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Confocal Stereology and Image Analysis: Methods for Estimating Geometrical Characteristics of Cells and Tissues from Three-Dimensional Confocal Images

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Summary

A short review of confocal stereology and three-dimensional image analysis is presented, pointing out the achievements accomplished in this field by the Department of Biomathematics (Institute of Physiology, Prague). One of the methods of confocal stereology, the fakir method for surface area estimation, developed by this laboratory, is described. Methods for automatic measurement of geometrical characteristics of microscopical structures, based on 3-D image processing or surface triangulation, are discussed and compared with interactive stereological methods. Three-dimensional reconstruction programs and software implementation of stereological and digital methods as well as their practical applications are presented. The future trends are discussed.

Key words

Confocal microscopy • Image analysis • Stereology • Three-dimensional reconstructions

Introduction

The present paper reviews achievements accomplished by this laboratory in the field of confocal stereology and three-dimensional (3-D) image analysis of confocal data during several past years. We have focused on development and software implementation of methods for measuring geometrical parameters of structural components of organs, tissues, cells or cell compartments. Such measurements are the main prerequisite for quantitative analysis in a number of studies in biological research, especially when the relationships between function and structure are analyzed. Taking into account that the information on the spatial organization of microscopical structures is also very useful to get a complex idea about such relationships, we have also been engaged in studies of 3-D arrangement of microscopical structures by relevant measurements and 3-D reconstructions. We have found confocal microscopy to be an invaluable tool in 3-D analysis of microscopical structures.

Confocal microscopy

Confocal microscopy is a special type of optical microscopy that enables to obtain perfectly registered

stacks of thin serial optical sections (having thickness from approx. 350 nm) within thick specimens (Fig. 1a). Digital images of such stacks represent suitable data for quantitative measurements as well as for computer 3-D reconstructions that can be made without having to solve the tedious problem of alignment of images of successive sections (Pawley 1995). The principle of a confocal microscope was patented by Marvin Minsky in 1957, but confocal microscopy became a useful and efficient tool not earlier than almost 30 years later, after the confocal microscope with a laser light source was introduced (confocal laser scanning microscope, CLSM, Åslund *et al.* 1983). The first commercially available system was Bio-Rad MRC-500 in 1986.

Recently, two-photon microscopy representing a new type of laser scanning microscopy providing images of thin optical sections has emerged (Denk *et al.* 1990), which is reported to be able to focus even deeper into the thick specimen (up to several hundred micrometers, see Svoboda *et al.* 1997).

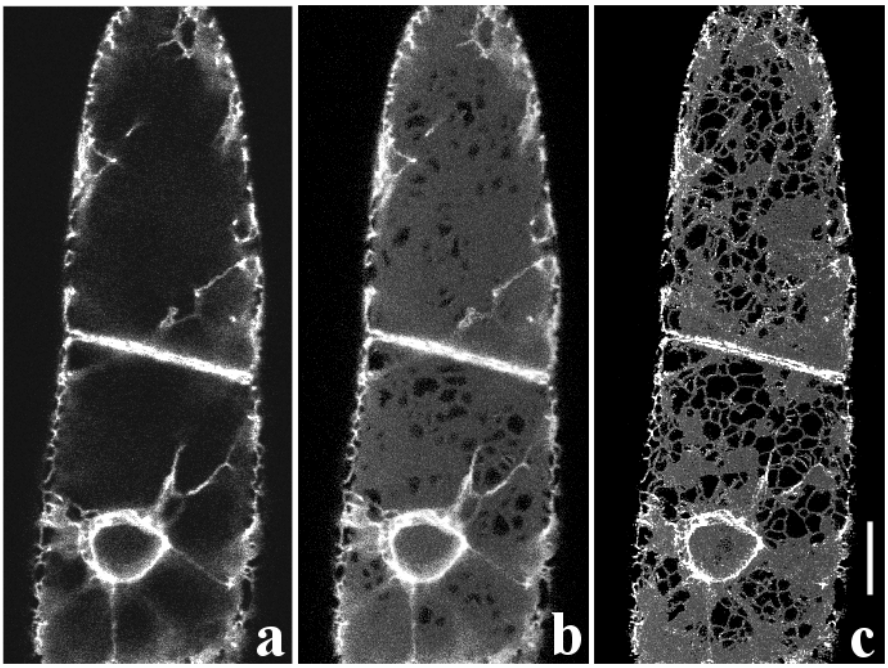

Fig. 1. Endoplasmic reticulum of *Nicotiana tabacum* BY-2 cell line transformed by a DNA construct with GFP sequence captured by a confocal microscope. a) A single confocal optical section. b) Maximum intensity projection of 42 confocal sections 0.2 µm apart. c) Maximum intensity projection of the same confocal series after applying a deconvolution algorithm. Scale = 10 µm.

