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THE USE OF STEREOLOGICAL METHODS IN NEUROSCIENCE: WHAT ARE WE LEARNING?

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ABSTRACT

Efficient, unbiased stereological methods for quantitating macroscopic and microscopic structures in the central nervous system have been developed since 1984, and are superior to the conventional assumption based methods previously used. The stereological method, the disector, a counting method, makes it possible to count particles e.g. cells and synapses, without bias, i.e. the selection of particles is not influenced by their size or shape. When the disector is used within a delineated region of the nervous system, whose volume is determined, the total number of particles is estimated. Even though over ten years has passed since the original description of the disector method, the disector is still the most important stereological method used in the field of neuroscience and when combined with specific labelling methods, such as immunohistochemical labelling or in situ hybridization, is a most powerful tool for quantitating structural and functional relationships. The application of stereological methods to non-invasive methods of imaging such as magnetic resonance imaging and computerized tomography, enables direct, quantitative study of the living human brain.

Key words: Cavalieri volume, disector, optical fractionator, stereology

INTRODUCTION

Over ten years ago in 1984, a method was introduced that enabled unbiased estimates of the number of objects in three dimensional space to be obtained from two dimensional images, namely thin histological sections. This method heralded the beginning of the era of stereology and a movement away from assumption based methods of quantitation. This paper aims to illustrate some of the major areas in which stereology has made an invaluable contribution to our understanding of neuroscience in recent years.

The disector method although first described in 1984, (Sterio, 1984) still dominates the contribution that stereological methods make to neuroscience. This stereological method allows an estimate of the number of particles e.g. cells,

of a specific type in a defined region of the nervous system, to be obtained without bias, i.e. the selection of particles is not influenced by their size or shape. The combination of the disector estimate of density with the volume of the area containing the specific population of particles, gives an estimate of absolute number that is independent of any tissue volume change that may affect density and of changes in particle form, both of which commonly occur during a number of normal and pathological biological processes and during tissue processing. Prior to 1984, the methods used to estimate particle number and size were dependent on assumptions about the shape, size and orientation of particles (e.g. Weibel, 1979) and were in general biased, and the extent of this bias was usually not determined (Coggeshall and Lekan, 1996). The reliability of the results obtained with the disector method ensures that the quantitative information on which findings are based is valid, especially as human data becomes more available and discrepancies between animal models or between animal models and human data become apparent (Harding et al., 1997).

The original disector method, now referred to as the physical disector (Sterio, 1984), used parallel section planes a known distance apart. A more efficient way however to generate disectors is by using successive focal planes within a single thick slice of tissue (Fig. 1). This led to the development of the optical disector method, where particles are counted in parallel optical planes within a single thick section, and later to the optical fractionator method, where the optical disector method is applied within a known fraction of the tissue. For a more comprehensive discussion of these methods see Gundersen (1986), West et al., (1991) and Mayhew and Gundersen (1996). These developments have increased the efficiency of using the disector method, but it is the disector method itself, as a means of obtaining an unbiased sample of particles, that has led to its very wide use in studies where, in particular, neurons and synapses have been counted (Braendgaard and Gundersen, 1986; Pakkenberg and Gundersen, 1988; Mayhew, 1992; West, 1993). In recent years the disector method has been combined with specific labelling techniques such as immunohistochemistry or more recently in situ hybridization techniques (Jansen and Moller, 1993; Aika et al., 1993, West et al, 1996). This makes it possible to count defined subsets of neurons or glial cells within a population. The combination of specific labelling techniques and stereological methods is very powerful as it allows estimates to be made of cells with specific functional roles.

The other major contribution that stereology has made to neuroscience in recent years, is the application of stereological methods to the living human brain (Lyden et al., 1994; Vogels et al., 1995; Garden and Roberts, 1996; Sheline et al., 1996). Images obtained with magnetic resonance imaging and computerized tomography imaging techniques have been used to assess specific volume changes in the human brain in vivo, which can be related to disease processes, functional states and to prognosis and recovery.

DETERMINATION OF TOTAL NUMBER - THE PHYSICAL DISECTOR

Using the disector method (Sterio, 1984) particles are counted when they appear within an unbiased counting frame, placed on one, of a set of two parallel section planes (the reference plane), but are not in the other of the two section

