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STESYS Software for Computer-Assisted Stereology

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Summary

A software system STESYS for interactive and flexible generation of stereological test systems is described. STESYS enables to implement many of the recent unbiased stereological methods applied to biomedical research and clinical diagnosis by using a simple personal computer. Advantages of the STESYS software are illustrated by several examples of stereological measurements for estimating the number, total and mean cross-sectional area, volume and surface area.

Key words
Axon profile area – Axon profile number – Computer graphics – Morphometry – Villus surface area

Introduction

It is desirable in a number of studies concerning biomedical research and clinical diagnosis to evaluate the structural components of biological tissues or cells. Such measurements are usually based either on conventional intuitive manual methods, or on automatic or semi-automatic image analysis. Conventional manual methods and other methods requiring tracing of the contours of structural components, e.g. by using a digitizer, are usually laborious and time-consuming (Gundersen *et al.* 1981, Mathieu *et al.* 1981, Bonnet *et al.* 1991, Zachařová and Kubínová 1995). Moreover, they usually introduce an operator-dependent bias into the measurement (see, e.g. Barba *et al.* 1992) and other biases, e.g. due to biased sampling of the biological objects (blocks, sections, fields) chosen for measurement. The rules of unbiased sampling are also often neglected in the implementation of automatic image analysis (e.g. Krekule and Saxl 1994) which is rapid but requires automatic identification (segmentation) of the components under study.

Both the above problems, i.e. biased sampling and difficult segmentation, can be solved by using stereological methods. The contemporary design-based stereological methods, based on rigorous mathematical theory, were shown to be efficient and unbiased, regardless of the shape and organization of the studied structure (Gundersen and Jensen 1987, Gundersen *et al.* 1988a,b, Cruz-Orive and Weibel 1990). They provide estimates of geometrical characteristics of three-dimensional objects and their components mainly by using measurements on sections (or projections) of sampled specimens when different test systems are superimposed and test points lying in profiles, intersections of profile contours with test lines, etc., are counted. A high efficacy of stereological analysis can be achieved by using an efficient sampling procedure when the optimal numbers of individuals, tissue blocks, sections, test points, etc. are evaluated.

The test systems are placed in the ocular of a microscope, on a projection screen or on a micrograph of the section. The measurements can be considerably facilitated and accelerated by using "customized" test systems generated by computer graphics and superimposing them either in the field of vision of the microscope (e.g. system Microvid Leitz), on the screen of a personal computer (Krekule and Gundersen 1989, Moss *et al.* 1989), or on video display (Krekule *et al.* 1995). Several commercial programme systems for computer-assisted stereology have already been developed, e.g. CAST-grid (Olympus DK A/S), Digital Stereology (Kinetic Imaging) or Stereology Toolbox (Morphometrix). They are relatively expensive because not only the software but also more sophisticated hardware (a camera attached to a microscope and a frame grabber connected directly to PC performing the measurements) has to be purchased. The present paper introduces a programme system STESYS which assumes that the digitized images have been captured independently. This means that STESYS does not operate on live images but can be run on a simple PC without special hardware or software requirements. It makes it possible to design and generate interactively and flexibly many stereological test systems used in design-based stereological methods. Several examples of STESYS applications are presented in the present report.

Description of STESYS

STESYS (Stereological TEst SYStems) is a compact, menu driven non-overlayed programme (Fig. 1a). It is run on IBM PC XT/AT and compatibles with the MS DOS operating system version 3.0 and higher. VGA/SVGA colour graphics is optimal for good performance but several lower graphical modes are also supported. The compiled code of STESYS (version 1.0) occupies about 220 KB, additional memory requirements depend on the test system and/or image complexity but are generally modest. Both keyboard and mouse control are provided. A variety of stereological test systems can be easily designed, previewed, saved, opened, superimposed on images, exported to different image formats and printed or plotted. The main STESYS features are briefly described below.

Calibration

There are three types of units used to scale STESYS test systems: pixels, real units (i.e. "external world" metric information) and auxiliary units. During the calibration procedure the user determines the relationships between the units and the size of the test system window.

