

SHTEREOM I SIMPLE WINDOWS[®] BASED SOFTWARE FOR STEREOLOGY. VOLUME AND NUMBER ESTIMATIONS

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ABSTRACT

Stereology has been earlier defined by Wiebel (1970) to be: “a body of mathematical methods relating to three dimensional parameters defining the structure from two dimensional measurements obtainable on sections of the structure.” SHTEREOM I is a simple windows-based software for stereological estimation. In this first part, we describe the implementation of the number and volume estimation tools for unbiased design-based stereology. This software is produced in Visual Basic and can be used on personal computers operated by Microsoft Windows[®] operating systems that are connected to a conventional camera attached to a microscope and a microcator or a simple dial gauge. Microsoft NET Framework version 1.1 also needs to be downloaded for full use. The features of the SHTEREOM I software are illustrated through examples of stereological estimations in terms of volume and particle numbers for different magnifications (4X–100X). Point-counting grids are available for area estimations and for use with the most efficient volume estimation tool, the Cavalieri technique and are applied to Lizard testicle volume. An unbiased counting frame system is available for number estimations of the objects under investigation, and an on-screen manual stepping module for number estimations through the optical fractionator method is also available for the measurement of increments along the X and Y axes of the microscope stage for the estimation of rat brain hippocampal pyramidal neurons.

Keywords: number estimation, morphometry, stereology, stereology software, volume estimation.

INTRODUCTION

In 1970, Weibel defined stereology as “a body of mathematical methods relating to three dimensional parameters defining the structure from two dimensional measurements obtainable on sections of the structure”. Three-dimensional estimations can be made with two approaches, namely “model-based” and “design-based”. Design-based techniques in stereology are unbiased since all parts of the material under investigation have an equal chance of being sampled. In design-based stereology, precise and accurate results are obtained through well-defined and efficient rules, regardless of the shape and organization of the structure under study while model based methods need too many measurements in order to obtain a result, which may be highly unreliable (Gundersen and Jensen 1987; Gundersen *et al.*, 1988a,b; Cruz-Orive and Weibel 1990; Howard and Reed, 2005). Thus, after an unbiased sampling procedure, two-dimensional measurements are obtained through the sectioning of the object under investigation (Oğuz, 2001).

For morphometric purposes in stereology, in order to perform two-dimensional measurements, different grids printed on transparencies can be placed on a personal computer (PC) monitor having a histological view from microscope ocular or the view can be reflected onto a table in a dark room and transparencies can also be placed on the two-dimensional view. There have been several methods described that operate either on a PC monitor (Krekule and Gundersen, 1989; Moss *et al.*, 1989) or on a video display (Krekule *et al.*, 1995). To support these methods, there are presently several image analysis software products on the market. After the description of both biased and manual conventional methods (Gundersen *et al.*, 1981; Mathieu *et al.*, 1981; Bonnet *et al.*, 1991; Barba *et al.*, 1992; Krekule and Saxl, 1994; Zachařová and Kubínova, 1995), a system known as the STESYS software (Karen *et al.*, 1998) was also reported as a digital design-based system. The main disadvantage of the STESYS system, however, was that it could not work on live images and that it needed already taken images. A later version of STESYS named STESYS 2 lacks an unbiased counting frame module.

Here, we introduce this simple Microsoft Windows®-based software named SHTEREOM I that can be used for stereological volume and particle number estimations. The software operates on live images that are transferred onto a 17-inch LCD monitor (Samsung SyncMaster 710v) via a CCD camera (VITEC, VCC - 3277, 1/3, PAL System, High Resolution, Taiwan) from an investigation microscope (Olympus, BX 51, Japan), where a dial gauge is used to measure the movements of the stage in the plane of the Z axis (Fig. 1).

