, and postfixation by immersion in 4% paraformaldehyde is required. Good fixation is mandatory for the technique to work; two weeks of postfixation suffice to render the cell nuclei extremely resistant to mechanical friction.

2. Tissue dissection

This step limits how reliable individual counts are, so the technique is best applied to easily dissectable structures. Whole brain can also be used, which requires little dissection.

3. Tissue dissociation

The dissected structures are homogenized by mechanical friction in a saline detergent solution, which dissolves the cell membranes while maintaining the nuclear membranes intact, thus turning the tissue into a suspension of free nuclei.

4. Counting total cell numbers

Samples of the diamino-phenyl-indol (DAPI)–stained nuclei suspension (made homogeneous by agitation) are counted in a hemocytometer. Typically, this step takes 15–20 min per structure to be counted.

5. Heat-induced epitope retrieval

This is a necessary step when tissue has been overfixed, that is, when it has been postfixed for more than 2 months.

6. Immunocytochemical identification of specific nuclear types

Neuronal nuclei in most structures can be identified by the expression of the NeuN antigen. Alternatively, morphological criteria can be used. Immunocytochemical processing takes 5–6 h, after which it takes ~30 min to determine the proportion of specific nuclear types in each structure. Alternatively, flow cytometry can be used in this step to speed up the determination of the proportion of immunolabeled nuclei (Collins et al. 2009). However, visual identification of immunolabeled nuclei has several advantages: (1) It ensures quality control over the samples, since stained debris is easily discernible from labeled nuclei under the microscope; (2) it allows the identification of specific cell types by their nuclear morphology; and (3) it is readily accessible to any laboratory equipped with an upright fluorescence microscope.

7. Storage of free nuclei

Since the immunocytochemical identification of specific nuclear types requires only a small aliquot of the nuclear suspension, the remaining volume can be stored in antifreezing solution for later reanalysis, for instance, when other antibodies become available, or for photographical documentation.

Materials

1. Tissue fixation
- Saline (NaCl, 0.9%)
- Paraformaldehyde (4% solution in 0.1M phosphate buffer)
- Peristaltic pump

2. Tissue dissection
- Surgical instruments
- Stereoscopic microscope
- Analytic balance

3. Tissue dissociation
- Tenbroeck tissue grinder (glass homogenizer, 2, 7, or 40 ml capacity, depending on the size of the tissue of interest)
- Dissociation solution: 1 l of 1% Triton X-100 in 40 mM sodium citrate
- Pasteur pipettes
- Centrifuge tubes (15 or 50 ml)
- PBS
- Clinical centrifuge

4. Counting total cell numbers
- DAPI (Molecular Probes, Life Technologies, Grand Island, NY), 10 mg/l
- Neubauer chamber (hemocytometer), improved to allow simultaneous visualization of DAPI-stained nuclei and the counting grid
- Micropipette, 10 µl
- Fluorescence microscope, upright (inverted microscopes will not do)

5. Heat-induced epitope retrieval
- 0.2 M boric acid, pH 9.0 (adjusted with lentils of NaOH)
- PBS
- 1.5 ml Eppendorf tubes (Eppendorf, Hauppauge, NY)
- Microcentrifuge
- Heating bath or oven at 70°C

6. Immunocytochemical identification of specific nuclear types
- Anti-NeuN mouse IgG (MAB377), or Cy3-conjugated polyclonal rabbit anti-NeuN IgG (in which case a secondary antibody will not be necessary; EMD Millipore, Billerica, MA)

- Normal goat serum
- Fluorescent antimouse secondary antibody
- DAPI (Molecular Probes, Life Technologies), 10 mg/l

7. Storage of free nuclei
- Sucrose, 30% in PBS
- Antifreezing solution (30% glycerol and 30% ethylene glycol in 0.024M phosphate buffer; use 10× dilution of 0.24M phosphate buffer, which is 30.8 g NaOH and 117.12 g NaH_2PO_4 in 4 l of distilled water)

Methods

Tissue fixation

1. Perfuse the animal of interest with saline followed by 4% paraformaldehyde. This step can be skipped for small tissues, such as embryonic mouse brains or invertebrate ganglia, which can be immersion-fixed.

