Stereological Quantification of Cerebellar Purkinje Cells

Literature Review and Description of a Variation of the Physical Disector Method Adapted to Confocal Laser Microscopy

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ABSTRACT

Estimation of cerebellar Purkinje cell number is an important evaluation parameter for the study of the development of the cerebellum as well as for investigating the changes occurring in various experimental and physiological conditions. The introduction of stereological techniques in biological and biomedical research has made quantification of Purkinje cells not only more reliable, because independent from morphology-related sources of bias, but also easier and less time consuming. In this paper, we will briefly overview the stereological techniques that have been used so far for estimation of Purkinje cell number in different animal species and we will describe a simple method (the confocal physical dissector) for Purkinje cell quantification on confocal microscope optical slices, based on a variation of the physical dissector technique that can be adopted without the need of any dedicated stereological workstation.

Key Words: cerebellum, Purkinje cells, design-based stereology, dissector, laser confocal microscopy, Cavalieri method

Introduction

Various cerebellar diseases involve the loss of Purkinje cells (PCs) and consequent severe cerebellar dysfunction. Today, the continuously increasing availability of transgenic mouse models of many clinical conditions (Tos et al., 2009) provides the researchers with a powerful tool for investigating etio-pathogenesis and find out effective therapeutic strategies for many diseases, including those affecting the cerebellum (Savill et al., 2005).

To reach this goal, researchers need to obtain reliable quantitative estimations of tissue changes occurring during disease progression and treatment and estimation of PC number is one of the most important, if not the single most important, predictor of disease and recovery for many cerebellar diseases. Yet, it also represents the key predictor of cerebellar development in physiological and pathological. Stereology has developed over the last decades as a group of investigation strategies which allow obtaining reliable quantitative information on the 3D structure of a biological structure (e.g. number and size of cells in a give organ or part of it) starting from quantitative information obtained on 2D images (Gundersen et al., 1988). Neuromorphology is definitely one of most important fields of application of stereological tools (West, 1999) and, in this context, quantitative assessment of cerebellar PCs has received much attention from stereologists since the new methods have started to spread within the neuroscience community.

The aim of this paper is to briefly overview some of the main applications of stereological techniques for PC number estimation. Yet, the paper also aims to describe a simple method for PC quantification.

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on confocal microscope optical disector slices based on a variation of the physical disector technique.

Quantitative estimation of Purkinje cell number: a stereological perspective
Quantitative assessment of PCs is probably one of the earliest applications of the emerging stereological techniques to neuromorphology. It was along the last two decades of the 20th Century that the first papers describing the use of the unbiased stereology for estimating PC number in normal and experimental conditions in the rat were published (Bedi et al. 1980; Harvey and Napper, 1988; Korbo et al., 1993). In 1989, Nairm et al. obtained estimates of PC number in twelve human brains using systematic random sampling and the fractionator technique followed by Andersen et al. (1992) who carried out stereological estimates of the numbers PCs in another five human brains by optical disector and Cavalieri methods.

Figure 1. An example of systematic random sampling of cerebellar serial sections. After starting section is selected at random (e.g., section 2 in this example), the following sections that will be analyzed are selected by systematically jumping at a given distance (10 sections in this example) from the former section. The distance between sections is decided based on the amount of sampling (i.e., the total number of sections that are required by each study design).

Since it is beyond the aims of this paper to describe the basic principles of stereological quantification on histological sections, the readers could refer to various extensive methodological papers (Gundersen et al., 1988; West, 1999; Geuna, 2000) including those published in this issue (Geuna et al., 2012; Kaplan et al., 2012), we will briefly overview some of the main papers that have been published dealing with studies which investigated, using stereological methods, the quantitative changes occurring in PC number in various physiological, experimental and pathological conditions, contributing to significant scientific advancements in this field.

One of the main fields that received a significantly benefit from stereological quantification of PCs is neurotoxicology. In fact, various studies allowed disclosing the effects of different chemicals, of environmental (including drugs) and alimentary origin, on the cerebellum, especially when exposure to substances occurs during development. These included acrylamide (Larsen et al., 1994; Sorensen et al., 2000), methyl mercury (Larsen and Braendgaard, 1995), zinc (Gokce et al., 2011; cocaine (Chen et al., 1996); diclofenac sodium (Ragbetli et al., 2007; Odaci et al., 2010), and nicotine (Chen et al., 2003). However, among potential toxic agents, the single substance that received the higher attention by researchers is definitely ethanol because of the high incidence and prevalence of alcoholism in many Countries. A number of stereology-based experimental studies allowed to demonstrate that exposure to ethanol during pre- and postnatal life causes a reduction in the total number of PCs (Napper and West, 1995; Pauli et al., 1995; Goodlett and Eilers, 1997; Goodlett et al., 1998; Thomas et al., 1998; Miki et al., 1999; Maier and West, 2001) thus emphasizing one of the most critical negative effects of alcohol consumption on the nervous system. Stereological quantitative data on PC loss after ethanol exposure were confirmed also in large animal models (West et al., 2001; Ramadoss et al., 2007) and in human patients (Baker et al., 1999; Andersen, 2004).