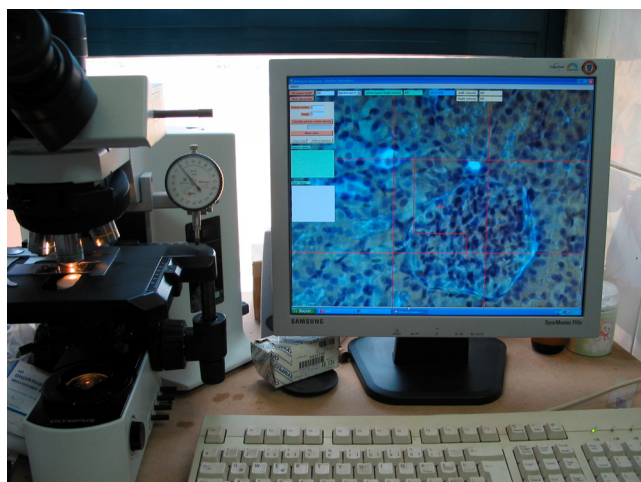


Fig. 1. *SHTEREOM System including LCD monitor, computer and a dial gauge attached OLYMPUS BX 51 Microscope.*

DESCRIPTION OF SHTEREOM I

SHTEREOM I is a compact, menu driven, overlaid programme produced in Visual Basic. The current modules and further modules under development are shown in the screen dump of the initial module selector window (Fig. 2). It can be run on any IBM compatible PC by Windows XP Operating System. The compiled code of SHTEREOM (Version 1.0) is of 2,800 Kb, and Microsoft NET Framework version 1.1 should be downloaded from the web. The software can be operated using keyboard and mouse, the point grid can be operated using a keyboard, and the unbiased counting frame and stepping module can be handled and operated using a mouse. Due to the convenience

of a larger screen, the software is already calibrated for a 17-inch LCD monitor (in micrometers), according to the magnification of the ocular used.

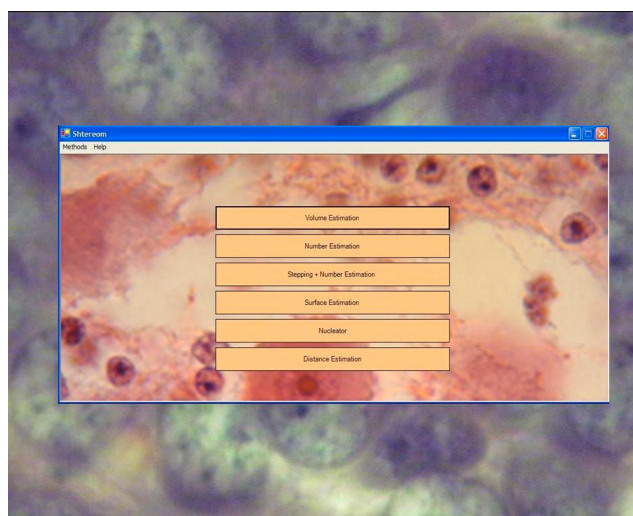


Fig. 2. *Main menu of SHTEREOM on computer monitor.*

CALIBRATION & TEST SYSTEMS

One system unit is used to scale the SHTEREOM I test systems: micrometer. The calibration procedure is initially set up by the user according to the relationships between the units and the size of the test system window, although the calibration of the system can be updated at any time before an investigation.

Three test systems are available: point grids (Fig. 3a), an unbiased counting frame (UCF) system (Fig. 4a) and a stepping plus unbiased counting frame system (Fig. 5a). These can all operate on both live and stored images (*e.g.*, TIFF, JPEG, BMP files). The test systems can be operated as full-screen only. The two-dimensional image is brought to the computer screen by either a frame capturing or a simple TV card.

The appearance of the full menu is optional; it can be hidden partly at any time so that a larger view is obtained for the counting. The image plus the whole test system can be exported at any time. The colours of the test systems (*e.g.*, of the crosses, UCF and X-Y step lines) can also be changed according to the colour of the material under investigation.