The calibration data are recorded in the test system file. It is possible to define default calibration to be assigned to a newly defined test system automatically. Default calibration data are saved in the options file. The calibration can be changed at any time during the test system development.

Test system types

Three different test system types are available: point grids, test line systems and systems of unbiased counting frames. They can be freely combined within an individual test system and, moreover, several levels of the same item type with different shapes, dimensions, origins, sizes and markers can be superimposed (Fig. 1b). Any test system can be shifted and/or rotated independently of the other. Miscellaneous shapes of test line system components (straight lines, circular, line or cycloidal segments, grid of lines) are available for various stereological methods.

There are several features that can be defined for the test system as a whole. This comprises options to shift and rotate the test system (either by defined values or randomly) and to enhance it by inserting horizontal and/or vertical scale and user comments.

The developed test system can be saved to a file (occupying less than 1 kB) for subsequent use and/or reediting.

Images

The particular test system is usually designed to evaluate a specific image or stack of images. STESYS makes it possible to load image files of various formats (e.g. TIFF, BMP, Bio-Rad PIC, etc.) and display them together with the test system superimposed on the computer screen (Fig. 1c). When loading a new image, the current calibration of the test system is examined. If it is different from the image calibration, the user is noticed and offered the option to accomodate the calibration to the new image.

Preview

The test system can be previewed at any time during its development. It is possible to view the opened image together with the test system. The user can view the entire test system window or its full-resolution portion (which can be moved rapidly across the image).

Export

It is possible to export the test system into a graphic file of a specific format (e.g. TIFF, BMP, Bio-Rad PIC, etc.) to enable subsequent application of miscellaneous image processing programmes. If the image is opened it can be exported together with the test system. Again, many options are provided to emphasize the test system superimposed on the image, including setting its colour.

Print

The test system can be printed for documentation purposes or for producing an overlay

transparency, ocular test system, etc. Several types of printers (e.g. 9- or 24-pin impact matrix, HP LaserJet and PostScript), resolutions and calibrations are supported.

Options

Many STESYS features, settings and defaults (e.g. video modes, preview options, printer types, calibration) can be stored and used in subsequent sessions to avoid user repetitive work.