Interestingly, stereological research, not only through a light on the damage occurring to the cerebellum after ethanol exposure, but also allowed to investigate the effects of potential therapeutic strategies aimed at preventing the negative effects of alcohol, such as antioxidants (Grigel and Chen, 2005), zinc supplementation (Chen et al., 2001), NGF (Heaton et al., 2000), and motor training (Klintsova et al., 1997; 2000; 2002)
Also the effects of the exposure various physical agents on PC number has been assessed by stereology, in various animal models. For instance, it has been recently shown, in rodents, that exposure to mobile phone electromagnetic fields (Rağbetli et al., 2010; Sonmez et al., 2010), X-irradiation (Li et al., 2002) and altered gravity (Sajdel-Sulkowska et al., 2005) induces a decrease in PC number. Stereological investigation also showed that that neonatal pinealectomy induces PC loss in the cerebellum of the chick (Tink et al., 2006).

The increasing availability of mutants and genetically-modified mice has also opened new perspectives for the adoption of the stereological quantification of PC number for investigating the mechanisms of cerebellar function in normal and pathological conditions. Using genetically-modified mice, as an example, stereological analysis have shown that PCs undergo a period of naturally occurring cell death that is mediated by the cell death proteins Bcl-2 and Bax (Fan et al., 2001). Yet the use of stereological methods in PC degeneration mutants as well as in transgenic mouse models of cerebellar ataxia have throw a light on the neurochemical changes occurring in cerebellar disease (Baurle et al., 1997) and on the potential effectiveness of innovative treatment strategies based on the tissue engineering (stem cell transplantation) approach (Chintawar et al., 2009).

Application of stereology to the study of the changes in PC number in the human cerebellum deserve particular mention. First, it has been shown that age-related selective loss of PCs does not occur (as it was previously thought) in the entire cerebellar cortex, but it occurs in the anterior lobe only (Andersen et al., 2003). In addition, stereological research has also focused on the relation between PC loss and neuropsychiatric disorders showing, partially unexpectedly, that the number of PCs is reduced in some forms of autism (Andersen et al., 2010) whereas in schizophrenia and Alzheimer’s disease it does not change significantly (Andersen and Pakkenberg, 2003; Whitney et al, 2008).

Noteworthy, stereology recently led to a significant methodological improvement in the study of PCs, both in animal models and in human brain samples, namely the demonstration that traditional Nissl staining may not be a reliable marker for PCs since it does not label all cells, while calbindin-D28k is a better alternative (Whitney et al., 2008b).

The confocal physical dissector: an adaptation of the physical dissector procedure to confocal optical slice galleries

Although the cost of stereological softwares and workstations is being progressively reduced over time, they are still not available in all laboratories thus partly limiting the spread of the use of stereological tools in quantitative neuromorphology. Recently, we have developed a simple method based on the employment of the physical dissector principle on confocal laser microscopy optical slices that could be thus named the “confocal physical dissector”. The method, that has already been successfully applied to the quantification of PC number in SCA1-mutant mice, with or without neuron stem cells grafting (Chintawar et al., 2009), is based on a three step procedure carried out on paraformaldehyde transcardially perfused mouse brains, embedded in ice, that were cryostat sliced at 18um and sections were immunostained by calbindinD-28 to reveal PCs soma and their dendrites in the molecular layer. The first two steps are aimed at respecting the equal opportunity rule (Geuna, 2005), i.e. that all histological sections of the entire cerebellum (first step) and then, in any selected section, all PCs (second step), have the same chance of being sampled. The third step is aimed at estimating the reference volume in order to eventually estimate the total PC number based on the PC density obtained in the sampled sections.

First stereological step: selection of sections by systematic random sampling

First, a systematic random sampling scheme is adopted to reach the goal all parts of the entire cerebellum are covered by sampling and have the same probability of being selected in the final sample. Systematic random sampling is carried out by the systematic selection of every n\textsuperscript{th} unit of the population from one randomly selected starting unit (Geuna, 2000). In the case of PC quantification, the units are represented by the serial sections of the cerebellum. In practical terms (fig. 1), all the serial sagittal sections including the whole cerebellar cortex are numbered and then, after
starting section is selected by chance, the following sections that will be analyzed are selected by systematically jumping at a given distance from the former section. The distance between sections (10 sections in fig. 1) is decided based on the amount of sampling (i.e. the total number of sections that are required by each study design). In our experience, a set of 6-8 sections is enough to get a sufficient sample of PCs in the mouse cerebellum.

