Journal of Neuroscience Methods 180 (2009) 77–81

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

A systematic random sampling scheme optimized to detect the proportion of rare synapses in the neuropil

Nuno Maçarico da Costa*, Klaus Hepp, Kevan A.C. Martin

Institute for Neuroinformatics, University of Zürich and ETH Zürich, Winterthurerstrasse 190, 8057, Zürich, Switzerland

ARTICLE INFO

Article history:
Received 28 August 2008
Received in revised form 27 February 2009
Accepted 3 March 2009

Keywords:
Synapse counts
Physical disector
Synapse density

ABSTRACT

Synapses can only be morphologically identified by electron microscopy and this is often a very labor-intensive and time-consuming task. When quantitative estimates are required for pathways that contribute a small proportion of synapses to the neuropil, the problems of accurate sampling are particularly severe and the total time required may become prohibitive. Here we present a sampling method devised to count the percentage of rarely occurring synapses in the neuropil using a large sample (~1000 sampling sites), with the strong constraint of doing it in reasonable time. The strategy, which uses the unbiased physical disector technique, resembles that used in particle physics to detect rare events.

We validated our method in the primary visual cortex of the cat, where we used biotinylated dextran amine to label thalamic afferents and measured the density of their synapses using the physical disector method. Our results show that we could obtain accurate counts of the labeled synapses, even when they represented only 0.2% of all the synapses in the neuropil.

1. Introduction

Identifying, describing, and sampling rare events is a problem common to many fields of science. In neuroanatomy, we often have to deal with this problem when we want to know the number of synapses formed by a specific pathway. Synapses can only be identified morphologically with the electron microscope (EM), but analyzing neuropil at the ultrastructural level is so labor-intensive and time-consuming that usually only a small volume of tissue is taken and only a small number of observations are made, and yet the result must be representative of the entire region of study. While the resurgent interest in mapping and quantifying neuronal circuits at the ultrastructural level is leading to high throughput methods to visualize larger samples (Denk and Horstmann, 2004; Micheva and Smith, 2007; Knott et al., 2008), synaptic quantification using more conventional methods also continues to improve through the development of new approaches based on stereological principles (Witgen et al., 2006; West et al., 2008).

Modern unbiased disector methods now provide a solid methodological base for counting a given element, whether cell or synapse. But even with disectors, the difficulties of obtaining accurate counts in a reasonable time are exacerbated when the structures of interest form a very small fraction of the volume of the neuropil, because extensive sampling is required to find sufficient numbers to give accurate counts. Examining large samples takes a prohibitively long time and, in our experience, a large amount of time is invested in photographing, identifying, and counting all the structures of interest (synapses in our case) at the sampling sites.

In order to deal with this problem we developed a strategy inspired by the bubble chamber used to study the interaction between sub-atomic particles. The chamber was photographed by several cameras at high resolution in order to reconstruct completely, in time and in space, the trajectories of the particles. While this produced vast numbers of photographs, the interactions of interest were only present in a small subset of frames. Many observers then scanned through the photographs to find the ones where a specific rare interaction occurred, and these were the only frames where measurements were taken. We face a very similar problem while counting very rare synapses in the neuropil, since the synapses of interest are only present in a very small subset of the ultrathin serial sections.

In the methodology presented in this paper, we use the unbiased disector for counting synapses in large numbers of sampling sites (~1000). We only photograph and make synaptic counts and measurements at sampling sites that have an axon or bouton (in this case labeled with a neuronal tracer) of the particular pathway being investigated. The density of labeled synapses in the neuropil is then calculated by taking into account the volume of all the sampling sites (i.e. photographed and not photographed). We can also calculate the density of all synapses in the neuropil by counting the number of labeled and unlabeled synapses on the photographed

* Corresponding author. Tel.: +41 446353044; fax: +41 446353053.
E-mail address: ndacosta@ini.phys.ethz.ch (N.M. da Costa).