2. Remove the brain from the skull; remove the dura mater and major superficial blood vessels from the fixed brain (for other body structures, remove any connective tissue and blood vessels).

3. Allow the tissue to postfixate by immersion in 4% paraformaldehyde. The ideal postfixation time is 2–4 weeks.

Tissue dissection

4. Under a stereoscope, dissect the regions of interest (ROIs).

5. Weigh the dissociated ROIs to be counted so that cell densities can be determined later.

6. Store the dissected ROIs in 4% paraformaldehyde if further postfixation is required (that is, if the tissue is not yet well fixed or has not been immersion-fixed for ≥2 weeks). Store them in PBS for immediate use, or in antifreezing solution for long-term storage until dissociation (see step 45, below).

Tissue dissociation

7. Place the dissociated tissue inside the homogenizer tube. Small tissues (approximately ≤50 mg, or 2 mm diameter) can be dissociated directly. Large tissues, such as whole cortical areas (≤1 g of tissue), need to be diced in a petri dish before homogenizing. Wash any leftovers in the dish and in your scalpel into the homogenizer tube using the dissociation solution. Note: It is fundamental that all tissue be homogenized. Much larger tissues (e.g., very large cortical areas, whole cerebella, spinal cords, or whole brains) must be divided into smaller portions of ~1 g to be dissociated separately and combined in a graduated cylinder.

8. Add dissociation solution to the homogenizer to a final volume that is approximately 10 times larger than the tissue to be dissociated, and large enough for the glass tube to be filled with fluid when the piston is fully inserted into the homogenizer. Use this volume to wash down into the tube any tissue fragments that stick to the walls of the homogenizer.

9. Insert the piston, and homogenize the tissue by making simultaneous up-and-down and rotating movements with the piston. Hold the tube upright at all times to avoid any spills. Use care not to let air into the tube, or foam will result.

10. Homogenization is over when no more tissue fragments are visible; this should take 10–30 min of grinding. To check for remaining tissue fragments, raise the homogenizer against the light, with the piston fully inserted, and look for small specks of tissue while gently rotating the piston. When no more specks are visible, proceed to the next step.

11. Wash the walls of the homogenizer. First, place the tube in a stand, remove the piston carefully, and use a fresh volume of dissociation solution to wash the walls of the piston into the tube. Once no material is left on the piston, place it on the bench and use another fresh volume of dissociation solution to wash the material left on the walls of the homogenizer into the tube. This collects into the main suspension any nuclei left behind on the glass walls.

12. Collect the nuclei suspension into a 15 ml or 50 ml graduated centrifuge tube. To do this, do not pour the contents of the homogenizer into the graduated tube. Rather, use a long Pasteur pipette to pick up the suspension (starting from the bottom of the tube, where the nuclei are densest) and transfer it to the graduated tube. Once all the suspension is transferred, use a fresh volume of dissociation solution to wash the walls of the homogenizer, collecting into the bottom of the tube any nuclei left on the glass walls. Transfer this volume to the same graduated tube. Repeat the wash approximately two more times or as needed, using small volumes in each wash, until the fluid in the transfer pipette is perfectly clear.
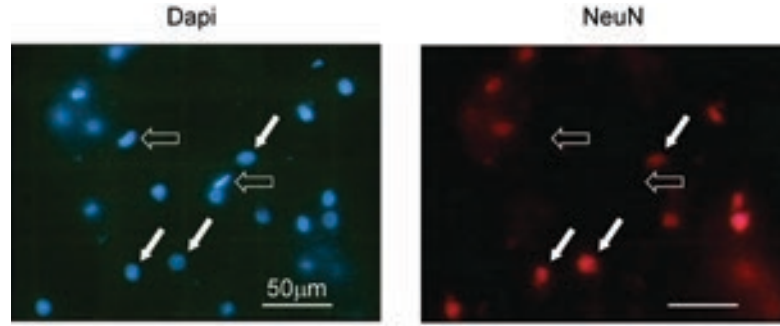
Figure 1. Appearance of isolated, DAPI-stained, and NeuN-stained nuclei. Left, Isolated nuclei have clearly delimited contours, indicating that the nuclear membrane is intact, and are brightly stained with DAPI. Notice that the nuclear morphology is preserved by the fixation. Right, Nuclei that are also stained by labeling with anti-NeuN antibody are readily identifiable against a dark background of virtually nonexistent, nonspecific staining. Filled arrows, examples of NeuN-positive nuclei; open arrows, examples of NeuN-negative nuclei. Scale bar, 50 μm.