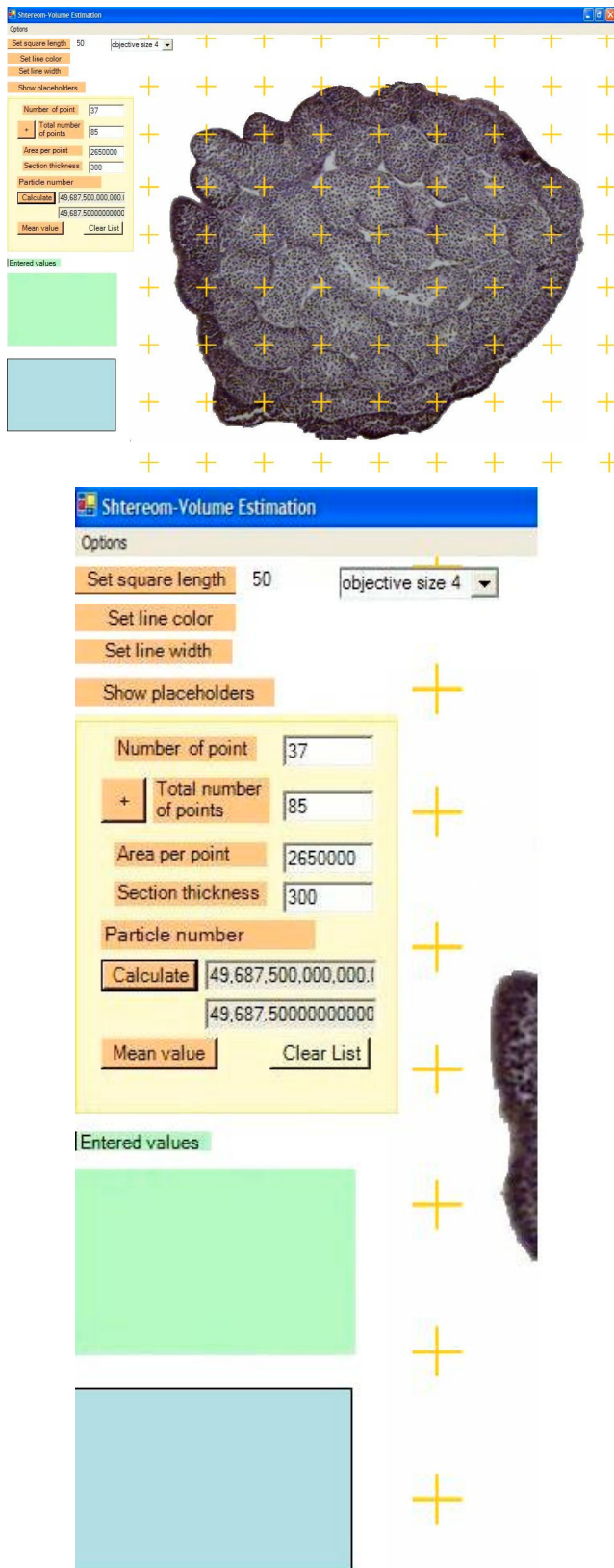


Fig. 3. a) Uniform point grid generated by SHTEREOM. b) Enlarged view of left of Fig. 3a showing data entry boxes and calculated results display boxes with objective size selector drop down menu.

APPLICATIONS OF SHTEREOM I

Volume Estimation

The Cavalieri principle for volume estimation (Cavalieri, 1635; Gundersen and Jensen, 1987) is a particularly old but well-known technique (Fig. 3a).

Volume Fraction Estimation

The volume of a sub-compartment in a material can be estimated by using the stereological ratio-estimator volume fraction (volume density). This tool can also be used to estimate the volumes of more than one sub-compartment in a material. Here, we can use points for the estimation of the subcompartment areas (Chalkley, 1943). Thus, a quadratic grid of points can be laid over systematic uniform random sections of the material under study, again, with the sampling of uniform random fields.

SHTEREOM requires the setting of the horizontal and vertical distances between test points, in micrometers. Data can be manually entered and summed in the data entry boxes on the upper left side of the screen, and with the height of the section known, the final calculations of volume estimation, mean volume estimation and volume fraction estimation of sub-compartments of the tissue under investigation can be made according to the magnification indicated by the objective size on the drop down menu (Fig. 3b).

As an example, Lizard total testis volume and seminifer tubuli volume through volume fraction can be estimated by our system (Fig. 3a).

Number estimations by optical disector

The number of objects within a reference volume can be estimated using the Disector Technique (Sterio, 1984). The use of an optical disector provides certain advantages (Braendgaard and Gundersen., 1986; Braendgaard *et al.*, 1990), which include the thicker histological sections that can be used, allowing an embedding medium to be used, such as metacrylate resin, where there is minimal shrinkage. Optical sectioning continues with the Unbiased Counting Frame (UCF) (Fig. 4a). For the counting of objects, the Gundersen 2-D counting rule (1977) and the UCF are used.