0165-0270/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2009.03.001

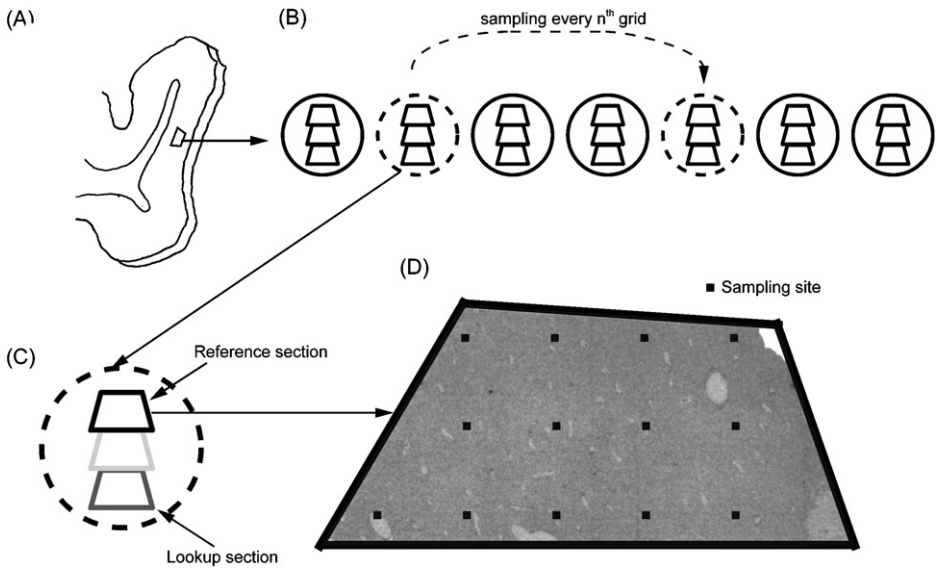

Fig. 1. Systematic random sampling scheme. (A) Drawing of coronal sections from the cat's brain. The trapezoid indicates the sampled region. (B and C) Schematic of copper grids with ultrathin sections. The dashed grids were a systematically random sample for the study. (C) Schematic of ultrathin sections in a copper grid. Unless there was some damage, the first section was always chosen as the reference (black) and the third as the lookup (dark grey). (D) Micrograph of the reference section with superimposed sampling sites represented as black squares.

sampling sites. This allowed us to calculate the proportion of the total synapse population labeled by our tracer.

We selected our sampling sites based on a systematic random sampling (SRS) scheme (Gundersen and Jensen, 1987; Slomianka and West, 2005). We will refer to this method as rare event systematically optimized random sampling (RESORS) throughout this paper. The physical disector method introduced by Sterio (1984) was used to perform the synaptic counts.

As an example, we applied our method to investigate the proportion of labeled thalamic afferent synapses in layer 4 of area 17 of the cat. Since we wanted to test our method in conditions where labeled synapses were very rare events (representing less than 1% of all the synapses), we sampled from regions of layer 4 where we had partially labeled the thalamic input and so only a few labeled boutons were present.

2. Methods

2.1. Surgical procedures

All experiments, animal treatment and surgical protocols were carried out with authorization and under license granted to KACM by the Kantonal Veterinaeramt of Zurich. Surgical procedures are described in Girardin et al. (2002). Thalamic axons were labeled by ionophoretic injections of biotinylated dextran amine (BDA, 10,000) (Molecular Probes, Leiden, Netherlands) in the A lamina of the dLGN. Details of perfusion and histological procedures can be found in Anderson et al. (1998).

2.2. Physical disector

Synapses and associated structures were classified using conventional criteria (Gray, 1959; Colonnier, 1968). The density of asymmetric synapses was estimated using the physical disector method (Sterio, 1984). Reference and lookup sections were separated by one intervening section. The density of synapses (N_V) was calculated using the following formula:

$$N_V = \frac{n}{V_{disector}}$$

where n is the number of synapses counted and $V_{disector}$ is the volume of a single disector.