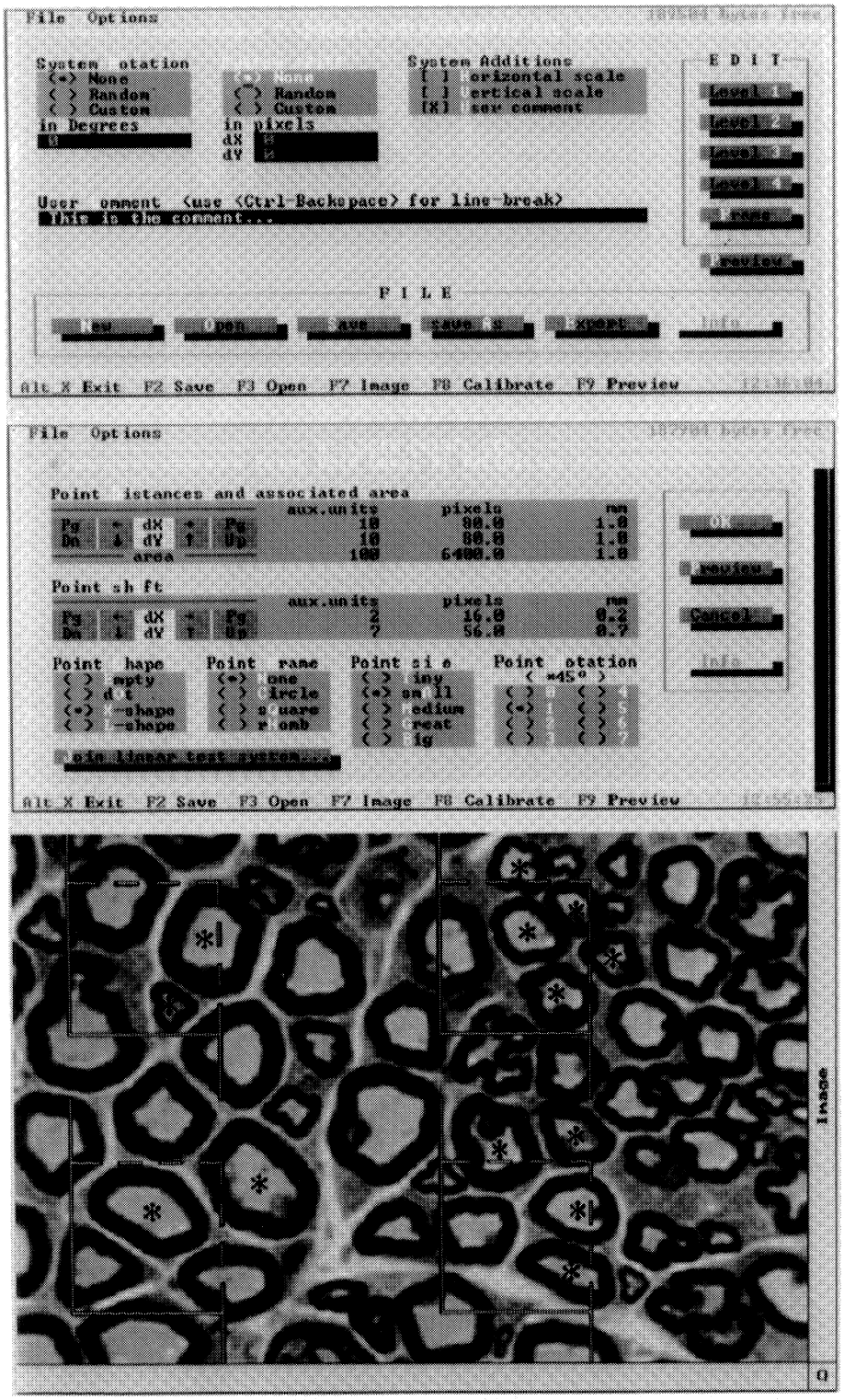

Fig. 1. *Examples of STESYS screens. (a) The main screen of STESYS. (b) Screen for generation of a point grid in level 1. (c) Screen showing the system of unbiased test frames superimposed on the image of nerve section. Number of selected axon profiles, n(ax), marked by *, is equal to 13 here.*

Applications

It should be stressed that proper sampling of the sections has to precede the measurements to obtain unbiased results. If the sections are larger than the field of view (i.e. image window), it is also necessary to sample quadrats (test system windows) from each section in an unbiased way (usually in a systematic manner) and the test system is superimposed on such quadrats.

Several examples of STESYS applications in stereological measurements are described below.

1. Number of axon profiles in nerve cross-sections

A system of unbiased sampling frames (Gundersen 1977) generated by STESYS is superimposed (at random) on the quadrats sampled from the nerve section (Fig. 1c). The number of those axon profiles that are lying at least partly within the frames, without being intersected by the full-drawn exclusion lines is counted. The total number of axon profiles in the entire nerve section (N(ax)) can be estimated by the formula:

$$\text{est } N(ax) = n(ax) \cdot \frac{a(as)}{a(fr)} \cdot \frac{1}{f} \qquad (1)$$

where n(ax) is the sum of profiles sampled by the unbiased sampling frames in all sampled quadrats of the nerve section, a(fr) is the area of a single sampling frame, a(as) is the area assigned to a single sampling frame (Fig. 2) and f is the fraction of plane area occupied by the test quadrats (for example, if f=1/4 then test quadrats occupy one fourth of the plane).

STESYS options: Setting the length and width of the frame and the rectangle assigned to the frame, position and orientation of frames. If the correct calibration is used, it is possible to read off the screen the values of a(as) and a(fr) for each setting of the frame system.