Confocal stereology

Confocal stereology represents a contemporary approach to evaluation of structures by using the combination of stereological methods and confocal microscopy.

Stereological methods are precise tools for the quantitative evaluation of the structure of three-dimensional objects (Weibel 1979, Howard and Reed 1998). The term of stereology as a new scientific discipline was coined in 1961, motivated by the need of investigators in material and life sciences to establish a rigorous theoretical basis for the solution of problems encountered in morphometry. Stereological methods are based mainly on observations made on 2-D sections or 3-D subsamples of tissue, applying test probes of different dimensions, i.e. zero-dimensional (0-D, i.e. points), 1-D (i.e. lines) or 2-D (i.e. planes) and counting the interactions of the probes with the structures under study. For example, the number of test points falling into the given structure or number of intersection points of test lines with the structure surface is counted.

The first application of confocal microscopy for stereological measurements was presented by Howard *et al.* (1985) in their concept of unbiased sampling brick. They used a special type of a confocal microscope – tandem scanning reflected light microscope (Petráň *et al.* 1968) for counting osteocyte lacunae. Yet, though mentioned by several authors (Gundersen 1986, Rigaut 1989), the unique features of confocal microscopy advantageous for stereological measurements of not only number but also other parameters such as surface area, have not been fully recognized earlier than during the last decade (Rigaut *et al.* 1992, Howard and Sandau 1992). Here, especially the possibility of a confocal microscope to capture series of optical sections within a thick

specimen and thus providing 3-D image data is exploited. This laboratory was among the first to develop implementations of different stereological methods using confocal microscopy including the development of new methods (Kubínová *et al.* 1995, 1996, Kubínová and Janáček 1998). Confocal microscopy proved to be especially useful in application of methods based on spatial estimators evaluating small 3-D samples of structure under study (Howard and Sandau 1992, Kubínová and Janáček 1998, Kubínová *et al*. 1999, Kubínová and Janáček 2001, Kubínová *et al.* 2002). A 3-D sample of examined tissue can be analyzed if a rectangle within a field of view of a microscope is focused through. By using a special software, it is possible to generate different virtual test probes with arbitrary pre-defined (e.g. random) position and orientation within the stack of sections and apply them directly to this 3-D image data. Such approach is used in estimation of surface area by a fakir method developed by this laboratory (Kubínová and Janáček 1998) and in length estimation by global spatial sampling (Larsen *et al.* 1998) where special planar "slicer" probes are used (Kubínová and Janáček 2001, Kubínová *et al*. 2001). Another examples of spatial probes are the spatial point grid used for efficient volume estimation (Cruz-Orive 1997, Kubínová and Janáček 2001) and the optical disector (Gundersen 1986) or unbiased sampling brick (Howard *et al*. 1985) used for counting or sampling particles (e.g. cells). We will describe in more detail fakir method, developed by this laboratory, that can be used for surface area estimation if series of confocal sections within a thick physical slice are available. Unlike classical stereological methods applied to thin physical sections, this method does not require randomizing the orientation of the section, hence the slice can be cut in arbitrary direction.

Fakir method

The surface area of, e.g. cell, S(cell), can be estimated using fakir probes. The fakir probe (named by Cruz-Orive 1993) is a systematic probe consisting of parallel test lines (resembling nails of a fakir bed piercing the surface, see Fig. 2). When estimating, e.g. the cell surface area, the intersections between the cell surface and the fakir probe are counted. We can imagine the cell is pierced through by the nails of the fakir bed and we are counting how many times the nails went into or out of the cell. The cell surface area *S(cell)* can be estimated by the following formula:

$$estS(cell) = 2 \cdot u^2 \cdot I \qquad (1)$$

where u is the distance between neighboring parallel lines of the probe, I is the number of intersections between the fakir probe and the cell surface.

