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Confocal Laser Scanning Microscopy and Chorionic Term Villi

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Introduction

The confocal laser scanning microscopy (CLSM) is a relative new biomedical technology and represents a good alternative to the conventional optical microscopy in the observation of static and dynamics events. CLSM is able to produce 3-dimensional images in high resolution of thick biological structures as tissues or single cells linked with immunofluorescent antibodies.
The mean difference with a traditional fluorescent light microscope is the use of a laser ray as light source and of optic and electronic systems which permit to perform, on the observed sample, different «optical sections» (i.e. scansions on defined levels) in order to evidence specific markers present into the sample or on its surface.

Confocal technology

In the conventional light microscopy, both the fire level of the objective lens and a great portion of the thickness of the biological sample is uniformerly and simultaneously lit up, and the final image is composed by the superposition of the light radiations originate by all levels which form the histologic sample. Obviously, this kind of technology needs of very thin histologic samples in order to obtain images with acceptable resolution and contrast.
On the contrary, the CLSM works with confocal procedure: the light field is restricted by a dot-like diaphragm, and the laser ray is focalized in a single point on a defined level within the sample. The radiation emitted from this point by the excitation of fluorochroms is captured by the objective lens, properly filtered by a pinhole diaphragm and deviated on a photomoltiplicator which transforms the light radiation in a proportionate electric signal.
All the light rays originate from different levels of the focal level are deviated by a dichroic mirror and they do not reach the revelation system. The image is scanned point by point up to the complete definition of the focal plane.
Moving the sample along the perpendicular axis (Z axis), it is possible to perform a series of different scansions which correspond to the different levels of the sample. Such scansions are named "optic scansions": their superposition, ordered by the software, permits the 3-dimensional reconstruction of the examined biological object. On the other hand, the images are acquired and stored as computer files and they can be analytically evaluated, artificially coloured, and modified in the contrast.
The maximum depth of the optical sections depends on several parameters: nature of laser ray used, type of lens, optical form and nature of the object, and type of fluorochrome. In presence of a fixed tissue marked with fluorescent probe the depth reach up 200-250 mm.
A contemporary use of two or more fluorochromes is favoured by the phenomenon of "colour merging".

Advantages of CLSM

As respect to the conventional microscopy the advantages of CLSM are the following:
Light source: the laser ray has a point-like surface, is monochromatic and less penetrating: it permits an image of higher quality, on the basis of the intensity and the coherence of the light source.
Sequential illumination: while in the conventional microscopy the structures present beyond the focal level interfere with the general vision, in the CLSM the emissions arising in areas different from the focal level are eliminate by the type of illumination, which is sequential and not simultaneous.
Optic sectioning: by the serial scansions the object is sectioned in very thin consecutive images, avoiding the technical artefacts. Moreover, with this procedure it is possible to examine singular cells *in vivo*.
Digital mixer: in the CLSM the presence of a digital control system, which filters the deep noise, provides of a better contrast and a higher resolution of the image.

Our experience

In the last months we applied the CLSM technology for the study of the placental villar tree and its vascolarization. It is well known that the 3-dimensional distribution of the chorionic villi is very complex and difficult to illustrate with conventional microscopy. On the other hand, the frequency and distribution of the capillary vessels present in each villous result of great importance for the materno-foetal exchanges in several pathological conditions. We think that the new CLSM technology may help the microscopic observations in this field. It is clear that any new technical approach needs a trial of practice. We refer in this paper