Second stereological step: selection of PCs in each selected section by confocal physical dissector probes

Once a sufficient number of sections have been sampled by systematic random sampling, the second stereological step is to define a sample of PCs in each section that is unbiased with respect to their size, shape and orientation. The definition of a set of inclusion/exclusion rules for clearly determining which PC profile fiber falls inside the sampling field is based on the axiom:

\[
N_{\text{profiles}} > N_{\text{cells}}
\]

where \(N_{\text{profiles}}\) is the total number of cell profiles on serial sections of an organ (or part of it) that is always higher than the true number of cells \(N_{\text{cells}}\) (since, independently of the size of the cells and the section thickness, at least some of the cells will be split into two sections, generating two profiles of one single cell). In 1984, a method named “dissector” was described as a set of rules for obtaining unbiased estimates of the true number of cells on histological sections (Sterio, 1984). In its original version (named physical dissector) method is based on the use of sets of pairs of parallel sections separated by a known distance and the counting of only those particles that appear in one of the two histological sections (named reference section) and not in the other (named look-up section). This goal is obtained by counting “tops”, i.e. the first part of the object that encounters the progressing plane of observation. The tops are localized by identifying the last-first profile of an object seen only in the reference section (and not in the look-up section) of the dissector pair of sections. In the optical dissector, top’s identification is carried out on single sections, not on section pairs, and the 3-D dissector probe is determined by focusing on the z-axis for a given distance and the cells are counted when they first come into focus within the sampling volume (Gundersen et al., 1988).

The confocal physical dissector that we describe here has already been used for PC number estimation in mice (Chintawar et al., 2009) and is a method which combines the physical and optical dissector procedures taking advantage of the possibility offered by laser confocal microscopy to obtain a set of serial optical slices inside the thickness of a histological section. Knowing the thickness of each optical slice (that can be set up by the investigator) a gallery of images can be used to define a optical reference section and an optical look-up section that can eventually be used to sample PCs according to the principles of the physical dissector (i.e. counting of only those PCs that appear in the reference section and not in the look-up section). Figure 2 illustrates how this is done in practical terms.

The number of PCs (\(N\)) which respects the inclusion rules inside each dissector probe volume (\(V\)) is then used to calculate PC density (\(Nv\)) according to the formula:

\[
Nv = \frac{N}{V}
\]

![Figure 2](https://www.neuroquantology.com)
**Third stereological step: estimation of the reference volume in order to estimate the final total number of PCs**

In order to obtain the final estimates of the total PC number from the average PC density assessed in the sampled sections, it is necessary to first estimate the reference volume (V_ref) of the structure under analysis. Provided that all PCs are included, the size of the reference volume could vary depending on the investigator choice: of course if a large reference volume (e.g. the entire cerebellum) is selected the average PC density will be lower, and vice versa, and thus size of this parameter is not expected to influence the final total number estimates. Therefore, selection of the reference volume should be guided by easiness in recognizing its borders on the histological sections in order to facilitate and speed up its measurement. In our experience, the best V_ref for PC number estimation is the whole volume occupied by PC layer and molecular layer together (Fig. 2). In practical terms, V_ref is estimated using the Cavalieri principle as follows: first the area (A) occupied by the PC and molecular layers is measured in each sections. Then, knowing the distance between sampled sections (calculated by multiplying the number of interval sections for the mean section thickness) the total reference volume is calculated according to the formula:

\[ V_{\text{ref}} = \Sigma A \times T \]  \hspace{1cm} (3)

where \( \Sigma A \) is the sum of areas occupied by the PC and molecular layers and \( T \) is the sum of all distances between sampled sections.

Finally the total number (N_tot) of PCs is estimated, based on the values obtained as described in the formulas (2) and (3), according to the formula:

\[ N_{\text{tot}} = V_{\text{ref}} \times N_v \]  \hspace{1cm} (4)

**Conclusions**

The spread among neuroscientists of the use of stereological techniques has made estimation of PC number, in various physiological and pathological conditions, not only independent from morphological bias and thus much more reliable than in the past, but also much easier and faster since the progress of stereological workstations have made them user-friendly and relatively simple to be used (Geuna, 2005). Yet, quantitative data from different studies can be more easily compared when unbiased stereological counting techniques are adopted.

Literature overview shows that the employment of stereological techniques to the study of PCs in various animals’ species, including humans, has contributed to significant advancements in scientific knowledge about cerebellar changes in various physiological and pathological conditions.

Therefore, always keeping in mind that there is no absolutely correct method for solving any problem that involves inductive reasoning (Smith, 1994; Geuna, 2005) and that any experimental approach should be always critically assessed, it can be concluded that, today, the adoption of traditional assumption-based morphometrical methods for PC number estimation, especially those based on simple profile counting, is no longer justifiable. Researchers in the field should thus primarily consider the adoption of design-based stereological approaches for quantitative morphology of the cerebellum, and especially of PCs.

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