13. Now that the entire suspension containing all cell nuclei in the original structure is in the graduated tube, add DAPI to it, diluting it 20–50 times from a 10 mg/l stock solution. The dilution required depends on how dense the nuclei suspension is; for a same-suspension volume, denser structures such as the cerebellum will require more DAPI (for instance, a 20× dilution only) to achieve sufficient labeling that is stable under UV illumination.

14. Use PBS to complete the suspension volume to a defined value in the graduated tube. The precise value is not important, as long as it can be read with precision on the tube.

Counting total cell numbers

15. Make the nuclei suspension homogeneous by inverting the graduated tube 10–20 times, taking care to avoid forming foam.

16. Immediately after making the suspension homogeneous, collect four 10 μl samples and place them in different chambers of two hemocytometers. Allow 1–2 min for nuclei to sink.

17. Under low magnification in the fluorescence microscope, verify the quality of the preparation. Check to see whether the nuclei are free and well distributed, without clumps (Fig. 1, left). Under high magnification (400×), verify that the nuclei are well labeled with DAPI, appearing brightly blue under UV illumination, and that the vast majority are intact, with preserved contours.

18. Count the number of nuclei in a same, known volume for each chamber. For instance, if nuclei in all 25 fields of the center grid are counted, that amounts to the number of cell nuclei in a volume of 0.0001 ml of the suspension. If there are ≥50–60 nuclei in the ensemble of the 25 central fields, then proceed to step 19. If not, then go to step 18A.

18A. Increase the density of nuclei in the suspension to be counted. This step is necessary to ensure that the counts will be reproducible and not subject to Poisson variation. To do this, spin down the graduated tube with the nuclei suspension in a clinical centrifuge (the exact time required depends on the diameter of the rotor; typically, 8 min at 3200 rpm suffice to ascertain that all nuclei are collected in a pellet). Remove the supernatant with a Pasteur pipette without disturbing the pellet; then add PBS to a precise final volume that is small enough to ensure that there will be >60 nuclei per 0.0001 ml of the suspension. Repeat step 18.

19. Average the number of nuclei counted in the same volume across the four samples, and verify whether the coefficient of variation (CV), which amounts to the SD of the values divided by the average value, is <0.15. Typically, the CV is <0.10. If it is not, make the suspension more homogeneous by agitation and count new samples. Additional samples can also be counted until CV <0.15.

20. Calculate the total number of nuclei in the suspension by simply multiplying the number of nuclei per ml by the total suspension volume. If you determined the average number of nuclei in

a volume of 0.0001 ml (that is, the 25 central fields of the chamber), then multiply that average by 10,000 to find the number of nuclei per ml of the suspension. Next, multiply that value by the total volume of the suspension.

21. Make the suspension homogeneous again by inverting the graduated tube and collecting 1 ml into an Eppendorf tube.

22. Spin down nuclei for 5 min in a microcentrifuge. Remove supernatant with a micropipette without disturbing the pellet.