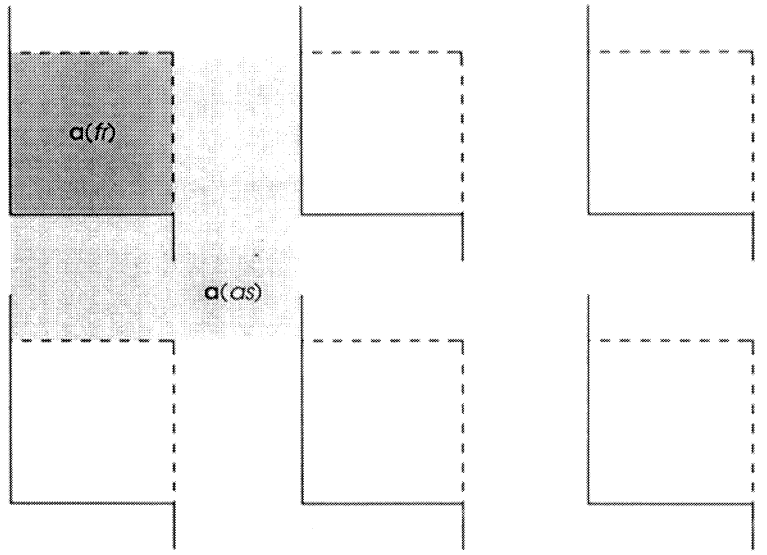

Fig. 2. *The system of test frames. a(fr) = area of the test frame; a(as) = area assigned to the frame.*

2. Total area of axon profiles in nerve cross sections

A point-counting method (e.g. Weibel 1979) can be used. A point test system generated by STESYS is superimposed on the quadrats sampled from the nerve section (Fig. 3). Test points falling in axon profiles are counted and the total area of axon profiles (A(ax)) is estimated by the following equation:

$$\text{est } A(ax) = P(ax) \cdot a(p) \cdot \frac{1}{f} \qquad (2)$$

where P(ax) is the total sum of test points hitting axon profiles in all sampled quadrats of the examined nerve section, a(p) is the actual area unit assigned to a single test point of the system and f is the fraction of plane area occupied by the test quadrats.

STESYS options: Setting horizontal and vertical distances between points, position and orientation of point grid, point 'size' and 'shape' (e.g. cross, X-shape, L-shape, dot) and optional combination of up to four different test point systems. If a correct calibration is used, the value of a(p) can be read off the screen for each setting of the point grid.

3. Mean area of axon profiles in nerve cross sections

The mean cross-sectional area of axons (a(ax)) can be estimated by the ratio of estimates of A(ax) and N(ax) (see above):

$$\text{est } a(ax) = \frac{\text{est } A(ax)}{\text{est } N(ax)} \qquad (3)$$

A combined test system of test points and frames can be used to perform both measurements simultaneously.