A high efficiency of the measurement can be achieved if we use a cubic spatial grid consisting of three mutually perpendicular fakir probes, halfway shifted with respect to each other (Fig. 2). In such case the average over the three fakir probes is considered in the surface area estimation:

$$estS(cell) = \frac{2}{3} \cdot u^2 \cdot (I_1 + I_2 + I_3) \qquad (2)$$

where *u* is the distance between neighboring parallel lines of the grid and I_j (j=1,2,3) is the number of intersections between the j-th fakir probe and the cell surface.

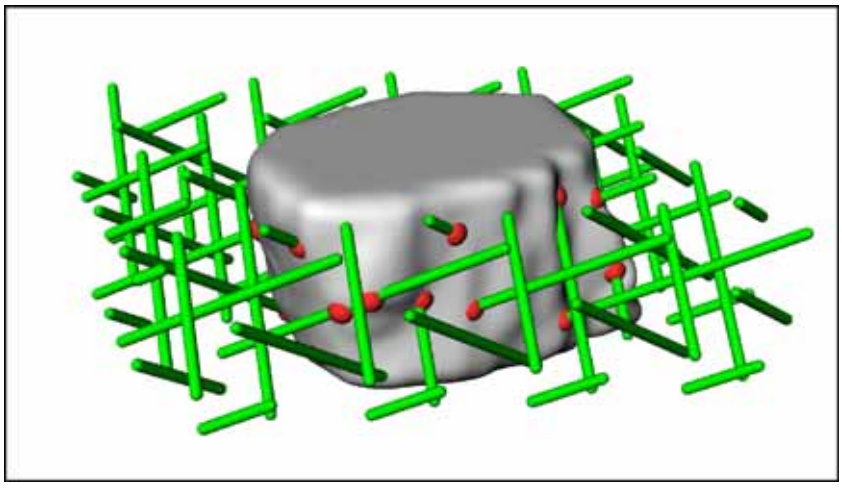

Fig. 2. A spatial grid consisting of three mutually perpendicular fakir probes, halfway shifted with respect to each other, applied to the measurement of the surface area of the side walls of a 3-D object (i.e. thick slice of a muscle fiber). The number of intersections (red) of the fakir probes (green) and object walls is proportional to the object surface area. For the sake of clarity, intersections are represented by "balls" and test lines by "pipes" here.

The measurement can be easily performed using our interactive FAKIR program (Kubínová and Janáček 2001; http://www.biomed.cas.cz/fgu/fakir/3dtools.htm for free download) or FAKIR module running in *Ellipse* (ViDiTo, Slovakia) environment. This software generates an isotropic set of virtual fakir probes and so it is not necessary to randomize the direction of the stack of sections.

Variance and efficiency of fakir method

In practical application of stereological methods, the question of efficiency is important, as it is always desirable to get sufficiently precise results with the least workload. Therefore, still more and more attention is being paid to the study of variances of stereological estimators. The variances of the estimators based on the Cavalieri principle, spatial grid of points and disector principle have been studied using Matheron's theory of regionalized variables (Matheron 1965) – see e.g. Gundersen and Jensen (1987), Cruz-Orive (1989, 1993, 1999), Kiêu *et al*. (1998) and Gundersen *et al*. (1999). For the estimators based on measuring intersections of the object with isotropic uniform random grids, such as the fakir method, their variance can be split into the component due to the grid orientation and to the residual component due to the grid position (Hahn and Sandau 1989). The first component depends on the mutual orientation of the fakir probes applied and on the shape of the object under study, namely the anisotropy of the surface, which can be expressed by the rose of directions of normals to the surface. The second, residual component of variance is dependent mainly on the grid density and arrangement, e.g. on the mutual shift of the fakir probes. The first component of variance is clearly the highest for totally anisotropic object, which rose of directions is represented by a single vector, i.e. for the flat surface in 3D. It can be proved that in this special, 'worst' case the coefficient of variance of surface area estimate by applying a single fakir probe is 57.74 % while by three orthogonal fakir probes (Fig. 2) it is only 10.16 % (Mattfeldt *et al.* 1985, Janáček 1999). For less anisotropic surfaces the first component of variance is decreasing, e.g. for the triple grid estimator of the surface area of ellipsoid with diameter ratios of 1:4:4 the coefficient of variance was calculated to be 4.90 % while for ellipsoid with diameter ratios of 1:1.6:1.6 it is already very close to zero (Hahn and Sandau 1989). The second, residual component of variance reflects the arrangement of the spatial grid applied. We have shown that the spatial grid consisting of three mutually perpendicular fakir probes, halfway shifted with respect to each other (Fig. 2), is much more efficient than non-shifted orthogonal triplet of fakir probes (Janáček 1999), requiring only about one half of the number of intersections for the same residual variance.