For SHTEREOM, the setting of the UCF area needs to be done in micrometers, with height of the disector and the particle numbers being entered. The numerical density can then be seen on the data display on the left of the screen, step by step, according to the magnification set on the objective size drop down menu (Fig. 4a,b).

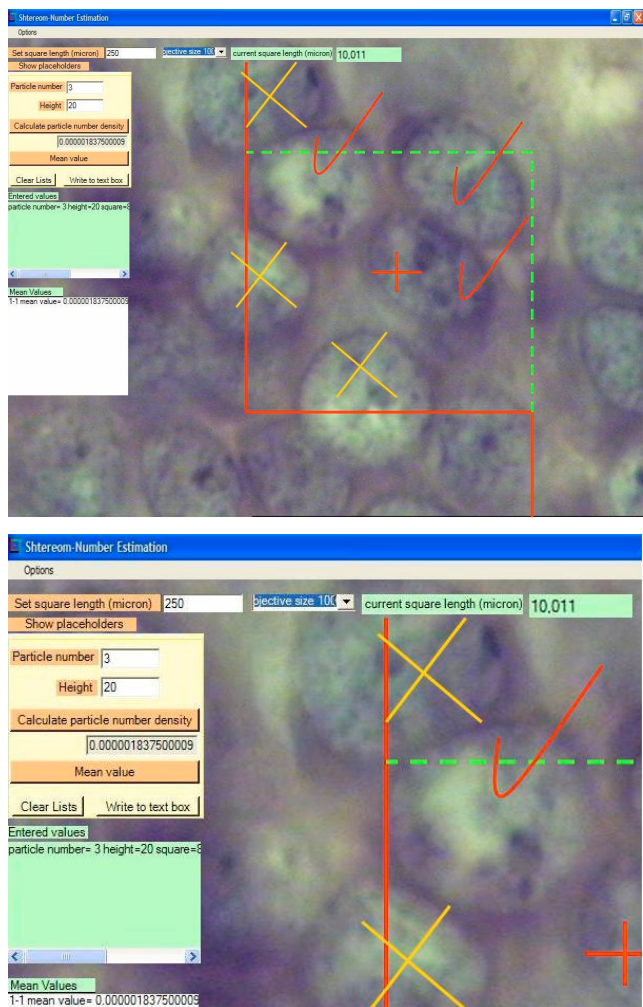


Fig 4. a) Unbiased counting frame generated by SHTEREOM. Cells checked are counted since they do not hit the continuous red line. Cells crossed cannot be counted since they hit the continuous red line. b) Enlarged view of left of Fig. 4a showing data entry boxes and calculated results display boxes with objective size selector drop down menu.

Number estimations by the optical fractionator

The optical fractionator (West *et al.*, 1991; Antunes, 1995) requires the counting of particles with optical disectors in a uniform and systematic sampling that constitutes a known fraction of the region under analysis. This estimator is free from any shrinkage problems.

In SHTEREOM I, there are two guidelines for the X axis and two guidelines for the Y axis. The distance between the lines on the same axis are shown in the upper data display. The intercepts of each of the X and Y axis guidelines overlap a structure appearing on the histological view on the monitor screen and can