2.3. Rare event systematically optimized random sampling

We used a systematic random sampling scheme (Fig. 1), but only took actual photographs of sample sites that had a labeled bouton in the reference section. The sites that did not have a labeled profile were noted, but no photograph was taken and no synapses were counted.

The disectors were collected from every nth grid (Fig. 1B). The starting grid was chosen randomly from 1 to "n", using the Matlab "rand" function initialized to a different state every time. On each copper grid the first section was chosen as the reference of the disector (Fig. 1C). A sampling grid indicating the location of the several disectors was then randomly positioned on a low power photograph of the reference section (Fig. 1D). The sampled interval within a section and between grids varied according to the number of sections we had in order that 1000 disector locations covered the entire sampling volume.

The mean density of labeled synapses ($\bar{N}_V^{labeled}$) was calculated using the volume of all the sampling sites, including those that were not photographed (where $n^{labeled}$ was equal to 0, and so was $N_V^{labeled}$). The mean density of all synapses in the neuropil (label and unlabeled, $\bar{N}_V^{all}$) could only be calculated using the sampling sites that were photographed.

Note that when counting unlabeled synapses, both sections can be used both as reference and as lookup in order to double the sample. However, this cannot be done when counting labeled synapses, unless every sampling site is also checked for the presence of a labeled synapse on the lookup section.

2.4. Systematic random sampling

The systematic random sampling scheme used was similar to the RESORS method described above, with the exception that asymmetric synapses were counted every nth disector instead of only sampling in sites with a label profile.

Table 1
Densities of asymmetric synapses in the neuropil using different sampling methods.

Cat (sample)	Disector size	Number of disectors	Density $\times 10^8$ (synapses/mm^3)	Sampling method
2003 (1)	5 μm × 5 μm × 0.12 μm	74	6.6 ± 0.6	RESORS
2003 (2)	5 μm × 5 μm × 0.12 μm	32	6.5 ± 0.9	SRS
0904 (1)	5 μm × 5 μm × 0.12 μm	58	5.1 ± 0.6	RESORS
1804 (1)	5 μm × 5 μm × 0.12 μm	92	5.9 ± 0.5	RESORS
1804 (2)	5 μm × 5 μm × 0.12 μm	46	5.5 ± 0.9	RESORS
1804 (3)	5 μm × 5 μm × 0.12 μm	168	5 ± 0.4	SRS

Table 2
Comparison between different SRS and RESORS samples in the same animal.

Cat2003	
Sample 1 (RESORS) *vs.* sample 2 (SRS)	$p \approx 1$
Cat1804	
Sample 1 (RESORS) *vs.* sample 2 (RESORS)	$p \approx 0.64$
Sample 1 (RESORS) *vs.* sample 3 (SRS)	$p \approx 0.31$
Sample 2 (RESORS) *vs.* sample 3 (SRS)	$p \approx 0.99$

p-Values of the two-sample Kolmogorov–Smirnov test between different SRS and RESORS samples.

3. Results

Sparse projections seem to be a common feature of the neocortex and quantifying their synaptic "weight" in the target area is important. To arrive at this number we need to know the density (or number) of synapses formed by labeled axons as well as the total density (or number) of synapses in the neuropil. Using the methodology proposed in this paper, only the disectors that have a labeled profile are considered for counting and so disectors that fell on blood vessels or cell bodies were not considered. This approach could lead to a slight overestimation of the number of synapses in the sampled region. Moreover, we needed to show that the density of synapses around a labeled profile is not different from any other randomly selected location in the neuropil. In order to test this, we compared the results obtained with RESORS with densities of synapses obtained when the sample of disectors was made using SRS, that is, not biased by the presence of a labeled profile. We then tested whether the densities of synapses calculated by the two methods were indeed the same, as the results given in Table 1 seemed to indicate.