Methods based on 3-D image processing

Automatic measurements of geometrical characteristics of 3-D objects can be applied directly to their binary images obtained by automatic segmentation of the grayscale images captured by a confocal microscope. Automatic segmentation is a procedure of processing the source digital grayscale image (defined as a data structure of numerical values in the spatial grid of image elements called pixels in 2D or voxels in 3D) resulting in a binary image, in which the foreground elements belong to the objects under study (Serra 1982). The validity of the results of such automatic measurement, i.e. their unbiasedness and precision, depends on how precisely the model describes the object under study. Appropriate spatial resolution and high image quality are necessary for geometrical measurements. High quality contrast images enable segmentation by simply thresholding the image values while image inhomogeneities due to uneven staining or heterogeneous acquisition conditions require more advanced techniques of segmentation based on region and edge detection techniques. The images distorted by noise must be pre-processed by filtration. A 3-D image may contain more complete spatial information on the object under study, e.g. separate 2-D sections do not contain information on gradients in the axial direction (i.e. perpendicular to the image plane). Further, topological properties, such as the continuity of objects, cannot be judged from a single section. Finally, the 3-D image processing, using more spatial information, can be more effective and robust than the 2-D processing of individual slices. Basic algorithms of 3-D image processing can be derived from those used in 2-D image processing in a straightforward way (Meyer 1992).

We have developed several 3-D image analysis algorithms for software implementation of automatic methods for measuring geometrical characteristics of 3-D structures captured by a confocal microscope (Kubínová *et al*. 1999, 2002): voxel-counting method for volume estimation and digital Crofton methods for surface area and length estimation.

Methods based on surface triangulation

Triangulated surfaces are currently used to model 3-D objects in computer graphics. Such surfaces can be obtained from the 3-D grayscale digital images by isosurface detection using the marching cube algorithm (Lorensen and Cline 1987) or by detecting the object contours in subsequent horizontal slices first and then connecting the contours by triangulated stripes (Oliva *et al*. 1996). The contours can be detected interactively or by (semi-) automatic tracking of the object boundaries in the slices (Baba 1991). Geometrical characteristics of the models delimited by the triangulated surfaces can be used as estimates of the characteristics of the objects under study (Guilak 1994). We have implemented methods based on surface triangulation for measuring volume, surface area, and length (Kubínová *et al.* 2002).

Three-dimensional reconstructions

The stacks of confocal images can have only a limited size, which means that confocal microscopy can be used in a straightforward manner to reconstruct only small structures like single cells or small pieces of tissue. It is often desirable to reconstruct larger tissue volumes with sufficient detail, so that the structure arrangement and organization can be revealed, e.g. arrangement of capillaries in different organs or tumors. From this reason we have developed a special GLUEMRC and LINKMRC software for composing stacks of confocal images (neighboring in lateral or axial direction) together into one large stack using algorithms for alignment of 3-D images (Karen *et al*. 2003, for free download contact karen@biomed.cas.cz). The surface renderings of microscopical structure under study can then be made. For this purpose, the structure contours in individual optical sections are obtained by image segmentation algorithms or, if the automatic segmentation is not feasible, the contours are outlined manually. Finally, the structure surface is rendered, after applying image processing algorithms like smoothing by 3-D Gaussian filtration. For example, see our 3-D reconstruction of human placental villi with their capillary bed in Figure 3.

