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An empirical analysis of the precision of estimating the numbers of neurons and glia in human neocortex using a fractionator-design with sub-sampling

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ABSTRACT

Improving histomorphometric analysis of the human neocortex by combining stereological cell counting with immunohistochemical visualisation of specific neuronal and glial cell populations is a methodological challenge. To enable standardized immunohistochemical staining, the amount of brain tissue to be stained and analysed by cell counting was efficiently reduced using a fractionator protocol involving several steps of sub-sampling. Since no mathematical or statistical tools exist to predict the variance originating from repeated sampling in complex structures like the human neocortex, the variance at each level of sampling was determined empirically. The methodology was tested in three brains analysing the contribution of the multi-step sampling procedure to the precision on the estimated total numbers of immunohistochemically defined NeuN expressing ($NeuN^+$) neurons and $CD45^+$ microglia. The results showed that it was possible, but not straight forward, to combine immunohistochemistry and the optical fractionator for estimation of specific subpopulations of brain cells in human neocortex.

1. Introduction

During the past two decades the use of stereological methods has provided important new insight in numbers of neurons (Braendgaard et al., 1990; Pakkenberg and Gundersen, 1997), synapses (Tang et al., 2001) and glia in the cerebral cortex of the normal adult human (Pelvig et al., 2008), and regional changes in numbers of neurons and glia in normal and pathological aging (Pakkenberg et al., 2003; West et al., 1994; Stark et al., 2004) and neuropsychiatric disease (Ongur et al., 1998; Pelvig et al., 2003; Dorph-Petersen et al., 2007; Kreczmanski et al., 2007). Despite the uniqueness of this type of information, the morphologically based classification of cells can pose problems, especially in the distinction between small neurons and glial cells or between different types of glial cells (Braendgaard et al., 1990; Pelvig et al., 2003; Davanlou and Smith, 2004; Stark et al., 2004). Unfortunately, the obvious solution to improve cell classification, using neuron and glial cell specific immunohistochemical markers, is not trivial in case of human brain (Evers and Uylings, 1997; Lewis, 2002; Schmitt et al., 2007).

So far, most stereological studies of the adult human neocortex have been based on the volume × density method (Gundersen et al., 1988a,b; Braendgaard et al., 1990), calculating the total cell number from the number of cells counted using the disector probe (Sterio, 1984) multiplied by the neocortical volume estimated by the principle of Cavalieri (Gundersen and Jensen, 1987; Regeur and Pakkenberg, 1989). When using the volume × density method, the result can be influenced by deformation of the tissue, such as shrinkage occurring during paraffin-embedding (Kretschman et al., 1982; Andersen and Gundersen, 1999; Dorph-Petersen et al., 2001) or staining and mounting of cryostate and vibratomic sections (Gardella et al., 2003; Wirenfeldt et al., 2003; Lyck et al., 2007). In practice, earlier studies reduced tissue shrinkage by use of long-term fixed brain material (Heinsen et al., 2000; Dorph-Petersen et al., 2004; Larsen et al., 2006) followed by embedding in resins (Reguer et al., 1994; Pakkenberg and Gundersen, 1997; Pelvig et al., 2003, 2008). This procedure is disadvantageous, since long-term fixation of tissue in formalin solution reduces tissue immunoreactivity (Evers and Uylings, 1997; Ikeda et al., 1998; Boenisch, 2005; Shi et al., 2007; Lyck et al., 2008).

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Table 1
Human brain tissue included in the study. Information about the sex, age, and diagnosis of donors A, B, and C, along with post mortem interval and fixation of the brain material. The volumes of neocortical regions and of white matter in the right hemisphere of the brains were determined by Cavalieri's principle.

Donor	A	B	C
Sex	F	M	M
Age	88	59	82
Diagnosis	Heart failure, ischemic heart disease	Heart failure after AMI	Heart failure, coronary heart disease
PMI	2 days	2 days	1 day
Fixation	Immersion in 4% Lillies PBFS for 24 hrs followed by immersion in 4% PFA at 4 °C for 2 weeks	Perfusion with 4% Lillies PBFS followed by immersion in 4% PFA at 4 °C for 2 weeks	Perfusion with 4% Lillies PBFS followed by immersion in 4% PFA at 4 °C for 2 weeks
Specimen number[a]	7a	6a	4a
Immunoreactivity score[a]			
NeuN	2–3	4	4
CD45	2	4	3–4
Volume data in cm^3			
Frontal neocortex	165	178	204
Temporal neocortex	95	105	106
Parietal neocortex	112	127	130
Occipital neocortex	48	76	72
Total neocortex	420	526	514
White matter	315	502	400

[a] Specimens from all three brains were included in the analysis of NeuN and CD45 immunoreactivity in Lyck et al. (2008). Immunohistochemical reaction was classified on a scale from 0 to 4, rating 0: no staining; 1: poor staining; 2: borderline staining; 3: good staining, and 4: optimal staining.