The samples Cat2003 (sample 1) and Cat1804 (samples 1 and 2) were taken using RESORS (from disector locations where there was a labeled bouton). As mentioned above, this could lead to an overestimation of the number of synapses. However, the results in Table 2 show that there is no significant difference between the results obtained with SRS (from disector locations where there was a labeled bouton and disectors sampled unbiasedly from the neuropil). The sample Cat1804 (3), even though not significantly different from the others, had a mean density that was lower than the other samples in this cat. This smaller mean may be due to a slight increase in zero counts, because some of the sampling sites were located over a blood vessel or a cell body. If these disectors with zero counts are removed, the mean density in Cat1804 (3) rises to 5.4×10^8 synapses/mm^3. This was not an issue for Cat2003.

If it is not known whether the density of labeled synapses around a labeled profile is the same as the average density in the neuropil, it is prudent to make a control study like the one presented here, in at least some of the animals. This control can be easily combined with RESORS, by simultaneously using an SRS scheme on the same material and using the same counting grid.

3.1. Using optimized systematic random sampling for rare events to calculate the proportion of labeled synapses

To test the method in a situation where label profiles are sparse, we deliberately chose a sample region where we have labeled a small proportion of the thalamic afferents (therefore the numbers presented in this study do not reflect in any way the total contribution of thalamocortical synapses in the primary visual cortex).

In Table 3 we show the results from four sections taken from three animals. The lowest density of labeled synapses for which we have tested our sampling method was 1.03×10^6 synapses/mm^3, and this represents 0.2% of all the synapses in the sampled volume. Detecting such small densities of synapses at the magnifications required to identify and classify synapses (~20,000×) is a daunting undertaking for both systematic and uniform random sampling. In the case of Cat1804 (2) a labeled synapse disappeared from the reference to the lookup section only in 3 out of 999 sampling sites. We would also like to note that overall there was a labeled profile in only 5–10% of sampling sites and of the labeled profiles, only a small proportion (4–16%) formed synapses that disappeared from the reference to the lookup section. In the remainder, the synapse could be in any one of a number of conditions: still present in the lookup section, located outside the counting frame (Figs. 2 and 3), or hitting one of the forbidden edges of the counting frame.

4. Discussion

We have presented a simple method for efficiently counting the number of synapses in the neuropil. This method is especially time-saving, in that it can detect very low densities of labeled profiles without resorting to prohibitively high numbers of disectors. This is of major importance in cases where the axons under investigation contribute very few synapses to the region of study.

4.1. Physical disector

We used the physical disector method (Sterio, 1984) to count synapses, but because we need to find the equivalent location in the lookup section and photograph it, this method is more time consuming than counting synapses in single sections (Beaulieu and Colonnier, 1985). However, counting synapses in single sections is a biased method and, several assumptions need to be applied for the

Table 3
Density of synapses using RESORS. The values are in synapse/mm^3.

Cat (sample)	Density of labeled synapses mean ± SEM (*n* disectors)	Density of unlabeled synapses mean ± SEM (*n* disectors)	Percentage of labeled synapses
Cat2003 (1)	$1.34 \times 10^6 \pm 0.67 \times 10^6$ (992)	$6.54 \times 10^8 \pm 0.48 \times 10^8$ (106)	0.21
Cat0904 (1)	$2.98 \times 10^6 \pm 1.21 \times 10^6$ (670)	$5.06 \times 10^8 \pm 0.57 \times 10^8$ (58)	0.59
Cat1804 (1)	$5.24 \times 10^6 \pm 1.34 \times 10^6$ (953)	$5.87 \times 10^8 \pm 0.49 \times 10^8$ (92)	0.89
Cat1804 (2)	$1.03 \times 10^6 \pm 0.58 \times 10^6$ (999)	$5.51 \times 10^8 \pm 0.9 \times 10^8$ (46)	0.19