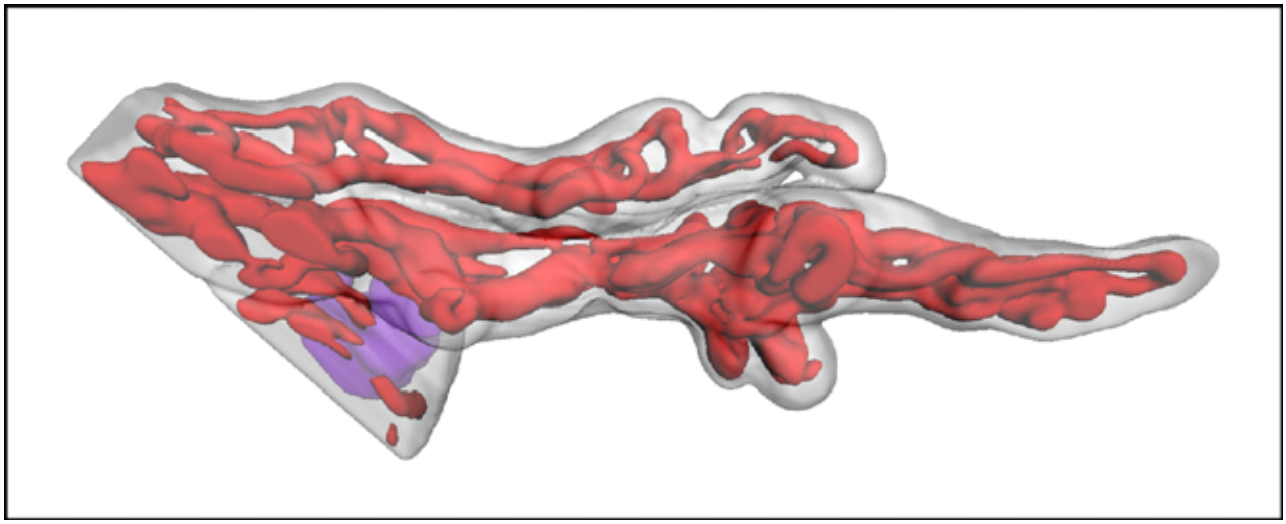

Fig. 3. 3-D reconstruction of human placental villi and their capillary bed. The confocal stack used for reconstruction had dimensions of 325x300x74 μm^3.

Software implementation of methods

The first programs for implementation of above methods that were developed by this laboratory were independent programs written in TurboPascal 6 for IBM PC under the MS DOS operating system (some of them still available as a freeware via the Internet at http://www.biomed.cas.cz/fgu/fakir/3dtools.htm). Other, mainly 3-D image processing algorithms, automatic methods and 3-D reconstructions, have been implemented as special modules of originally IRIS Explorer (SGI, USA, UNIX operating system, later NAG, UK, Windows NT operating system) visualization environment (for free download of our modules contact janacek@biomed.cas.cz). Following the further hardware and software development we have gradually turned to

building a system of mutually compatible modules, written in C++, running under *Ellipse* (ViDiTo, Slovakia) software image analysis environment. So far, a number of such modules have been developed, e.g. for implementation of fakir, slicer (global spatial sampling), disector, spatial point grid methods, contour delineation, and surface rendering algorithms for 3-D reconstructions.

Comparison of methods

We have compared many of the above mentioned stereological and image analysis techniques from the point of view of their applicability, efficiency and precision (Kubínová *et al.* 1999, 2002). It should be stressed that there is no absolutely universal method which would be optimal for all types of structure. In general, the automatic methods are faster than interactive stereological methods but require automatic segmentation of analyzed objects from the images or at least a high contrast between the object and the background. They also require careful testing and adjusting to the given type of microscopic structures. For surface area estimation, the fakir method appears to be the most universal of all tested methods and so it might be recommended for testing the applicability of other, less time-consuming methods. The method using spatial grid of points is very good for interactive volume estimation. If the object segmentation is feasible, the voxel-counting method is suitable for the volume measurement. Methods for volume estimation usually give precise results and they are not sensitive to voxel size and degree of smoothing. The triangulation method applied to grayscale images appears to be suitable for measuring the volume and surface area of isotropic as well as anisotropic objects, provided a high contrast between the object and the background is achieved. Digital methods for surface area estimation, especially triangulation method are more sensitive to image processing. Therefore, it is necessary to be cautious with noisy images, taking into account that noise increases the surface area measured. It can be reduced by smoothing, but a suitable degree of smoothing should be found and carefully tested.