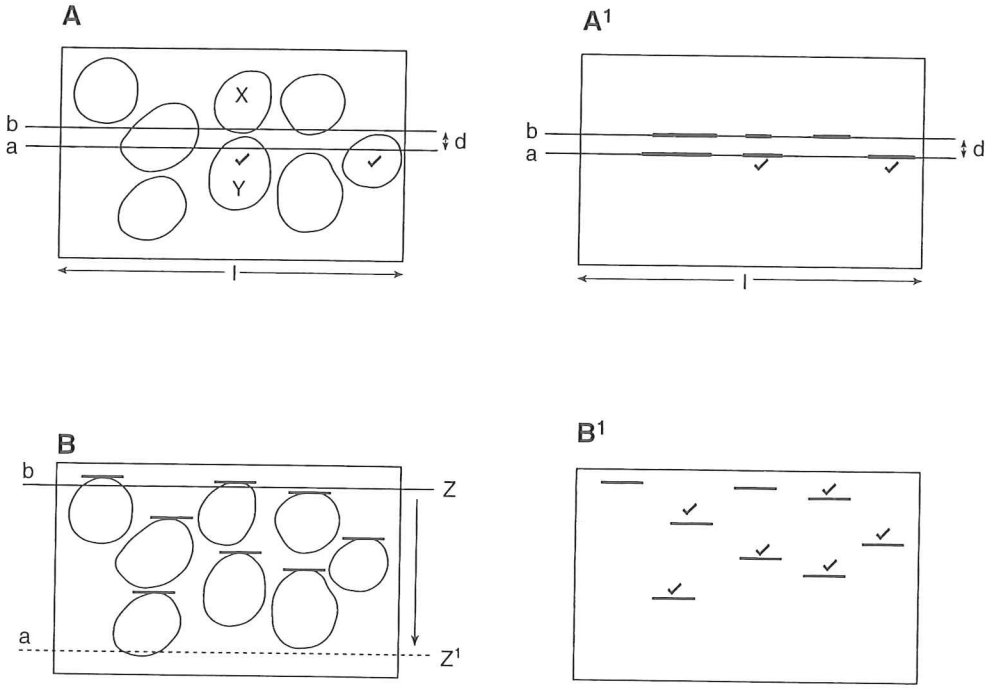

Fig. 1.
An illustration, in 2 dimensions, of the physical and optical disector principles. (A) Eight profiles, the nuclei of densely packed neurons with scant cytoplasm, are contained in a defined plane. The parallel lines a and b, each of length l, represent the 'reference' and 'look-up' planes of a physical disector. The 2 planes are separated by a distance d, which is smaller than the mean profile height at right angles to the lines. (A^1) Line a transects 3 profiles. Two of these profiles are not transected by line b, and so are counted (✓). The uppermost pole of each transected profile (the counting particle) occurs in the space l x d, lying between the lines. The position of profiles X and Y and their transects in line a and b, demonstrates how difficult it can be to identify transects in a 2D image as belonging to different structures.

(B) Imagine a line b, sweeping across the plane from position z to z1.(B^1) It will encounter 8 profiles in its travel, 6 of which have an identifiable point, the counting particle, e.g. the first sharply focused image of the nucleus, which can be unambiguously dissociated from its nearest neighbours. This is analogous to the optical disector in which a series of focal planes sweeps through a volume of tissue and encounters the identifiable points of 3D particles contained within the volume. The existence of the parent particle in the volume of the disector removes the potential problem of identifying the origin of the profile transects as occurs in the physical disector.

planes (the lookup plane). The pair of sections that comprise the disector should be randomly located within the specimen and be a known, measured, distance (less than profile height in the direction orthogonol to the section planes), apart. The presence of a particle is not dependent on its size or shape but only on the fact that each particle has a topmost point, a fact which is true of all particles where all profiles can be identified as being part of their parent particle (see Gundersen, 1986; Mayhew and Gundersen, 1996). This method produces a density, which although unbiased, is not of great value because cell densities do not equate to cell number, although in the past, and unfortunately still in the present, this has been considered to be the case (Navaroo et al., 1996). The product of density and reference volume, the volume within which the particles occur, gives an estimate of total number. This estimate, if the density and volume estimates are determined in the same material, is independent of any differential tissue volume changes that may occur during preparation of the material, which would affect density and render density meaningless for all practical purposes, if knowledge of the reference volume change is not available. Total number, obtained either by the combination of disector method and the Cavalieri estimator to obtain reference volume or with the fractionator method, employing the disector method to sample particles, is where the power of the disector method lies (see Mayhew and Gundersen, 1996). It must also be remembered that the disector method does not only lead to an estimate of total number via an unbiased estimate of density, but that it is the only way to select an unbiased, sample of particles for further stereological analysis, such as the estimation of mean nuclear volume (Janson and Moller, 1993; Aika et al., 1994; Licht et al., 1994; Mouton et al., 1994; Larsen and Braendgaard, 1995; Madsen and Schroder, 1996).

The physical disector method, as used on parallel section planes, although currently less widely used in light microscopical studies than the various variants of the disector method, continues to make a significant contribution to our neuroscience knowledge when used with conventional transmission electron microscopy to quantify ultrastructural features of nervous tissue (Carlton and Coggeshall, 1996; Navarro et al., 1996).

Modern stereological methods allow reliable estimates of the total number of synapses in a region of the brain or along a particular pathway to be determined (Peterson et al., 1994; Poduri et al, 1995; Klintsova et al., 1997). Although the disector method has been available for this type of determination since 1984, and an earlier unbiased method based on a stack of serial sections was described in 1980 (Cruz-Orive, 1980), the literature describing changes in synapse number is full of seemingly contradictory data and the information on synaptogenesis is not conclusive (e.g., see Geinisman et al., 1995). Changes in the numerical density of synapses are widely published (Calverley and Jones, 1987, 1990; Hunter and Stewart, 1989; Geinisman et al., 1992) but these cannot be interpreted unambiguously to represent equivalent changes in total number. However, several researchers have used modern stereological methods to determine the total number of synapses rather than density (Siklos et al., 1990; Madeira and Paula-Barbosa, 1993; Geinisman et al., 1996; Kleim et al., 1997; Klintosova et al., 1997). This is of extreme importance as total number of synapses is an important parameter when the functional capacity of a region or pathway is being assessed. Although the efficacy of synapses may differ,

especially at different locations on the postsynaptic element, the total number of synapses is a valuable measure of neural input to the neurons in a specific region and may be related to functional changes (Black et al., 1990; Kleim et al., 1997; Klintsova et al., 1997).