the working procedures and the technical difficulties.
We retrospectively studied 3 normal term placentas (37-38 weeks) and 3 preeclamptic placentas (34-37 weeks), formalin fixed and paraffin embedded. For each archival paraffin block we obtained 8 mm thick sections. According to the general immunohistochemical techniques the sections were de-waxed, hydrated, treated in a pressure cooker for 30', and incubated with primary antibody against CD34 (QBEND 10). After an incubation with a secondary biotinilate antibody, the sections were dipped in a solution of fluoresceine-avidine at room temperature for 45' in darkness. Finally, the sections were mounted in Vecta Shield (Vector).
The CLSM evaluation was performed on 10 terminal villi selected for each placenta at 400x magnification, with a total number of 30 villi in normal and 30 villi in pre-eclamptic placentas. The laser analysis of the sections revealed an effective thickness of 10-12 mm, and a series of 28 scansions was programmed for each section (1 scansion every 0.4-0.5 mm). In the first attempts we noted a relevant activity of spontaneous fluorescence meanly generated by the red blood cells, which interfered with the fluorescent signal of the linked fluorescein. To distinguish between natural and induced fluorescence, we used a double scansion for each optical level using sequentially two laser rays: Ar/Kr laser ray with a wave length of 488nm, which excites the fluorescein linked to CD34 and reveals the endothelium, and Gre/Ne laser ray with a wave length 543nm, that excites the spontaneous fluorescence of red blood cells and trophoblast. The double scansion may be superimposed by the software, generating an image whose vessel contour is easy localized in the villar architecture.
The same software provides a series of morphometrical evaluations about the images acquired for each optical level and for the sum of all the 28 levels of each villous. The morphometrical parameters are the following:
Arithmetical mean value of immunofluorescence for each field.
Arithmetical mean amplitude of immunofluorescence.
Maximum value of amplitude of immunfluorescence.
Minimum value of amplitude of immunfluorescence.

Results

Most of researches performed with the CLSM technology used thick sections (10-20mm) of fresh tissues or large sections (100-150mm) of tissue fixed in glutaraldeide/formalin obtained by the vibrotome. This kind of sections are able to assume reactive substances and antibody complexes. We decided to work with archival material routinely processed and paraffin embedded. We used thick sections of this material and we prolonged the time of the antibody incubation to permit its complete penetration. We remarked that the mean thickness of 10mm (programmed thickness 8mm) is available for the antibody penetration and it is sufficient for a general 3 dimensional idea of vessel distribution in chorionic villi in normal and pathological conditions.
The presence of the spontaneous fluorescence, meanly related to red blood cells, with a large emission band, interferes with signal of the fluorescein and it disturbs the evaluation of the vessel structure. The use of two different laser rays, with a different wave length, allows two signals which may be composed for the spatial reconstruction of the image. It is clear that the morphological and morphometrical evaluation of the vessels was only performed in the signal generated by the Ar/Kr laser ray which described the localization of endothelium.
In the normal placentas the terminal villi showed an unique capillary loop, with a large lumen filled of red blood cells and a regular wall.
The terminal villi of placentas in pre-eclamptic pregnancies had hyper-ramified vessels, with small lumen, often devoid of red blood cells. The vascular wall had very irregular contour, as observed in the sequence of scansions and in the average projection of each villous.
The morphometrical evaluation confirmed the morphological data. In fact, all the parameter values of the normal villi were significantly higher than the values of the villi present in placentas of women with pre-eclampsia. The statistical analysis has not been completely performed.

Comment

The application of CLSM technology to the study of vascular architecture in the term placental villi permits to have a very satisfied comprehension of the form and distribution of vessels. What is supposed in the bi-dimensional observation with conventional microscopy, is clearly illustrated by the reconstruction performed by the confocal microscope. This technology is very useful in the study of complex structures as the chorionic villar tree.
A previous study on vascularization of chorionic villi with the use of CLSM was performed by Jirkovska et al (1), on normal term placentas, paraffin embedded, by recording the spontaneous fluorescence in Hematoxylin-Eosin very thick sections (100mm). Our study represents an attempt of technological improvement, using a specific and selected signal generated by the immuno-reaction of the endothelium with the CD34 antibody: the obtained images are very clear and defined in contrast with all the other spontaneous fluorescence which is electronically suppressed. Moreover, our technical procedure permits a rapid and easy utilization of archive and routine material.
The differences found with CLSM between normal and pre-eclamptic placentas confirm and illustrate the observations suggested by the conventional microscopy. In fact, pre-eclampsia is considered a condition characterized by an utero-placental hypoxia in which a "branching angiogenesis" of villi is present, according to the classic "Kaufmann's triangle" of villous maturation (2). In addition, confocal observation shows that in pre-eclamptic condition the vessels of terminal villi present minimal and frequent irregularities of wall, probably as a consequence of multiple and continuous stimuli of angiogenetic factors: this minimal perturbations are hardly visible in light microscopy.
The morphological observation with CLSM and the

morpfometrical parameters we will performed may supply a more precise definition of villar vessel characteristics in pre-eclampsia and other pathological conditions of pregnancy.

Key words

confocal microscopy, chorionic villi, technology

References

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