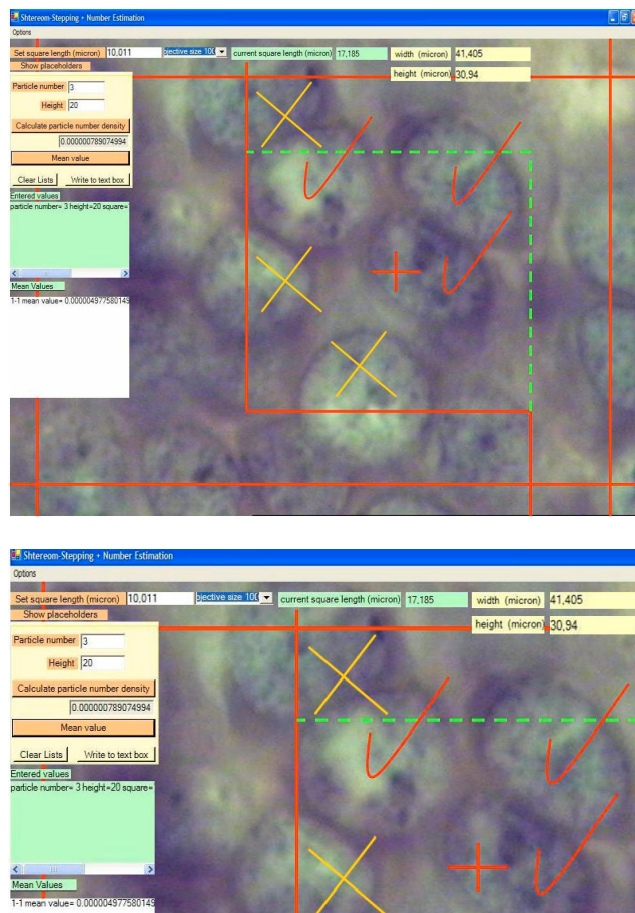


Fig. 5. a) Step lines on X (on the left and right), step lines on Y axes (on the top and bottom) and unbiased counting frame generated by SHTEREOM . Cells checked are counted since they do not hit the continuous red line. Cells crossed cannot be counted since they hit the continuous red line. b) Enlarged view of left of Fig. 5a showing data entry boxes and calculated results display boxes with objective size selector drop down menu.

be taken as reference points. Using the microscope stage screw, area fractions can be obtained by the distance preadjusted with cursors on the X and Y axes guidelines. Thus, in using our system an area fraction is easily obtained without the need of an expensive motorised stage (Adiguzel *et al.*, 2003). The area of the UCF making up the optical disectors (a/f) must be known, as must the area associated with each x, y movement (Δx multiplied by Δy). The area sampling fraction can then be calculated from this information. The height of the disector relative to the mean thickness of the sections should also be known, which can be estimated either with a microcator or a dial gauge (Howard and Reed, 2005).

SHTEREOM I provides the setting of the fraction

step distances on the X and Y axes (Adiguzel *et al.*, 2003) and the area of the UCF in terms of micrometers, with the height of the disector and particle numbers entered; the numerical density can be seen on the menu according to the magnification determined from the objective size drop down menu (5a). For example, rat hippocampus CA region neuron numbers can be estimated by optical fractionator (Onem *et al.*, 2005) (Fig. 5b).

DISCUSSION

Here, we have described the main features of the SHTEREOM I software that is used for generating different stereological test systems, and we have indicated a few of its initial applications that are aimed at the estimation of various morphometrical characteristics of biological structures. Through the use of SHTEREOM I, it is relatively easy to implement some of the recent design-based stereological methods that have no requirements in terms of the shape and organization of the sample under study.

There are other stereology image analysis systems that are available commercially, one of which is known as Mercator (Nova Explora, La Rochelle, France), although these systems generally need expensive attachments, such as a digital microcator, a frame grabber and a motorised stage. There are also more advanced programme systems for assisted stereology (*e.g.* newCAST [Visiopharm, Denmark], Stereo Investigator (MBF Microscience, USA) (Glazer & Glazer, 2003), BIOQUANT Stereology Toolkit (BIOQUANT, USA), (<http://www.bioquant.com/products.php?page=stereology&content=overview>), DISECTOR (Tomori *et al.*, 2001). All of these need expensive microcator and motorised stages or detailed sophisticated image mapping systems or only consists of only one module such as disector. However, in our system SHTEREOM I we have simple dial gauge and the X, Y fraction steps on the screen are handled with pre-placed cursors manually by utilising the on-screen stepping module shown in Fig. 5a as discovered by Adigüzel *et al.* (2003) and our system will be open for upgrades with more than one module integrated into a common user interface (Fig. 2). The use of SHTEREOM I avoids the need for expensive attachments, such as a digital microcator and an X-Y stepping motorised stage. Another advantage is that SHTEREOM I can operate on both live and stored images that have been captured by different microscopy systems, including on confocal and transmission electron microscopes.

The development of SHTEREOM I has provided a cost-efficient tool for the design of easily tested systems that are suitable for specific user needs and sample types. It is written in Visual Basic, such that new test systems and options can be included quickly and easily. The Nucleator; particle volume estimator, a surface area estimator, and a two-dimensional distance measurement tool will be included in the next step, in SHTEREOM II.

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