A very elegant piece of work by Geinisman et al., (1996) used unbiased stereological methods to determine the total number of synapses in the striatum radiatum of the CA1 region of the rabbit hippocampus. This involved a two stage process; the estimation of the volume of the striatum radiatum at the light microscope level using the Cavalieri method, and the determination of synaptic density using the disector method at the electron microscopic level. Both estimates were made on the same tissue samples to avoid the problem of differential shrinkage that occurs between material embedded in different media, as is generally the case for light and electron microscopy. The stereological design used in this study also overcomes the need to use the very elegant, but very technically demanding, double disector method (Braendgaard and Gundersen, 1986; Gundersen et al., 1988). Random systematic sampling within the entire CA1 region of the hippocampus was used to ensure that all parts of the synapse containing region and all synapses within the region, had an equal chance of being sampled.

This study demonstrates the use of an optimal stereological design and as the sampling and counting procedures used are unbiased, provides an estimate of the true, total number of synapses in the stratum radiatum of the female New Zealand albino rabbit. However, the true value of this paper to neurostereologists lies in its description of a uniform, random, sampling scheme that could be used in any defined brain region and in the calculation of and description of the variance at different levels of the multilevel sampling scheme. The authors demonstrate that only 28% of the total variance of the estimate of the total number of synapses (N(syn)) is due to the coefficient of error of the estimate of N(syn), but that it is the inherent biological variability among the five rabbits used that contributed most to the total variance of the estimates. This analysis of the contributing sources of variance in the estimate allowed the authors to determine that, with similar variance of N(syn), a minimum of eight animals per group should be sufficient to demonstrate that group mean differences in total synapse number of 20% are significant at the 0.05 level. A design, such as that used by Geinisman et al., (1996), may not always be technically possible, so it is important to remember that random samples from within the total synapse containing region may be used and will be unbiased, but may be somewhat less efficient at obtaining an estimate of the true value.

An earlier study by Madeira and Paula-Barbosa (1993) has also made an important contribution to our neuroscience knowledge by illustrating the type of information that can be obtained using modern stereological methods. The authors address the controversial problem of the nature of the synaptic reorganization that occurs when there is a selective but partial deafferentation of a neuronal population. They investigated the effects of neonatal hypothyroidism on the contact between mossy fibres and dendritic excrescences of CA3 pyramidal cells where the number of postsynaptic pyramidal cells is normal but the presynaptic granule cell numbers are reduced to around 50% of normal. Previous studies have inferred that neonatal hypothyroidism disrupts synaptogenesis, but as this conclusion is based on numerical density data, a

quantitative parameter that cannot be assumed to be reliable (Coggeshall and Lekan, 1996), the inference may in fact not represent the real situation. A combination of the Cavalieri and disector methods was used to determine the total number of mossy fibre-CA3 pyramidal cell synapses in a systematic random sample from the suprapyramidal bundle of the mossy fibre system, a zone that broadly corresponds to the stratum lucidum. Within this design, vertical sections (Baddeley et al., 1986), that retain layered information within a region, were used to estimate the ultrastructural data. This allowed not only synaptic density but other parameters of the synaptic connectivity between the two cell populations to be determined. Specifically, the use of vertical sections, allowed estimation of the surface area of the mossy fibre terminal plasmalemma from the surface density (surface area per unit volume, Sv) and the volume of the terminals (obtained from volume density of mossy fibre terminals combined with the volume of the reference space i.e. the volume of the suprapyramidal part of the mossy fibre system) (Baddeley et al., 1986).

Despite the decrease in the number of granule cells with hypothyroidism, the total number of synapses had returned to normal within 180 days. Interestingly, the numerical density of synapses was significantly greater in hypothyroid animals compared with controls, which indicates the unreliable nature of such quantitative measures. The surface area of the mossy fibre terminals was the same in hypothyroid and control animals because the area of the terminal membrane per unit volume of mossy fibre terminal was greater in the hypothyroid rats. Stereological methods enabled the more irregular appearance of the mossy fibre terminals in hypothyroid rats to be reliably quantified and have provided information on the mechanistic change that compensates for the reduction of presynaptic input to a normal postsynaptic counterpart. This study is valuable in that it shows that stereological methods can be used to provide mechanistic information, not only static descriptive information.

Stereological methods have been generally applied to synaptic populations that are identified on morphological criteria, as seen in conventional transmission electron micrographs. However, the identification of functional subgroups of synapses within a given population using immunohistochemical and in situ hybridization methodologies is possible (Umbriaco et al., 1994). Combination of specific labelling methods to identify synapses and stereological methods, will allow valuable quantitative information on synaptic function to be obtained in the future.

