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

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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 dissectors, 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 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.
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:

$$N_v = \frac{n}{V_{\text{disector}}}$$

where $n$ is the number of synapses counted and $V_{\text{disector}}$ 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 $n$th 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 ($\bar{N}_{\text{labeled}}$) was calculated using the volume of all the sampling sites, including those that were not photographed (where $n_{\text{labeled}}$ was equal to 0, and so was $N_{\text{labeled}}$). The mean density of all synapses in the neuropil (label and unlabeled, $\bar{N}_{\text{all}}$) 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 $n$th disector instead of only sampling in sites with a label profile.
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 $1.03 \times 10^5$ 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 ($\sim 20,000\times$) 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 dissectors. 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 1

<table>
<thead>
<tr>
<th>Cat (sample)</th>
<th>Disector size</th>
<th>Number of disectors</th>
<th>Density $\times 10^6$ (synapses/mm$^3$)</th>
<th>Sampling method</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003 (1)</td>
<td>5 $\mu$m $\times$ 5 $\mu$m $\times$ 0.12 $\mu$m</td>
<td>74</td>
<td>$6.6 \pm 0.6$</td>
<td>RESORS</td>
</tr>
<tr>
<td>2003 (2)</td>
<td>5 $\mu$m $\times$ 5 $\mu$m $\times$ 0.12 $\mu$m</td>
<td>32</td>
<td>$6.5 \pm 0.9$</td>
<td>SRS</td>
</tr>
<tr>
<td>0904 (1)</td>
<td>5 $\mu$m $\times$ 5 $\mu$m $\times$ 0.12 $\mu$m</td>
<td>58</td>
<td>$5.1 \pm 0.6$</td>
<td>RESORS</td>
</tr>
<tr>
<td>1804 (1)</td>
<td>5 $\mu$m $\times$ 5 $\mu$m $\times$ 0.12 $\mu$m</td>
<td>92</td>
<td>$5.9 \pm 0.5$</td>
<td>RESORD</td>
</tr>
<tr>
<td>1804 (2)</td>
<td>5 $\mu$m $\times$ 5 $\mu$m $\times$ 0.12 $\mu$m</td>
<td>46</td>
<td>$5.5 \pm 0.9$</td>
<td>RESORS</td>
</tr>
<tr>
<td>1804 (3)</td>
<td>5 $\mu$m $\times$ 5 $\mu$m $\times$ 0.12 $\mu$m</td>
<td>168</td>
<td>$5 \pm 0.4$</td>
<td>SRS</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Cat2003</th>
<th>Sample 1 (RESORS) vs. sample 2 (SRS)</th>
<th>p = 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1 (RESORS) vs. sample 2 (SRS)</td>
<td>p = 0.64</td>
<td></td>
</tr>
<tr>
<td>Sample 1 (RESORS) vs. sample 3 (SRS)</td>
<td>p = 0.31</td>
<td></td>
</tr>
<tr>
<td>Sample 2 (RESORS) vs. sample 3 (SRS)</td>
<td>p = 0.99</td>
<td></td>
</tr>
</tbody>
</table>

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

Table 3

<table>
<thead>
<tr>
<th>Cat (sample)</th>
<th>Density of labeled synapses mean $\pm$ SEM (n dissectors)</th>
<th>Density of unlabeled synapses mean $\pm$ SEM (n dissectors)</th>
<th>Percentage of labeled synapses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat2003 (1)</td>
<td>$1.34 \times 10^5 \pm 0.67 \times 10^5$ (992)</td>
<td>$6.54 \times 10^5 \pm 0.48 \times 10^5$ (106)</td>
<td>0.21</td>
</tr>
<tr>
<td>Cat2004 (1)</td>
<td>$2.98 \times 10^5 \pm 1.21 \times 10^5$ (670)</td>
<td>$5.06 \times 10^5 \pm 0.57 \times 10^5$ (58)</td>
<td>0.59</td>
</tr>
<tr>
<td>Cat1804 (1)</td>
<td>$5.24 \times 10^5 \pm 1.34 \times 10^5$ (953)</td>
<td>$5.87 \times 10^5 \pm 0.49 \times 10^5$ (92)</td>
<td>0.89</td>
</tr>
<tr>
<td>Cat1804 (2)</td>
<td>$1.03 \times 10^5 \pm 0.38 \times 10^5$ (999)</td>
<td>$5.51 \times 10^5 \pm 0.9 \times 10^5$ (46)</td>
<td>0.19</td>
</tr>
</tbody>
</table>
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 × 10⁶ synapses/mm³) 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
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.

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