A simple and efficient alternative to implementing systematic random sampling in stereological designs without a motorized microscope stage

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Summary
When properly applied, stereology is a very robust and efficient method to quantify a variety of parameters from biological material. A common sampling strategy in stereology is systematic random sampling, which involves choosing a random sampling relevant objects start point outside the structure of interest, and sampling at sites that are placed at pre-determined, equidistant intervals. This has proven to be a very efficient sampling strategy, and is used widely in stereological designs. At the microscopic level, this is most often achieved through the use of a motorized stage that facilitates the systematic random stepping across the structure of interest. Here, we report a simple, precise and cost-effective software-based alternative to accomplishing systematic random sampling under the microscope. We believe that this approach will facilitate the use of stereological designs that employ systematic random sampling in laboratories that lack the resources to acquire costly, fully automated systems.

Introduction
Stereology is being increasingly recognized as a very efficient method to obtain robust quantitative data from sectioned biological materials. This approach is based on sound mathematical and statistical sampling foundations, facilitating both theoretically unbiased and precise estimates of biologically important parameters such as cell number (Mouton, 2002; Baddeley & Vedel-Jensen, 2005; Howard & Reed, 2005).

The efficiency of stereology is a consequence of the sampling scheme employed (Gundersen & Jensen, 1987; Gundersen et al., 1999); specifically, the use of systematic random sampling (SRS) (Mouton, 2002; Baddeley & Vedel-Jensen, 2005; Howard & Reed, 2005). SRS involves beginning the sampling process with a random start point, after which subsequent sampling points occur at pre-determined, equally spaced intervals. Although both simple random sampling and SRS can obtain precise estimates, SRS designs tend to converge on the true value of the estimated parameter with much less effort than simple random sampling (Mouton, 2002). In fact, the variance of a sample mean tends to be proportional to \(1/n\) for \(n\) independent samples, but \(1/n^2\) for \(n\) samples obtained using SRS (West et al., 1991). Thus, less sampling is required to obtain a given level of precision using SRS.

Theoretical unbiasedness in stereology is accomplished through the use of geometric probes that ensure that size, shape, and orientation do not influence the estimate (Mouton, 2002; Howard & Reed, 2005). In the case of obtaining theoretically unbiased estimates of the total number of cells in a particular structure, the disector probe was introduced (Sterio, 1984). This three-dimensional probe, with its associated counting rules, allows the estimation of cell number within a defined volume in such a way that the estimates obtained are proportional only to absolute number, without the influence of cell size, shape, and orientation. Placing disector sampling sites across a given tissue section in an SRS manner thus facilitates a theoretically unbiased and efficient method to estimate total cell number in a given structure. A common stereological design used to obtain total cell number estimates is the optical fractionator (West et al., 1991). This technique is based on the use of the optical disector as a counting probe, and a fractionator-sampling scheme, which takes sub-samples...
of the entire structure of interest (referred to as the reference space) in an SRS fashion.

SRS at the microscopic level involves beginning the sampling process with a random start point outside the relevant reference space, after which subsequent sampling sites occur at pre-determined, equally spaced intervals (Mouton, 2002; Howard & Reed, 2005). This results in a raster-like pattern of sampling sites across the tissue section, covering the structure of interest. In practical terms, this generally involves the use of a motorized stage that can move in both the x and y planes with high precision and reproducibility (Glaser & Glaser, 2000). In combination with motorization in the z plane, the optical fractionator can be conducted very efficiently.

One practical limitation of such set-ups is their cost. An automated system, complete with integrated hardware and software to accomplish SRS, can cost tens of thousands of dollars. This puts the implementation of such systems out of reach for many laboratories. Here, we report a software-only-based approach to achieving SRS at the microscopic level, thereby avoiding the expense of a motorized stage.

The software and its capabilities

The software was written in C++, using the Microsoft Visual C++ 6.0 integrated development environment. This environment was chosen because of its compatibility with our camera’s software development kit. The QCam software development kit was used to obtain live images from the QImaging Retiga EXi 12-bit monochrome camera (QImaging, Burnaby, British Columbia, Canada). WxWidgets, a cross-platform Graphic User Interface and tool library, was also used in order to create the user interface (http://www.wxwidgets.org).

A one-time measurement of a stage micrometer under each objective allows for the calibration of the number of pixels per unit distance. This information is then applied to the relevant magnification manually via a drop-down menu. The user can set the grid area at any value (the step size lengths along x and y axes is then calculated as the square root of this value). Once these values are set, the grid system can either be thrown randomly or placed at a particular desired location. In our particular system, we have added additional features that facilitate the use of the optical fractionator, such as the ability to apply disector probes of a defined size, as well as auto exposure controls. As these aspects are not inherent to the method described in this paper, we will not discuss the details of these features further.