Application of methods

The methods of confocal stereology and 3-D image analysis can be applied for evaluation of a large variety of structural components of organs, tissues, cells or cell compartments. This laboratory contributed to successful application of such methods in different fields of biological research, e.g. embryology and histology. The number of capillary connections in terminal villi of human diabetic placenta was proved to be higher than in normal placenta (Jirkovská *et al.* 1998, 2002). The length of capillaries going along fibers of a rat muscle per fiber length was found to be larger in soleus muscle than in extensor digitorum longus muscle while the capillary length per fiber surface area was not different in both muscle types (Kubínová *et al.* 2001). The number of satellite cells per fiber length and per the number of all myonuclei was lower in old human vastus lateralis muscles than in the young ones (Sajko *et al.* 2002, 2004). Other applications of our methods comprise diverse scientific fields, such as plant anatomy (Albrechtová *et al*. 2001) or radiobiology (Kubínová *et al.* 2003).

Practical considerations

Whether interactive stereological or automatic image analysis techniques are applied, it is always necessary to follow proper sampling, i.e. the fields of view chosen for evaluation must be selected in a representative, unbiased manner. Usually, systematic sampling is a good and efficient way to select the sections and sampling frames for analysis (Gundersen and Jensen 1987). It is also necessary to define precisely conditions and aims of the study. The above methods as any other technique lead to reliable results only if the reference space and structures under study can be unambiguously identified. Further, it is necessary to take into account possible bias due to technical processing of the tissues under study, especially deformation caused by the tissue shrinkage during fixation, embedding and cutting of material (Dorph-Petersen *et al.* 2001). Such deformations should be minimized by developing suitable processing techniques. The possible deformations should be measured and controlled in different steps of technical processing. This requires to calibrate the microtome or vibratome used for cutting the tissue and to measure lateral and axial deformations – a confocal microscope enabling to measure axial and lateral distances in different steps of tissue processing can be a very useful tool for such evaluation.

Confocal microscopy, like any other technique, has some drawbacks. Sometimes it can be difficult to find a suitable fluorescence staining of structures to be examined. It should be noted that in histological specimens a non-specific staining like eosin can often be

used (Jirkovská *et al*. 1998) while the cell surface can be visualized by immunofluorescence techniques when antibodies are bound to integral membrane proteins of cellular plasma membranes and labeled by a common fluorescent dye. Another drawback consists in the axial resolution (though higher than in a conventional optical microscope) being lower than the lateral resolution. The shape of the point spread function of a confocal microscope is elongated in the direction of z-axis (Shaw 1994) which causes defocusing that can possibly result in an overestimation of the surface area and volume of the examined objects. This can be eliminated by applying special deconvolution algorithms to perform 3-D deblurring of the images before the measurements (Fig. 1). In two-photon excitation fluorescence microscopy, the axial resolution is higher than in confocal microscopy (Denk *et al.* 1990, Nakamura 1999, Diaspro 2002) and it is also possible to penetrate more deeply into the specimen with decreased bleaching of fluorescence dyes. However, possible deblurring of 3-D images should be considered even here. Aberrations, especially the axial displacement due to the unmatched refractive indices (Sheppard and Török 1997), should also be taken into account in the measurements of microscopical structures.

Glimpse into the future

It was demonstrated how useful confocal microscopy in connection with stereology and 3-D image analysis can be for estimating different geometrical parameters of microscopical structure and for its three-dimensional visualization. In comparison with a conventional optical microscopy, confocal and especially two-photon microscopy offers not only higher resolution and examination of thicker specimens but also a better possibility to analyze living, fresh or more easily prepared specimens.

In the years to come, further development of confocal stereology and its applications can be anticipated with the spreading of usage of confocal microscopy and the ever increasing demands for objective measurements of different types of biological structures. This laboratory will follow this trend exploiting confocal and two-photon microscopy available. One of our topics will be the development of methods of spatial statistics for measuring second-order properties of biological structures, analyzed by a confocal microscope, characterizing their arrangement and mutual relationships. We have already developed and implemented methods of spatial statistics for evaluation of clustering of one type of marker or colocalization of two types of markers in electron micrographs of immunostained ultrastructures of the cell nucleus (Philimonenko *et al.* 2000). Another direction in the evaluation of 3-D structure arrangement is the study of its orientation and texture, e.g. of fibrous structures like capillaries, microtubules or endoplasmic reticulum (Fig. 1). Here, stereological as well as image analysis methods can be useful. In our opinion, the future development in quantitative evaluation and 3-D visualization of structures will proceed in the direction of combination of stereological and digital, image analysis based methods, applying both interactive and automatic methods. This laboratory, being engaged in all types of these techniques, is prepared to search for efficient combination of techniques leading to a complex evaluation of the 3-D microscopical structures under study.

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