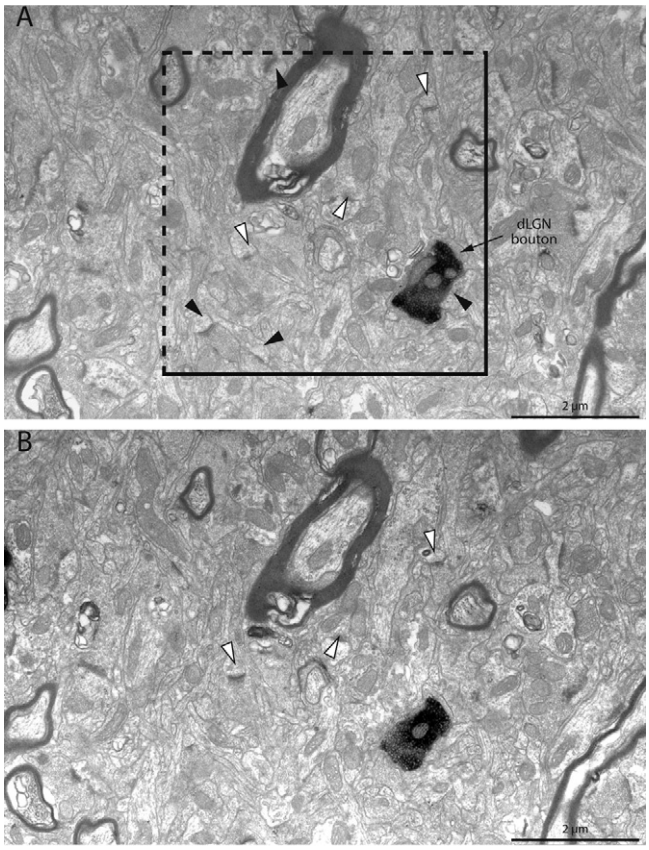

Fig. 2. Electron micrographs used for a disector. (A) Reference section with labeled bouton forming a synapse. The disector frame is show in black, the solid lines represent the forbidden edges and the dashed lines the acceptance edges. Synapses disappearing from the reference to the lookup section are indicated by black arrowheads. Synapses present in both sections are indicated by white arrowheads. (B) Lookup section.

estimation of density (review by Mayhew, 1996). On the other hand, the only requirement of the disector method is that objects can be identified unequivocally in both sections. The physical disector is also unbiased for particle size, shape and orientation, even though it does not completely avoid the problem of "lost caps" (reviewed in Geinisman et al., 1996; Mayhew, 1996; Mayhew and Gundersen, 1996; Howard and Reed, 2005). As pointed out by Guillery and Herrup (1997), the disector method is also not completely free of assumptions, for example, it assumes that the sections are of uniform size and that the top and bottom of the sections are smooth planes. The section size problem can be dealt with by measuring sections with more sophisticated sampling strategies (Mayhew and Gundersen, 1996). It has also been argued that both methods, the disector and the model-based method used by Beaulieu and Colonnier (1985) produce similar results, with the latter being more efficient and with less variability in the results (DeFelipe et al., 1999). Others have found the disector method to be more efficient, even though it did produce similar results to that of single section methods (de Groot and Bierman, 1986; Calverley et al., 1988).

One important factor in calculating densities with the disector method is the volume of the sample. With the RESORS method the density of labeled synapses is compared with unlabeled synapses from the same sections, and so section thickness is not a problem.

4.2. Sampling of disectors

When tested in the same animal, all the disector sampling methods gave results that were not significantly different from each other. This was true even in cases where the location of the disector was biased by selecting sampling sites that contained a labeled thalamic bouton, or where an overestimation of synapse density was expected, simply because disectors that contain a blood vessel or a cell body were excluded. There is some indication of such overestimation in only one case, even though the difference does not reach statistical significance. This suggests that finding a blood vessel or a cell body in a disector is also a rare event.

