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STEREOLOGY AND CONFOCAL MICROSCOPY: APPLICATION TO THE STUDY OF PLACENTAL TERMINAL VILLUS

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ABSTRACT

The possibilities of combining confocal microscopy with stereology are demonstrated on human placental terminal villi. The volumes and surface areas of individual villi and their capillary bed are estimated and the Euler number of the capillary bed is counted. Finally, the advantages and limitations of confocal microscopy are discussed.

Key words: capillary bed, confocal microscopy, human placenta, stereology, terminal villus.

INTRODUCTION

The confocal microscope, which allows us to obtain thin, perfectly registered serial optical sections of entire cells or other objects, can be advantageous in stereological studies.

The combination of confocal microscopy and stereology will be shown in the case of the study of human placental terminal villi. Terminal villi form a predominating structure in the placenta at term. They contain majority of placental capillary bed which plays a key role in fetomaternal transport. At their level some pathological changes also take place, e.g. in diabetes. Therefore, information about their capillary bed surface area, volume and topological characteristics is valuable in the studies of normal and pathologic placenta.

Based on the above example, some advantages and limitations of confocal microscopy are analyzed.

MATERIAL AND METHODS

Samples of normal human term placentas were fixed in 4% formalin solution and embedded in paraffin wax. Approximately 100 μm thick sections were cut by a sledge microtome, stained by 0.15% eosin solution and mounted in entelan (for details see Jirkovská et al., 1994).

Terminal villi were studied, i.e. villous segments containing no vessels other than capillaries (Kaufmann et al., 1985). Several individual villi (Fig.1) lying completely inside the thick sections were sampled using the disector principle (Sterio, 1984). Digitized images of thin serial optical sections (1μm apart) were captured by a Bio-Rad MRC 600 confocal laser

scanning microscope (CLSM) connected with the inverted fluorescence microscope NIKON Diaphot using a planapochromat oil immersion objective (60x, N.A. = 1.4).

Four of the sampled villi were evaluated as follows:

a) The volume density of the capillary bed in the terminal villus was estimated by the point-counting method (see, e.g. Weibel, 1979).
b) The volumes of the villus and capillary bed were estimated by Cavalieri's principle (see, e.g. Michel and Cruz-Orive, 1988).
c) The villus outer surface area and the inner surface area of the capillary bed were estimated by the method of the spatial grid (Sandau, 1987).
d) The Euler number ($\chi(cap)$) of the capillary bed in the villus characterizes its topological properties, namely its connectivity ($c(cap)$), i.e. the number of redundant connections in the capillary bed. Taking into account that the capillary bed is connected and that it does not contain any enclosed cavities, the following equation is valid in our case:

$$\chi(cap) = 1 - c(cap) \tag{1}$$

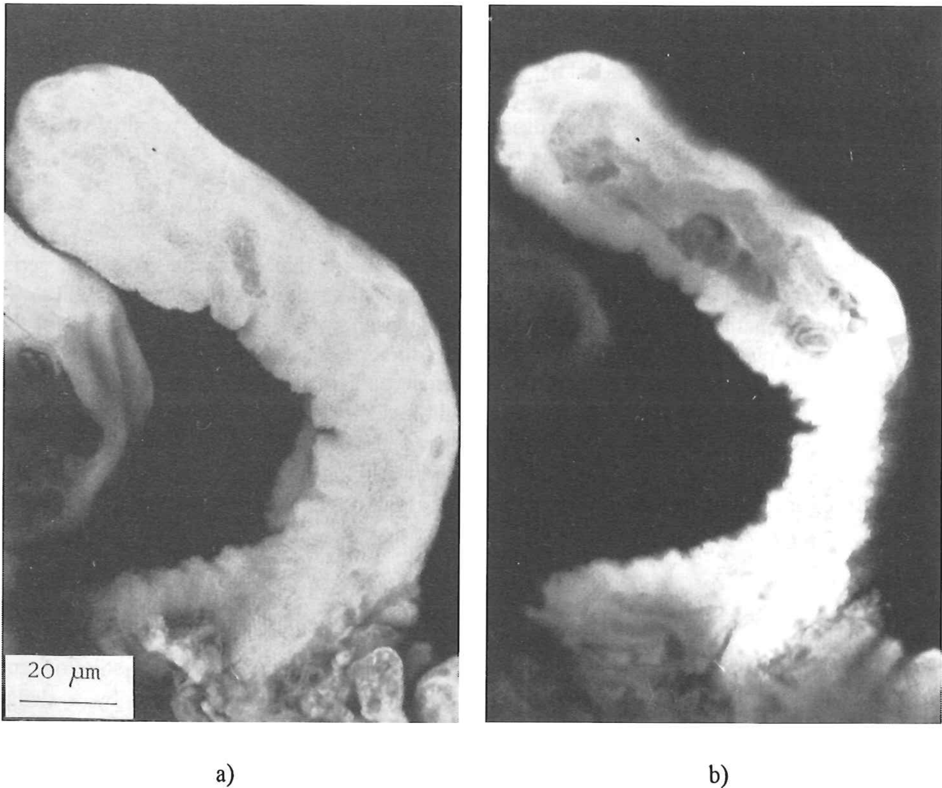

Fig.1. Terminal villus of human placenta. a) Projection of 65 optical sections of the villus. b) A single (40th) optical section of the villus.

Euler number was measured by the "sweeping plane" method (DeHoff, 1968) when the profiles of the capillary lumen were observed in subsequent optical sections and two types of events were counted : Firstly, the number of luminal appearances (i(cap)) and secondly, the number of luminal connections (b(cap)). The Euler number (χ(cap)) was then evaluated by the formula:

$$\chi(cap) = i(cap) - b(cap) \quad (2)$$

RESULTS

The results for the villi chosen for illustration of the methods are shown in Table 1. No serious conclusions can be made from the limited number of villi studied but the reported results show that there is a high variance in all studied characteristics of the villus and its capillary bed.

Table 1. The estimated villus volume (V(vil)), capillary bed volume (V(cap)), volume density of capillary lumen in villus (V_V(cap)), villus outer surface area (S(vil)), capillary inner surface area (S(cap)), ratio of capillary surface area to villus surface area (S_S(cap)), and Euler number of the capillary bed (χ(cap)).

villus number	V(vil) μm^3	V(cap) μm^3	V_V(cap)	S(vil) μm^2	S(cap) μm^2	S_S(cap)	χ(cap)
1	124 600	32 800	0.379	16 000	12 800	0.80	-1
2	43 600	12 200	0.279	6 300	4 400	0.70	1
3	73 800	8 000	0.110	7 800	3 700	0.47	0
4	79 000	24 000	0.315	10 100	8 950	0.89	1

DISCUSSION

The presented example has shown that the application of CLSM in stereology is feasible and enables estimation of many characteristics of the studied object. However, it should be noted that there exist some problems which limit the implementation of confocal microscopy in stereological studies:

1. A suitable staining of the tissue components to be studied must be used. Contemporary confocal microscopes have an epi-illumination design, and so dyes enabling us to observe the specimen in fluorescence or reflectance are needed. In the above example we have found the unspecific staining by eosin to be satisfactory. Eosin staining is also suitable for staining placental tissue embedded in glycol methacrylate instead of paraffin. The tissue shrinkage, occuring in our case, when we have assumed that it is uniform in all villi, thus can be minimized. Sometimes, especially in botanical specimens, the autofluorescence of tissue components (e.g. plant cell walls, chloroplasts, erythrocytes) can be exploited.
2. The depth of observation of CLSM is limited, thus it may be difficult to evaluate larger objects. The depth of observation is given by different factors, ranging from the working distance of the objective used up to the optical density of the material under study. In the present example, in order to obtain sufficient resolution, the planapochromat oil immersion objective with N.A. = 1.4 and working distance of 170 μm was used. It was possible to

make optical sections within a 100 μm thick slice.

It should be noted that some of the new types of confocal microscopes (e.g. 2-photon) are claimed to achieve a considerably increased depth of observation.