THE OPTICAL DISECTOR

The disector method evolved from its original description, known as the physical disector, to the optical disector (Gundersen 1986), where different focal planes within a single physical section are used as the disector planes (see Fig. 1). In essence one counts particles, according to the two dimensional counting rule (Gundersen, 1978) as they come into view within a known, measured depth of tissue, within the physical section. Reasons of efficiency, and the difficult task of determining the absolute section thickness, as used in the physical disector calculation of density, have been instrumental in facilitating the use of the optical disector as the most widely used disector method in light microscopic

studies (Gundersen, 1986; Bjugn, 1993; Bjugn and Gundersen, 1993; Janson and Moller, 1993; West, 1993; Tandrup and Braendgaard, 1994; Regeur et al., 1994). Absolute section thickness is not required for the optical disector method where density and reference volume are both obtained from the same sections. Any tissue shrinkage that has occurred during tissue embedding and thus affected the measured density determined with the optical disector, will be incorporated into the estimate of mean section thickness used in the calculation of reference volume, ensuring that the estimate of total number will not be altered by tissue shrinkage/expansion. The optical disector method has also been combined with the fractionator method, to become the optical fractionator method (West et al., 1991). This method involves counting neuronal nuclei, or any identifiable particle, with optical disectors in a uniform, systematic manner, within a known fraction of the region within which the particle of interest occurs. This method increases efficiency by not requiring a separate determination of the volume of the region of interest and overcomes the problem of determining the volume of narrow layers of neurons (Goodlett and Lundahl, 1996; Pilegaard and Ladefoged, 1996; Madeira et al., 1997).

Estimates of total number, obtained with either the combined disector/Cavalieri method or the optical fractionator, have contributed to our knowledge in a number of areas, including our understanding of the relationship between neuronal number within specific brain regions and neural circuits, the pathology of a number of neurological illness and in normal aging (Pakkenberg, 1993; West, 1993; Pakkenberg and Gundersen, 1997). The appreciable use of quantitative information in neuroscience was demonstrated by Coggeshall and Lekan (1996), in a survey, carried out in 1994, of four major neuroscience journals. They found that around 60% of all published papers used histological sections and that of these, 30% reported quantitative data. Unfortunately only 5% of these papers had used stereological methods. Clearly information about stereological methods has, over the ten years since the description of the disector method (Sterio, 1984) penetrated only slowly and incompletely into the neuroscience community (Saper, 1996). It appears that this penetration rate has increased and hopefully will continue to do so, although methods that may obtain unreliable data are still being used (Paskavitz et al., 1995).

THE IMPORTANCE OF TOTAL NUMBER

A classic paper by Pakkenberg and Gundersen (1988) demonstrated that cell density is a very unreliable method for determining the true change in cell number that occurs within a structur. They found that despite the fact that the density did not change, the dorsomedial thalamic nucleus of schizophrenic patients had around half the number of neurons as the controls. The disparity between density and total number as a quantitative measure, is also demonstrated by the finding that there is a significant reduction in the total number of neurons in leucotomized schizophrenics as compared to non-leucotomized schizophrenics with no difference in the density of these cells (Pakkenberg, 1993).

These and many other papers have been instrumental in the realization that to develop meaningful models of normal development and aging, of

neuronal connectivity and of neurodegenerative diseases, reliable information on the number of neurons in different parts of the nervous system is essential (Bjugn and Gundersen, 1993; Korbo et al., 1993; Holman et al., 1996; Rasmussen et al., 1996).

Neurostereology has helped to elucidate whether cell loss occurs in the brain with aging and if so, has established reliable estimates of the pattern of cell loss that may occur during normal aging in the brain. Previous studies have suggested that there is a loss of Purkinje cells in the human cerebellum with increasing age, while in the rodent both a decrease in and no change in this cell population has been reported (Hall et al., 1975; Rogers et al., 1984; Sturrock, 1989; Bakalian et al., 1991). Assumption based methods have been used in these studies, thus it is not possible to determine how these findings relate to what may be the real changes in cell number that occur during aging. The use of the disector method however allows the real picture of the neuronal loss that occurs in the brain with aging to be described. Dlugos and Pentney (1994) used unbiased stereological methods to show that in the rat cerebellum there is no loss of Purkinje or granule cells with increasing age from 3 to 27 months. The total number of pigmented neurons in the locus coeruleus, determined using unbiased stereological methods, also shows no decline with increasing age in non demented males (Mouton et al., 1994), whereas, approximately 10% of all neocortical neurons are lost over the life span, from 20 to 90 years, in both sexes (Pakkenberg and Gundersen, 1997).

It has been suggested that neurological symptoms, performance deficits on particular tasks and the pathological changes seen in brain damage and in a number of disease states are consequent to cell loss in the central nervous system. Establishing the relationship between an alteration in brain function and neuronal number is an essential part of establishing the structural basis of a range of well established functional changes seen in the brain.

Performance on the spatial reference memory version of the Morris water maze was found to be impaired in 2 year old male Ico:WIST rats compared to 2.5 month old animals. However there was no significant difference in the mean total numbers of neurons, determined with unbiased stereological methods, in various subdivisions of the hippocampus and subiculum in these aged compared to young rats (Rasmussen et al., 1996). This stereological data allowed the authors to conclude with confidence, that the structural correlates of impaired spatial memory, on this specific task, with increased age are other parameters than a decline in total neuron number as measured above.