4.3. Accuracy of assessing rare events

In one of the datasets investigated, only three labeled synapses that disappeared from the reference to lookup section were found in 999 disectors. Photographing, searching for the location of the counting site in the lookup section, and counting synapses in 999 disectors would have taken several months work, even with the efficient disector method, but with the method presented here, this was reduced to a few weeks. Clearly 3 hits out of 999 is still a very low rate and this is of course reflected in the reduced accuracy of the mean and on a high SEM. However, when we look at the SEM (0.58×10^6 synapses/mm^3) as a percentage of the density of synapses in the neuropil, it is just above 0.1%. In order to obtain a more precise measurement, more samples from the same

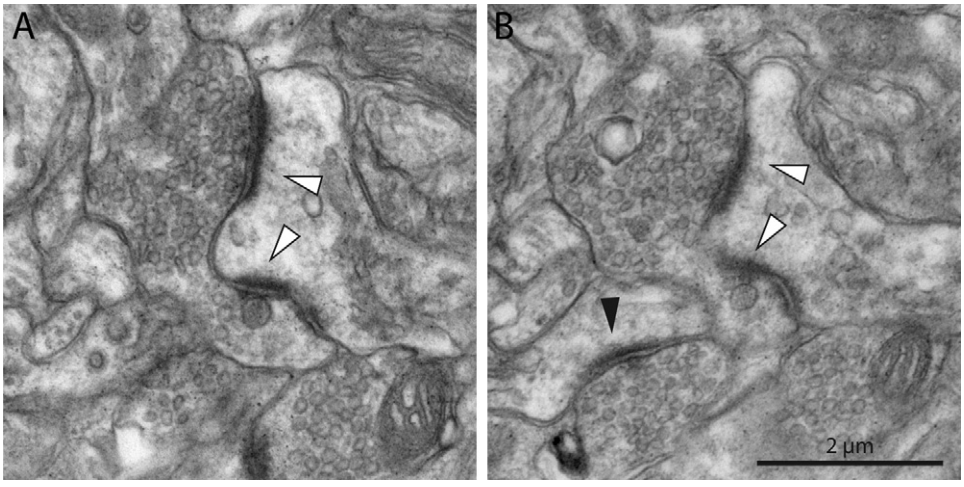

Fig. 3. Electron micrographs showing synapses in a disector. A synapse disappearing from the reference section (A) to the lookup section (B) is indicated by a dark arrowhead. Synapses present in both sections are indicated by white arrowheads.

portion of the tissue could be taken. However, since the density of synapses in the neuropil is already known, the sampling could focus on sites where a labeled synapse disappears from the reference to the lookup section and so avoid photographing sampling sites (and counting synapses) where there is a labeled profile, but no disappearing synapse.

In stereological studies using SRS it is common to estimate what proportion of the observed variance is generated by the sampling method. This is done by calculating the precision of the stereological procedure, the coefficient of error (CE), and then comparing it with the variance in the results (discussed in Slomianka and West, 2005). The usefulness of the CE in a sampling scheme, like the one presented here, is limited. Given that we are counting very rare events, the variance is inevitably large and, even when the CE is large, it represents a small proportion of the observed variance. Moreover, most sections have no disappearing synapses and so both consecutive and alternate sections tend to have the same number of counts. This will lead to a small CE using some of the most common methods (Gundersen et al., 1999; Cruz-Orive and Geiser, 2004; Slomianka and West, 2005). Sample sizes that produce a more meaningful CE will have to be much larger and for each case a decision would have to be made between the workload and the need to estimate the contribution of the sampling scheme to the observed variance.

4.4. Conclusion

That counting is a time-consuming and often onerous task is a strong disincentive to do quantitative anatomy. However, in studies of neural circuits quantification is increasingly an essential component and thus any method that shortens the time spent in counting will increase the likelihood of these important data being produced. RESORS is a time-saving method for sampling low densities of identified synapses in EM data without compromising the accuracy and quality of the data.