The principle of this software is very simple. The sampling begins by having the software display a live image of the tissue section on a computer monitor (Fig. 1A). The size of the sampling grid can be set and thrown randomly across the image. After choosing a particular grid area (Fig. 1A, arrow), which is the product of the x and y step sizes (Fig. 1A, arrowhead), the user can then switch to a higher magnification that can be used, if necessary, for counting. The relevant objective can then be selected from a drop-down menu so that the original grid size is now applied to the new magnification (Fig. 1A, concave arrow). Notice that, in the illustrated example, our x and y step sizes were 100 µm, corresponding to a grid area of 10 000 µm². Within the live field of view, the user simply outlines some distinct features (Fig. 1B, arrowheads) using the incorporated drawing tools (Fig. 1B, arrow). Then, by clicking a button in the chosen direction, in our example, left (Fig. 1C, arrow), the tracings are moved left by the exact amount set in the x step size (Fig. 1C, arrowheads). Note that movements in all four compass points are possible in our system. The user then simply moves the stage manually in the required direction until the originally traced elements fall back under the displaced tracings (Fig. 1D, arrowheads). Repeated over the total area of the reference space, a raster-like SRS pattern can be accomplished with ease.

Applications

Several stereological methods employ SRS as a critical element. For many experiments, determination of the number of particles (i.e. cells) is often the desired objective, for which the optical fractionator is often an ideal choice. However, other important biological parameters, which also employ an SRS design, can also be estimated stereologically. For example, the nucleator estimates cell volume by using SRS in combination with disector probes to indicate which cells are selected to measure (Gundersen, 1998; Howard & Reed, 2005); those cells that fall within the disector and fulfil the rules for inclusion are selected for volume estimation. This results in a number-weighted average of cell volume within a particular reference space (Howard & Reed, 2005).

The limitations of the approach

The method described in this paper represents a valid alternative to implementing SRS using a non-motorized microscope stage. Of course, we do not mean to imply that our system is equally as efficient as fully automated systems, but we feel that in a wide variety of stereological designs, it represents a much less costly alternative. Using our system, we can quantify the number of granule cell neurons in 10 adult dentate gyrus hemisections in 6–8 h with a coefficient of error of less than 6%. This time scale, we believe, is not unreasonable, given that users with fully automated systems can take 3–4 h per structure (Keuker et al., 2001). Other researchers who choose to count more than the generally quoted 100–200 particles in a fractionator experiment can spend one full day per structure (Schmitz & Hof, 2000). Even though our approach is less efficient than fully automated methods, we believe that the extra time required is not prohibitive, given the benefits that SRS affords.
Fig. 1. A software-based approach for achieving SRS across live microscopic images. Sampling begins by choosing a grid area (A, arrow) that has been calibrated to the relevant objective (A, concave arrow). Note that the grid area determines the step sizes in the $x$ and $y$ planes (A, arrowhead). The grid is then thrown randomly across the image by hitting the button labelled ‘random’. Using the incorporated drawing tool (B, arrow), some cells can be traced by using the mouse (B, arrowheads). The tracings can then be displaced in the desired direction (left, in this case: arrowheads in C), by hitting the relevant direction button (C, arrow). Finally, the stage can then be moved manually until the originally traced objects fall back under the tracings. This results in a movement of the specimen by 100 μm to the right across the $x$ axis.
One obvious limitation of our approach is when the required step sizes exceed the dimensions in the field of view. Of course, this will depend primarily on the objective being used. Even when using a 100× objective, we get a y-axis field of view of about 100 µm, and using a 63× objective, about 168-µm step sizes that are not unrealistic in many studies. If a larger step size is needed, then the user would have to trace and move the stage two or more times to accommodate the interval. However, in our experience, stepping twice in any given direction to land on a single counting site adds very little extra time, and is thus still feasible.

Discussion

We describe a simple solution to achieving SRS at the microscopic level without the aid of a motorized stage. This approach involves relatively simple software that is designed to trace distinct features of a live video image in a given field of view. The tracing is then shifted in the required direction by the specified step size. The investigator then simply moves the stage manually until the traced objects fall back into register with the original tracing.

This method of conducting SRS is highly reliable, efficient and, importantly, cost-effective. In the authors’ experience, many investigators are aware of the advantages of stereological designs such as the optical fractionator, but simply do not have the financial resources to obtain commercially available systems that automate the SRS component. We believe that this simple software approach will encourage other researchers to use stereology as a standard quantitative technique.

Other methods of achieving SRS at the microscope without a motorized stage have been published (e.g. Kaplan et al., 2001). This study employed the use of dial indicators attached to the stage of the microscope to measure stage displacement in the x and y axes. Although not necessarily better for all users (e.g. in designs requiring longer step sizes), we believe that our system has some advantages over that described by Kaplan and colleagues (2001). To begin with, our system does not require the addition of any hardware components to the microscope; such elements could be rather cumbersome to mount and work around on many microscopes. Also, because mechanical devices are used, regular monitoring and possibly calibration would be critical to ensure their accuracy and reproducibility. Although the authors claim that no stage backlash was experienced in their system, backlash is never an issue in our software-based system, as one can always land very accurately on the required location simply by ensuring the alignment of the displaced tracings with the originally traced components. Thus, with our system, the accuracy of the step size can be immediately confirmed visually.

The software system we describe in this paper allows the user to benefit from the SRS design, without the need for expensive hardware such as a motorized microscope stage. A system with the same essential features described here is relatively straightforward for someone with computer programming knowledge to develop for their particular hardware components. In conjunction with a stage that is motorized in the z plane (common on many research microscopes currently sold by major manufacturers), the optical fractionator can be employed to efficiently and precisely estimate the total number of objects within a given structure.

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References