Alzheimer's disease (AD) is a progressive, neurodegenerative disease of the central nervous system that occurs with an increased frequency with increasing age and is characterized by personality changes and cognitive deficits not seen in normal aging. It has been suggested that neuronal loss may play an important role in the generation of dementia in patients with Alzheimer's disease (Terry et al., 1981; Paskavitz et al., 1995; Paula-Barbosa et al., 1986; Davies et al., 1987). Stereological methods were used by West et al., (1994) to determine how the pattern of cell loss seen during normal aging in specific subdivisions of the hippocampus (West, 1993) relates to cell loss in Alzheimer's disease. They found the greatest AD-related neuronal loss was seen in the CA1 region, that does not exhibit normal age-related neuronal loss, where an average reduction of 68% of its neurons occurred in the AD group. Two other regions of the

hippocampus, the dentate hilus and subiculum, that normally exhibit cell loss with increasing age, showed reductions of 47% and 25% respectively, that were in excess of the loss attributable to normal aging. The selective pattern of neuronal vulnerability in AD is also demonstrated by a severe loss of neurons in the entorhinal cortex, even in mild cases of AD where clinical symptoms of dementia are only just apparent (Gomez-Isla et al., 1996; Giannakopoulos et al., 1997). It is interesting to note that neuronal density, even though obtained with the disector method, was not able to detect this cell loss due to an appreciable decrease in the volume of the entorhinal cortex in those individuals with AD. Stereological methodology also enables this neuronal loss to be investigated on a laminar basis with layers II and IV exhibiting dramatic cell loss that correlates with the susceptibility of the cells of these layers to form neurofibrillary tangles (Gomez-Isla et al., 1996). The comparison of the pattern of cell loss in AD patients with that of normal aging, on the basis of absolute numbers in very precise brain regions, allowed these authors to conclude with confidence that AD is not an inevitable consequence of aging but that different patterns of cell loss are occurring that contribute to memory impairments (West, 1993; Gomez-Isla et al., 1996).

Neurostereological methods are also being used to investigate the type of neurodegeneration that occurs in acquired immunodeficiency syndrome (AIDS). Atrophy of the brain has been reported in radiological studies (Pedersen et al., 1991; Rainiko et al., 1992) and a stereological study of volume on formalin fixed brains found a significant cortical and central atrophy (Oster et al., 1993). Although brain atrophy is considered to be a generalized phenomenon of advanced human immunodeficiency virus (HIV) infection, it is unclear whether atrophy is specifically related to dementia or if atrophy in specific CNS locations is responsible for HIV dementia (Oster et al. 1993). In an attempt to elucidate the relationship between atrophy in specific brain regions and HIV associated-dementia, Subbiah et al., (1996) used stereological methods to estimate volume changes in postmortem brains of prospectively followed patients that were clinically characterized into various categories dependent on an assessment of the presence and severity of HIV associated dementia. There was a significant reduction in mean neocortical volume in patients with AIDS (with and without dementia) compared to seronegative controls that was increased when only AIDS patients with dementia were compared to controls. There were no differences in cerebral atrophy between AIDS patients with or without dementia. Interestingly there were no significant differences in the mean volumes of the hemispheres, white matter or basal ganglia. Oster et al., (1995) used stereological methods to determine whether neuronal loss may be one of the mechanisms of brain atrophy in patients with AIDS as recent data has suggested a role of programmed (apoptotic) cell death in the neuropathological changes seen in AIDS brains (Petito and Roberts, 1995). They found that the mean total number of neurons in the entire neocortex was reduced by 37% with this loss occurring, to roughly the same extent, across all four neocortical lobes (Oster et al., 1995). The loss of neurons seen in this group of AIDS patients was not accompanied by neurological deficits although this may be attributable to the small number of patients with dementia in the study. The neuronal loss determined in this study cannot be directly compared to the cell loss found in earlier studies that reported cell densities in limited areas. It is important to note that Oster et al., (1995)

report that tissue swelling, occurring during postfixation processing, was significantly different in the brains from AIDS patients compared to controls and would thus affect the apparent density of cells. This highlights the superiority of total number as a reliable measure of change.

It is clear that the unbiased determination of total number is making and will continue to make significant contributions to our neuroscience knowledge. However it must be remembered that the accuracy of an estimate of the total number of a specific population of cells is dependent on the choice of reliable criteria for cell classification (Korbo et al., 1993; Tandrup, 1993; Pakkenberg and Gundersen, 1997). In cell counting in the central nervous system the distinction between the smallest neurons and the largest glial cells is not a trivial problem and thus the identification of neurons depends on the ablility to distinguish glial cells from neurons (Skoglund et al., 1996). Large neurons are usually distinguished morphologically on the basis of a large spherical nucleus which typically has a diffuse and even chromatin pattern with a prominent nucleolus and the presence of distinct Nissl bodies in the well defined cytoplasm. However, small neurons may have scant cytoplasm without distinct Nissl bodies (Palay and Chan-Palay, 1977) and if the physical disector counting method is used, the nucleolus may not be present in the portion of the nucleus contained within the section. It may therefore be very difficult to distinguish small neurons from astrocytes. It is also very difficult if not impossible to identify different functional subgroups of neurons within a parent population on morphological criteria (Tandrup, 1993). Immunohistochemical or in situ hybridization labelling methods, that identify specific groups of cells on a functional basis, combined with stereology is potentially a very powerful tool for the estimation of cell numbers (Aika et al., 1994; Moffet and Paden, 1994; Carlton and Coggeshall, 1996; Giannakopoulos et al., 1997; Madeira et al., 1997).