Acknowledgments

We thank John Anderson for his helpful comments on the manuscript. We also thank Rita Bopp and German Koestinger for their expert technical assistance. This work was supported by EU Daisy grant FP6-2005-015803 an EU SECO grant 216593 to K.A.C.M. N.M.C. was a fellow from Fundação para a Ciencia e Tecnologia in the Gulbenkian PhD Program in Biology and Medicine grant SFRH/BD/2724/2000.

References

Anderson JC, Binzegger T, Martin KA, Rockland KS. The connection from cortical area V1 to V5: a light and electron microscopic study. J Neurosci 1998;18:10525–40.
Beaulieu C, Colonnier M. A laminar analysis of the number of round-asymmetrical and flat-symmetrical synapses on spines, dendritic trunks, and cell bodies in area 17 of the cat. J Comp Neurol 1985;231:180–9.
Calverley RK, Bedi KS, Jones DG. Estimation of the numerical density of synapses in rat neocortex. Comparison of the 'disector' with an 'unfolding' method. J Neurosci Methods 1988;23:195–205.
Colonnier M. Synaptic patterns on different cell types in the different laminae of the cat visual cortex. An electron microscope study. Brain Res 1968;9:268–87.
Cruz-Orive LM, Geiser M. Estimation of particle number by stereology: an update. J Aerosol Med 2004;17:197–212.
de Groot DM, Bierman EP. A critical evaluation of methods for estimating the numerical density of synapses. J Neurosci Methods 1986;18:79–101.
DeFelipe J, Marco P, Busturia I, Merchan-Perez A. Estimation of the number of synapses in the cerebral cortex: methodological considerations. Cereb Cortex 1999;9:722–32.
Denk W, Horstmann H. Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. PLoS Biol 2004;2:e329.
Geinisman Y, Gundersen HJ, van der Zee E, West MJ. Unbiased stereological estimation of the total number of synapses in a brain region. J Neurocytol 1996;25:805–19.
Girardin CC, Kiper DC, Martin KA. The effect of moving textures on the responses of cells in the cat's dorsal lateral geniculate nucleus. Eur J Neurosci 2002;16:2149–56.
Gray EG. Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. J Anat 1959;93:420–33.
Guillery RW, Herrup K. Quantification without pontification: choosing a method for counting objects in sectioned tissues. J Comp Neurol 1997;386:2–7.
Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. J Microsc 1987;147:229–63.
Gundersen HJ, Jensen EB, Kieu K, Nielsen J. The efficiency of systematic sampling in stereology—reconsidered. J Microsc 1999;193:199–211.
Howard CV, Reed MG. Unbiased stereology. Oxon: Garland Science/BIOS Scientific Publishers; 2005.
Knott G, Marchman H, Wall D, Lich B. Serial section scanning electron microscopy of adult brain tissue using focused ion beam milling. J Neurosci 2008;28:2959–64.
Mayhew TM. How to count synapses unbiasedly and efficiently at the ultrastructural level: proposal for a standard sampling and counting protocol. J Neurocytol 1996;25:793–804.
Mayhew TM, Gundersen HJ. If you assume, you can make an ass out of u and me': a decade of the disector for stereological counting of particles in 3D space. J Anat 1996;188(Pt 1):1–15.
Micheva KD, Smith SJ. Array tomography: a new tool for imaging the molecular architecture and ultrastructure of neural circuits. Neuron 2007;55:25–36.
Slomianka L, West MJ. Estimators of the precision of stereological estimates: an example based on the CA1 pyramidal cell layer of rats. Neuroscience 2005;136:757–67.
Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 1984;134(Pt 2):127–36.
West MJ, Bach G, Soderman A, Jensen JL. Synaptic contact number and size in stratum radiatum CA1 of APP/PS1DeltaE9 transgenic mice. Neurobiol Aging 2008.
Witgen BM, Grady MS, Nyengaard JR, Gundersen HJ. A new fractionator principle with varying sampling fractions: exemplified by estimation of synapse number using electron microscopy. J Microsc 2006;222:251–5.