Only a few studies have estimated the cell numbers in the human neocortex using the optical fractionator (Heinsen et al., 2000; Samuelsen et al., 2003; Larsen et al., 2006; Dorph-Petersen et al., 2007). The optical fractionator has been successfully used for estimation of immunohistochemically stained neurons and glia in animal models (Muller et al., 2001; Wirenfeldt et al., 2003; Lyck et al., 2007). With the exception of the study by Dorph-Petersen et al. (2007) these studies have based their fractionator design on a Cavalieri sample sectioning the entire hemisphere exhaustively (Gundersen, 1986; West et al., 1991; Cruz-Orive, 2004). However, if the aim is to obtain information about global changes in specific subpopulations of immunohistochemically stained neurons and glia in, e.g. skizophrenia, Alzheimer's dementia, or in multiple sclerosis, sampling by exhaustive sectioning of the cerebral hemispheres not only becomes time consuming (Heinsen et al., 2000; Kreczmanski et al., 2007), but also expensive, because sections shall be stained as free-floating sections to achieve penetration of the staining (Wirenfeldt et al., 2003; Lyck et al., 2006, 2007).

To reduce the workload and expenses, we employed a fractionator sampling protocol with several steps of sub-sampling, that could be applied to short-term fixed human brain, allowing for immunohistochemical labelling of subsets of brain cells. Since there was no method to predict the statistical properties of sampling from complex structures like the neocortex, the variation in the sequential steps of systematic uniform random sampling (SURS) was analysed empirically. The methodology was applied to three human brains in which the total number of morphologically and immunohistochemically identified neurons and glia was estimated.

2. Materials and methods

2.1. Tissue

The three human brains were obtained from donations to the Department of Anatomy and Neurobiology at the University of Southern Denmark. Sex, age and cause of death are given in Table 1. The use of human brains for the study was approved by the Danish Biomedical Research Ethical committee for the Region of Southern Denmark (permission number S-20070065). Autopsy was performed with a post mortem interval up to 48 h. In the case of donor A both hemispheres were fixed by immersion in 4% paraformaldehyde (PFA: Fluka, Sigma–Aldrich, Brøndby, Denmark) diluted in 0.15 M phosphate buffer, pH 7.4 (PB: 3.71 g KH_2PO_4, 21.84 g $Na_2HPO_4{\cdot}2(H_2O)$ in 1000 ml H_2O, pH 7.4) for 2 weeks at 4 °C. The brains from donors B and C were initially perfused through the internal carotid artery with 4% Lillies phosphate buffered formaline solution (PBFS), pH 7.0 (Sygehusapotek Fyn, Odense University Hospital, Odense, Denmark) followed by fixation by immersion, as described for the hemispheres from donor A (Table 1). Specimens from these brains have been part of a previous study on immunohistochemistry on human brain material (Lyck et al., 2008), and the scored immunoreactivity of the specimens have been included in Table 1. Neuropathological examination showed no evidence of neurological disease. In all brains the right hemisphere was selected for cell counting and the left hemisphere of the brains from donors A and C was used to analyse the variance of the sampling design. The selection of hemisphere for analysis was not randomised for this methodological study, but should be randomised in future applications of the methodology.

2.2. Sampling and processing of the right hemispheres for cell counting

2.2.1. Anatomical delineation

The neocortex was delineated using anatomical landmarks, and the pial surface of the specific lobes marked using waterproof ink (Scarlet, Blue, and Orange, Calli Calligraphy ink, Daler-Rowney, Bracknell, England), as has been described in earlier stereological studies of human neocortex (Fig. 1A) (Regeur and Pakkenberg, 1989; Braendgaard et al., 1990; Pelvig et al., 2003).

2.2.2. Fractionator sampling

The right hemisphere was embedded in 5% agar (BBB 10030, Bie & Bernsen, Denmark) in a rectangular metal mould and cut at 4.25 mm (donors A and B) or 6.00 mm (donor C) intervals using a Teflon-coated (Star*Cote 7055, ICS Technologies, Sarnia, Ontario) Zwilling knife and a metal cutting guide (Fig. 1B), generating coronal slices with a random starting point. The volume of the neocortex and grey and white matter was estimated for individual lobes using the Cavalieri's principle (Table 1). By SURS, every second slice was sampled, resulting in a slice sampling fraction (*ssf*) of 0.5. Then sampling was conducted by a series of SUR sampling steps, subsampling series of transcortical wedges, which were cut into bars,