THE DISECTOR AND SPECIFIC LABELLING METHODS.

An early study where immunolabelling and stereological methods were combined was carried out by Ren et al., (1992). The physical disector method was used to count cells in the barrel area of rat somatosensory cortex and postembedding immunocytochemical methods were then used on adjacent sections to characterize the phenotype of the counted cells. Specific labelling of a subpopulation of cells, allows them to be quantified and a change detected, that may be masked within the parent population, and this quantitative change in the subpopulation may relate to a functional change.

Unfortunately immunohistochemical staining is often not possible or may be compromised on tissue that has been embedded in paraffin or plastic resin, as commonly used in stereological studies, as a result of conformational changes that occur to the antigenic sites during tissue processing. Frozen sections are well suited to immunohistochemical staining. However they are not ideal for studies using the optical disector/Cavalieri estimator combination because shrinkage occurs, particularly in section thickness, due to section collapse, during the preparative procedures resulting in a real section thickness significantly less than the mean section thickness at which the sections were cut (Goodlett and Lundahl, 1996). The optical fractionator method is better suited for immunolabelled tissue, as the optical disector estimation is carried out in a

known fraction of the tissue which includes a known fraction of the thickness of the shrunken/collapsed section thickness.

Janson and Moller (1993) combined immunocytochemical and stereological methods to examine the putative protective action of chronic nicotine treatment against lesion-induced degeneration of nigrostriatal dopamine neurons. They found that the lesion induced decrease (65%) in the total number of neurons showing tyrosine hydroxylase-like immunoreactivity was significantly counteracted by nicotine treatment, but the decrease (20%) in Nissl stained neurons and the increase in non-neuronal cells was not affected. Immunohistochemistry and stereology have also been combined to study the effects of aging on the hippocampal formation of the Brown Norway rat (Cintra et al., 1994). Using the optical fractionator, these workers determined that the total number of glucocorticoid receptor immunoreactive neurons in CA1 and CA2 did not differ in the 3 and 36 month-old rat. Although there was no change in the number of astroglia in the CA1-CA2 area with increasing age, there was a significant reduction in the total number of basic fibroblast growth factor immunoreactive astroglia between 3 and 36 months of age. This finding is important, not only as it suggests a mechanism that may underlie reduced nerve cell survival with increasing age, but it also demonstrates that specific changes within particular cells can be quantified and although a change in total cell number may not occur, functional changes within the cell population may occur which affect the overall function of the structure.

A combination of the optical disector and immunolabelling has also been used by Petersen et al., (1996) to determine the characteristics of the population of entorhinal cortex layer II neurons that die following lesion of the perforant path, in order to elucidate the mechanisms that contribute to neuronal death. Cells, identified as neurons based on thionin staining, were counted using the optical disector and the phenotype then examined with fluorescent immunolabelling. This allowed multiple labels to be resolved individually and then be combined to provide the details of cell phenotype. Unfortunately, poor penetration of the antibodies into the 60μm thick vibratome sections did not allow direct counting of the labelled cells. However, ratios of immunolabelled neurons expressing certain other markers e.g. calbindin-D_{28k} were used to determine which subpopulation of neurons was susceptible to lesion induced death.

The use of immunolabelling techniques coupled with unbiased counting is clearly an important development in the field of neurostereology and one that will continue to make a major contribution to our understanding of the function of particular cell circuits, brain regions and the entire brain, in both normal and pathological states, in the coming years.

A further extension of the idea of counting very specific cell populations has been published by West et al., (1996) where in situ hybridization was used to label a specific neuronal population, thus providing a link between molecular neurobiology and stereology. West et al., (1996) used the optical fractionator method to estimate the total number of neurons expressing mRNA for somatostatin in the striatum of the rat brain. These cells form a subgroup of the highly variable population of aspiny interneurons that constitute around only 10% of the total striatal neuronal population. The striatum on one side of the brain, in the five rats studied, was found to contain on average 21,300 neurons expressing somatostatin mRNA. It is of interest to note that the methodological

design used in this study would allow a difference of 15% in the group means to be detected as statistically significant. As the somatostatin containing neurons are only a small percentage of the total neuronal population, this very specific labelling allows a change to be detected that may not be detectable when interneurons alone are quantified. The somatostatin containing neurons contain more transmitters and co-transmitters than other striatal neurons suggesting different roles under different physiological conditions (Dawson et al., 1991). Being able to quantify this population of neurons and presumably the other subpopulations, will allow a more rigorous analysis of the circuitry of the striatum to be undertaken as a very important step in understanding the functional circuitry of the striatum in both normal and diseased states.