23. If the tissue of interest has been in fixative for <6 weeks, then proceed to step 28. If not, proceed to step 24.

Heat-induced epitope retrieval
24. Add 1 ml of PBS to the pellet and dissolve it completely, using the micropipette if necessary. Do not vortex. Spin down nuclei again and remove supernatant.

25. Repeat step 24 a total of 3 times (that is, wash nuclei 3 times in PBS).

26. Add 1 ml of 0.2 M boric acid, pH 9.0, to the pellet (to break down the excess of aldehydes and the autofluorescence they cause), and dissolve the pellet completely, using the micropipette if necessary. Do not vortex.

27. Incubate at 70°C for 45 min, then spin down nuclei to remove supernatant.

Immunocytochemical identification of specific nuclear types
28. Add 1 ml of PBS to the pellet and dissolve it completely, using the micropipette if necessary. Do not vortex. Spin down nuclei again and remove supernatant.

29. Repeat step 28 a total of 3 times (that is, wash nuclei 3 times in PBS).

30. Add 199 μl of PBS to the pellet and 1 μl of anti-NeuN primary antibody (1:200 dilution). Alternatively, use the appropriate dilution of the desired antibody. Dissolve the pellet completely, using the micropipette. Do not vortex.

31. Incubate at room temperature for 2 h, preferably under agitation.

32. Spin down the nuclei for 5 min in a microcentrifuge. Remove supernatant with a micropipette without disturbing the pellet.

33. Add 1 ml of PBS to the pellet and dissolve it completely, using the micropipette if necessary. Do not vortex. Spin down nuclei again and remove supernatant.

34. Repeat step 33 a total of 3 times (that is, wash nuclei 3 times in PBS).

35. Add the secondary antibody in the required dilution in the presence of 10% normal goat serum and 10% DAPI. Dissolve the pellet completely, using the micropipette. Do not vortex.

36. Incubate at room temperature for 2 h, preferably under agitation.

37. Spin down the nuclei for 5 min in a microcentrifuge. Remove supernatant with a micropipette without disturbing the pellet.

38. Add 1 ml of PBS to the pellet and dissolve it completely, using the micropipette if necessary. Do not vortex. Spin down nuclei again and remove supernatant.

39. Repeat step 38 a total of 3 times (that is, wash nuclei 3 times in PBS).

40. Add 1 ml of PBS to the pellet and dissolve it completely, using the micropipette if necessary. Do not vortex.

41. After agitating the tube, remove a 4 μl sample and place it in the Neubauer chamber. Allow 1–2 min for the nuclei to set.

42. Under 400× magnification, determine the percentage of DAPI-labeled nuclei that also show labeling with the antibody (Fig. 1). The total number of nuclei that needs to be counted depends on the frequency of antibody-stained nuclei; the smaller the percentage of stained nuclei, the larger the total number of nuclei that must be counted. Given that usually 30%–90% of nuclei in any brain structure are NeuN-positive, a minimum of 500 nuclei should be counted.

43. Determine the total number of neurons (that is, NeuN-positive nuclei) in the structure using the formula (% NeuN$^+$ nuclei × total number of nuclei)/100.

44. Determine the total number of nonneuronal cells (that is, NeuN-negative nuclei) in the structure as (total number of cells – NeuN$^+$ cells).

Storage of free nuclei

45. For later reanalysis of nuclei (e.g., with another antibody or morphological criterion), collect the remaining nuclei for storage by spinning them down in a centrifuge.

46. Discard the supernatant and resuspend the nuclei in 30% sucrose in PBS. Allow ≥2 h for the nuclei to equilibrate and start to descend in the tube.

47. Spin down the nuclei, discard the supernatant, and resuspend the nuclei in antifreezing solution. Allow ≥2 h for the nuclei to equilibrate and start to descend in the tube.

48. Store at –20°C until the next use. Frozen nuclear suspensions can be utilized for immunocytochemistry. In that case, agitate the frozen suspension before taking a 1 ml sample for processing, spin down the nuclei, and proceed either to step 24 (if epitope retrieval is required) or to step 28.

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