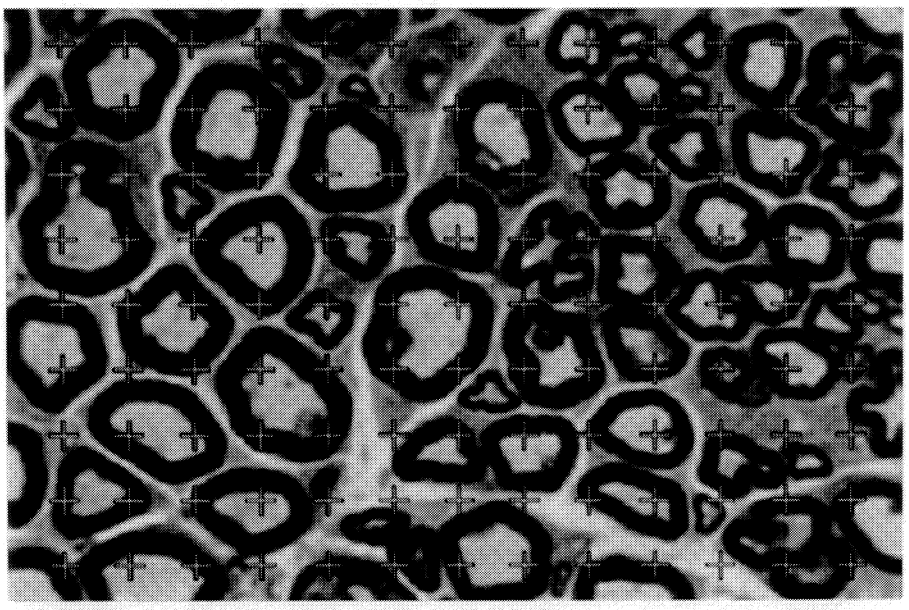

Fig. 3. *Point test system superimposed on the image of nerve section.*

STESYS options: Possibility to combine the frame system with summation of up to four different test point systems and setting their mutual position.

4. Volume of placental terminal villus

Cavalieri's principle (see e.g. Gundersen and Jensen 1987, Michel and Cruz-Orive 1988, Roberts *et al.* 1994) can be used. It is based on summing up the areas of systematic parallel sections of the object multiplied by the distance between the sections. Firstly, systematic parallel sections of villus (with random position of the first section) are cut and the images containing entire sections are captured. A point grid is then superimposed (at random) on the section images and the villus volume (V(vil)) is estimated by the formula:

$$\text{est } V(\text{vil}) = T \cdot a(p) \cdot \sum_{j=1}^{n} P_j(\text{vil}), \qquad (4)$$

where T is the constant distance between two consecutive sections, a(p) is the actual area unit assigned to one test point, n is the number of sections, and $P_j(\text{vil})$ (j=1,2,...,n) is the number of test points falling into the j-th villus section.

STESYS options: Setting the horizontal and vertical distances between points, position and orientation of point grid, point 'size' and 'shape' (e.g. cross, X-shape, L-shape, dot).

5. Surface area of capillary bed in placental terminal villus

Different stereological methods can be applied, e.g. the method of vertical sections (Baddeley *et al.* 1986). Vertical uniform random sections of the villus must be available (i.e. sections cut parallel to a chosen vertical axis being randomized around this axis). Images containing entire sections are captured. A cycloidal test system combined with a point grid (Fig. 4) is superimposed on each section image so that the minor axes of cycloids are parallel to the vertical axis. Intersections of the capillary surface and the cycloidal test system are counted, as well as the number of test points falling in the villus. The surface area of capillary bed in the villus (S(cap)) can be then estimated by the formula:

$$\text{est } S(\text{cap}) = 2 \cdot \frac{p}{l} \cdot \frac{\sum_{j=1}^{n} I_j(\text{cap})}{\sum_{j=1}^{n} P_j(\text{vil})} \cdot V(\text{vil}) \qquad (5)$$

where p/l is the number of test points per unit length of cycloidal test system, n is the number of examined vertical sections, $I_j(\text{cap})$ (j=1,...,n) is the number of intersections between the cycloidal test system and the contours of capillaries in the j-th section, $P_j(\text{vil})$ (j=1,...,n) is the number of test points falling in the villus profiles, and V(vil) is the villus volume which can be estimated, e.g. by Cavalieri's principle (see paragraph 4).

STESYS options: Setting the horizontal and vertical distance between test points and/or cycloidal arcs, optional combination of up to four different point and cycloidal systems. The system orientation can be changed so that the minor axes of cycloids are parallel to the vertical axis which is the prerequisite for the

validity of equation (5). The value of p/l which is fixed for the given test system and calibration can be read off the STESYS screen.

Many other contemporary design-based stereological methods can be implemented by using STESYS, e.g. the surface area estimation by orientator principle (Mattfeldt *et al.* 1990) when the systems of linear segments or quarters of circles can be used or by the method of spatial grid (Sandau 1987) using the grid of lines. The mean volume-weighted volume of particles can be estimated by the point-sampled intercepts method (Gundersen and Jensen 1985), when the system of parallel lines is used and the number and mean volume of particles by the method of the disector (Sterio 1984, Gundersen *et al.* 1988a) when the unbiased sampling frames are superimposed on two parallel sections.