It cannot be disputed that this paper by West and co-workers (1996) is very exciting and will become a fundamental paper in the development of stereological methods to quantify neuronal populations on the basis of the expression of mRNA. There are however a number of methodological issues that must be considered if stereological investigations are to be undertaken on immuno- and in situ labelled tissue. Such issues and how they may affect the interpretation of data obtained from in situ hybridized tissue, have been discussed thoroughly by West et al., (1996) but I consider it is important to briefly mention some of the major points. Conditions that are optimal for applying optical disectors to a tissue sample e.g. thick sections, at least 20μm thick when mounted, are however, not optimal for hybridizing techniques which generally require relatively thin sections, i.e. 4-20 microns, to ensure that the probe penetrates the full depth of the section, allowing all cells of a particular type within the section to be potentially identifiable. Such sections may also not be suitable for the immunohistochemical labelling of tissue (Petersen et al, 1996). If this methodology is to be used, it must be demonstrated that in fact the probe/label does penetrate the entire depth of the section. It is also important when counting with the optical disector that the particle to be counted can be clearly identified in a consistent manner within the unbiased sampling frame. This is most likely to be the case when the simple form of a cell nucleus is the counting particle. It is therefore important to counterstain in situ/immuno labelled tissue to provide reliable visualization of the nuclei of the specifically labelled cells. There is also a possibility of ambiguity in the definition of the entity being counted in in situ hybridized and immunolabelled cells. The amount of labelling required before a cell is defined as labelled must be specified as this may differ between experimental groups if there has been a change in the antigenicity of some cells in a population or a down-regulation/changed expression of the messenger RNA in other cell populations (Fig. 2). The efficacy of the labelling may vary between preparation, investigator and with the specific label, potentially introducing a significance source of variance. These factors suggest that caution is required in interpreting the reliability of results based on the specific labelling of neurons within a population. It must also be remembered that many proteins, enzymes etc., considered to be specific to a certain cell population may in fact not be specific and that not all cells in a population may contain a particular protein (e.g. see Goto et al., 1986a,b; Schlaepfer, 1987; Lowe and Cox, 1990). It is thus important that any estimate of cell number is conditional on the criteria used for identification (Bjugn and Gundersen, 1993). However, since a decrease in labelled cells may represent a

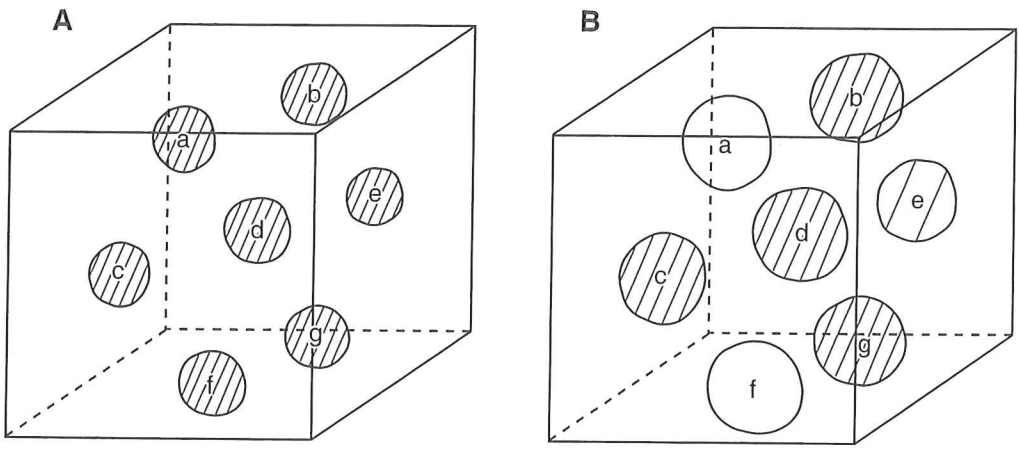

Fig.2.
An illustration of the disector principle using immunohistochemical stained tissue. (A) The diagram shows a block of tissue containing 7 identified particles (e.g. astrocytic glial cells) all of which would fall within the unbiased sampling volume of the disector used in the optical fractionator method. All the particles (glial cells) express glial fibrillary acidic protein (GFAP) ( ) and basic fibroblast growth factor (bFGF) (▨).

(B) The diagram shows that the same 7 identified glial cells still fall within the disector volume but that each individual cell has increased in volume. This gives a greater apparent density of particles emphasizing the importance of using the disector method to determine particle number. All the glial cells express GFAP immunoreactivity but cells a and f do not express bFGF immunoreactivity. Immunolabelling allows quantitation within a subgroup of astrocytic glial cells, while total glial cell number remains constant (see Cintra et al., 1994). Note that particle e shows a small amount of bFGF immunoreactivity. It is important that the degree of labelling required to be defined as immunoreactive must be specified and that labelling efficacy may vary between investigations introducing a potential source of variance.

downregulation of expression of messenger RNA or a change in antigenicity, rather than an actual loss of neurons in a population where the total number of neurons is determined, this methodology does provide some insight into the anatomical basis of observed functional changes. (West et al., 1996).