3. The field of view of CLSM is limited which can be circumvented by examining several neighbouring, aligned frames.
4. The axial displacement due to unmatched refractive indices (e.g. Visser et al., 1992) results in biased measurements along the axial direction. It can be minimized by matching the refractive index of the specimen to that of the objective and the medium between them as was done in our example.
5. CLSM, like any other type of microscope, has resolution limits which can affect especially the surface area estimation. However, in comparison with the conventional light microscope it has a higher lateral and above all axial resolution. It is important to choose the right distance between the sections in the series. In normal placentas we have found the distance of 1 mm sufficient. However, in diabetic placentas, capillaries with diameter of 1 mm were found. Therefore, the serial sections 0.5 mm apart might be more appropriate, to avoid errors in Euler number evaluation.

   Problems analogous to those listed above must be naturally addressed also when using a conventional light microscope in stereological studies using optical sections.
6. Special attention should be also paid to the proper application of stereological methods to avoid bias in estimated values. In our case the unbiased, number weighted sampling of villi should be ensured. If all villi lying completely inside the thick tissue slice were sampled, the villi with smaller diameter would be sampled with a higher probability than the thicker ones. Further, if the orientations of the main axis of villi were not distributed isotropically and the orientation of the thick slice were fixed, the villi with the main axis more or less parallel to the slice surface would be more likely to be sampled. Therefore, slices with isotropic orientation should be cut and the unbiased sampling of villi could be ensured by using the disector principle (Sterio, 1984). Even then there will remain the problem with sampling villi having their calliper diameter larger than the thickness of the slice minus the disector height. The solution of this problem will be sought in our future studies, although it seems that most villi are not thicker than 80 mm.
7. In order to obtain unbiased estimates of surface areas of villi and capillaries by the method of the spatial grid (Sandau, 1987), the orientation of the sections in the stack should be isotropic. However, the isotropic orientation of the thick slice does not ensure the isotropic orientation of the optical sections with respect to the villus because mainly villi having their longitudinal axis approximately parallel to the slice surface would be sampled for the surface area estimation. Nevertheless, according to results of Sandau & Hahn (1994), if the restricted randomization (i.e. isotropic orientation of the test grid only within the sections) is used, only a low bias can be expected. There are also other possibilities how to tackle this problem, e.g. to use the method of vertical spatial grid (Cruz-Orive & Howard, 1994) with the vertical axis chosen parallel to the projection of the villus axis to the slice surface. An analogous method could be also used. The vertical axis would be chosen perpendicular to the slice surface and so all sections perpendicular to the stack of optical sections would be vertical sections. The intersections of special cycloidal test systems (with the orientation of the main cycloid axes perpendicular to the vertical axis, as usual) contained in two mutually perpendicular systems of vertical planes would be counted.This method itself does not need random orientation of serial sections. Another possibility is to use the method based on the computer generation of a spatial grid (Sandau, 1987) with an isotropic orientation in 3D.

Further, the isotropic serial sections of the villus, derived from the digitized images of the stack of its optical sections, can be made and different stereological methods (e.g. spatial grid or vertical sections method) can be then implemented in such rendered stacks of object sections.

8. Let us mention the advantages of CLSM for stereological studies. Above all it is the possibility of capturing a stack of perfectly registered serial sections of the studied object which means that new stereological methods (e.g. spatial grid method) can be easily implemented. Moreover, 3-D rendering of the object is feasible, as well as the study of its topological properties (e.g. precise determination of Euler number), without the actual need to reconstruct the object.

   The possibility to obtain a relatively thick stack of registered optical sections which are not blurred by the out-of-focus regions, as it is in conventional optical microscopes, makes the unbiased sampling of particles easier. The sampling by the disector principle (Sterio, 1984) as well as by the unbiased brick (Howard et al., 1985), followed by the estimation of their volume (using Cavalieri's principle) or their surface area can result in information not only about their mean volume or mean surface area, but also about their size distribution.

   Taking into account that digitized images of optical sections are obtained by CLSM in a non-destructive way which makes it possible to generate optical sections of chosen positions and orientations within the studied specimen, CLSM provides possibilities for testing efficiency of different stereological methods and sampling designs in different types of biological material.

   CLSM can also make it easier to study the 2nd order properties. The mutual spatial relationships of two (or more) components can be studied because a simultaneous observation of differently labelled components is possible.

   The enhanced possibilities which CLSM provides in studies of living material enable to include the 4th dimension in some stereological studies, exploiting the time series of stacks of sections.

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