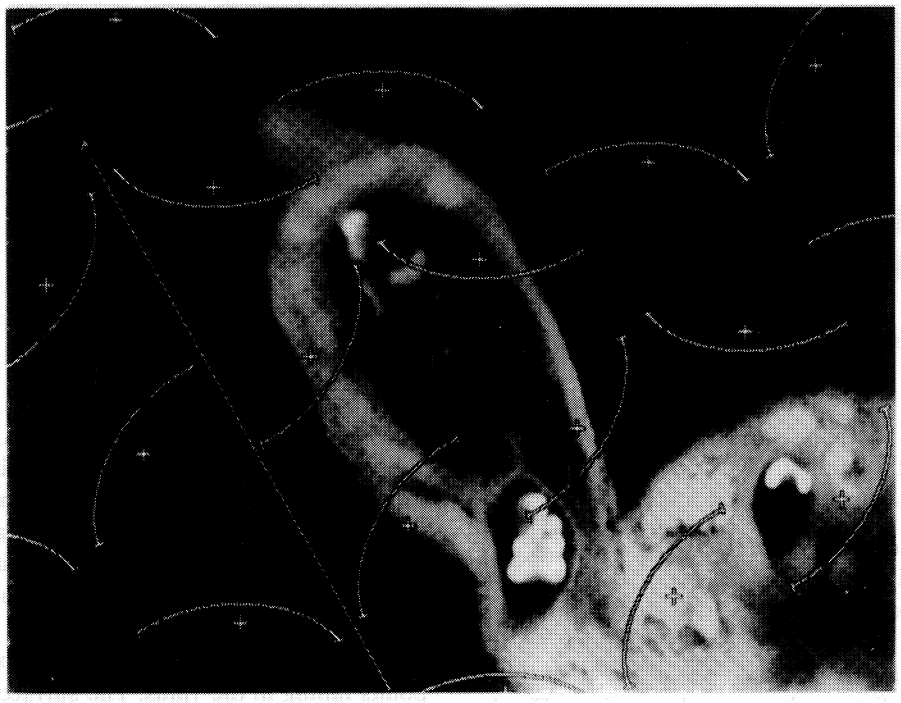

Fig. 4. *The cycloidal test system superimposed on the image of section of a placental terminal villus. The direction of vertical axis is indicated by the arrow.* $I_j(cap) = 3$, $P_j(vil) = 4$ *here.*

Discussion

The features of STESYS software system for generating different stereological test systems were described and several of its applications aimed at the estimation of various morphometrical characteristics of biological structures were shown. STESYS makes it possible to implement easily and comfortably many of the recent design-based stereological methods which have no requirements regarding the shape and organization of the studied structure. For example, there are no assumptions on the distribution and shape of axon cross-sections as it is in other morphometrical methods such as the "line-sampling" technique (Harman *et al.* 1991). Unlike digital methods, stereological methods do not require segmentation of the structural component under study which is often a difficult task, especially in the case of biological tissues. Moreover, if properly applied, design-based methods lead to practically unbiased estimates of morphometrical characteristics and they are more efficient than methods based on tracing structure contours.

Unlike some other programme systems for computer-assisted stereology (e.g. CAST-grid (Olympus DK A/S), Digital Stereology (Kinetic Imaging), Stereology Toolbox (Morphometrix), OpenStereo (Abrams *et al.* 1994), STESYS cannot operate on live images (advantageous especially when using the method of optical disector, see Gundersen 1986, Gundersen *et al.* 1988a), and it does not support the automatic digitized stereology (applicable to segmented images, see Zhao and Browne 1992). The above features were not included due to their higher hardware requirements.

The STESYS software system was developed as a cost-efficient tool to design easily test systems suitable for the users' specific needs and type of the material to be evaluated. It is written in a way allowing new types of test systems and options to be included fast and easily and so it will be possible to implement new stereological methods by using STESYS. The only

requirements of STESYS are the availability of digitized images of the structure under study (obtained usually *via* a camera attached to conventional light, electron or confocal microscopes and a frame grabber) and very modest hardware which means that the measurements can be made easily using a simple personal computer in the office as well as at home.

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Reprint requests

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