Irrespective of the interpretation caveats that must be applied to both immunolabelled and in situ hybridization labelled cells, the combination of these labelling techniques with the optical fractionator methods will make a major contribution by enabling the molecular biological basis of nervous system function to be quantified under both normal and pathological conditions. However it is most important that we do not forget the original description of the disector method (Sterio, 1984) based on a comparative analysis of the presence or absence of particles in parallel section planes. It is very likely, as neurobiologists continue to use immunohistochemical and in situ hybridization techniques to identify specific subpopulations of cells in the central nervous system, that the physical disector method will be used on sets of serial thin sections to count labelled cells. This may result in a decrease in efficiency but this may well be counteracted by increased confidence in the penetration of the specific labels and thus in the labelling of cells. Additional stereological estimators can then be applied to this unbiased population of clearly labelled cells.

STEREOLOGICAL METHODS APPLIED TO THE LIVING HUMAN BRAIN.

Normal brain function and pathology must finally be studied and understood in the human brain and the relatively non-invasive methods of imaging, computer assisted tomography and magnetic resonance imaging, allow investigation of human brain morphology to be carried out in living normal and pathological subjects. Stereological methods can be used on this type of image to obtain unbiased estimates of volume (Vogels et al., 1995), and if certain criteria are fulfilled surface areas and thicknesses can also be determined (Pakkenberg et al., 1989; Mayhew and Olsen, 1991). Volume estimation is straightforward as it is not affected by the natural constraints that exist when a brain is imaged in situ, such as the preferred plane and angle of image collection, because the collection of images for volume estimation, must only be uniform random in location in any convenient direction (Gundersen and Jensen, 1987).

Stereological methods have been used to measure volumes of structures from routine brain computerized tomography images in clinical stroke trials (Lyden et al., 1994). Volume changes within a number of compartments of the intracranial space including infarctions, estimated by three observers independently, showed strong inter observer agreement and significant volume changes between the stroke and control groups. The methods were fast, reliable and unbiased and could also be used to obtain prestroke data form 'at risk' patients and to monitor pre and poststroke in clinical trials (Lyden et al., 1994). Stereological methods, namely the Cavalieri method used with point counting techniques, has been used to measure volume changes on MRI scans. Sheline et al., (1996a) used stereological methods to measure frontal lobe volume form MRI scans in 17 adults and then compared these estimates with those obtained using traditional edge tracing methods. The stereological methods, applied by

three independent raters, gave very repeatable results when both inter- and intra-rater results were compared and were more time efficient than conventional edge tracing methods. These methods were also considered to be very suitable for use on small, complex structures such as the hippocampus and may be able to be used to estimate the small differences in frontal lobe volume that are postulated to occur in certain neurologic and psychiatric disorders (Sheline et al., 1996b; Haller et al., 1994). In some circumstances it may be of more value to determine cortical thickness, or this may be the only measurement possible from the images available as the images have not been obtained from slices that are random in slice position and also in 3 dimensional space (isotropic), something that is difficult to obtain in living humans subjects. A study by Mayhew et al., (1996) has however demonstrated using stereological methods to estimate the volumes, surface areas and thickness of the cerebral cortex in a number of mammals, that apparent cortical thickness (measured directly on slices) is a satisfactory estimate of true thickness (estimated from cortical volume divided by the mean of outer and inner cortical thickness). This may be important for medical slice imaging where only apparent local thickness can be obtained but it must be remembered that changes may only be local which may or may not be clinically important. A combination of stereology and MRI has also been used to measure fetal growth and the volume of the brain, liver and lungs in utero (Garden and Roberts, 1996). Fetal movement resulted in some image degradation but this methodology may have clinical applications for monitoring 'at risk' fetuses to detect any abnormalities of fetal growth.

The use of stereological methods to obtain unbiased estimates of volume on living human subjects rather than on post-mortem material, where the issue of tissue shrinkage and its variability with age, brain compartment and disease process is complex, allows us to directly compare data from humans and animal models. A recent study by Harding et al., (1997), using stereological methods on post-mortem material, has demonstrated that the decrease in hippocampal volume seen in alcoholics occurs exclusively in the white matter with no neuronal loss in any of the subregions of the hippocampus. Chronic alcohol abuse also results in a significant loss of white matter in the brain but no loss of neocortical neurons (Jensen and Pakkenberg, 1993). However, neuronal loss has been shown to occur in the hippocampus in a rat model of alcoholism, when unbiased methods were used to quantify the extent of this cell loss (Lundquist et al., 1995). These contradictory findings in the human and rat, question the relevance and in fact the validity of using rodent models of neuronal loss caused by alcoholism or potentially by other disease processes. Stereological studies carried out on post-mortem material to determine total number will be important in validating the extent of cell loss in animal models of human disease processes. However, the use of images obtained by computerized tomography or magnetic resonance imaging techniques, especially as image resolution increases, will be instrumental in our understanding of such processes.

Neurostereological methods are clearly making a very valuable contribution to neuroscience that has grown in diversity and sophistication over recent years. However, the methods that clearly dominate this contribution are methods that have been available for more than a decade; the Cavalieri method, especially as it is applied to living human subjects and the disector method, in its

various forms. The combination of these two methods, or use of the disector principle within the fractionator method, allows reliable quantitation of discrete populations of cells within the central nervous system, increasingly on a functional basis, allowing a more precise understanding of the links between brain function and brain structure to be developed.

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