Field Emission Scanning Electron Microscope (FE-SEM) is a useful tool to study thin sections of vertebrate retina

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The vertebrate retina is a layered structure formed by several neuronal and glial cell types. Numerous retinal research, deal with the characterization of the different layers of the retina and the ultrastructural study of their components. The most common techniques employed for these studies include light microscopy and transmission electron microscopy (TEM). The light microscopy is very useful to map the retinal-layered structure. However, it can not be used to probe structural details into each layer. On the other hand, it is not possible to have a panoramic view of the different layers of the vertebrate retina using the TEM. Often, a combination of these two techniques is being used to cover the complete study of the whole retina. Field Emission Scanning Electron Microscope (FE-SEM) has been used, as an approach to visualize nanoscale biological structures, like cell organelles and DNA material. However, in the present work, the simultaneous use of FE-SEM for both panoramic view of the laminar distribution of the retina, with a high morphological and ultrastructural resolution detail of different layers' components can make of them a useful tool to study vertebrate retina.

Keywords: field emission scanning electron microscopy; FE-SEM; vertebrate retina; retinal ultrastructure; light microscopy; transmission electron microscopy (TEM).

1. Introduction

The vertebrate retina is a layered structure formed by several neurones and glial cell types [1, 2]. Numerous retinal research, deal with the characterization of the different layers of the retina and the ultrastructural study of their components [3-5]. The most common techniques employed for these types of studies include the combination of light and transmission electron microscopes [5-8]. The light microscopy is very useful to map the layered retina structure (8-9). However, it cannot be used to probe structural details into each layer. On the other hand, it is not possible to have a panoramic view of the different layers of the vertebrate retina using only the transmission electron microscope (TEM).

The combination of electron microscopy (EM) with light microscopy (LM) has been employed for decades to generate molecular and structural identification. An example of the progress in this methodology has been the combining the capabilities of two typically separate microscopy platforms LM (or fluorescent) and EM named Correlative Light Electron Microscopy or CLEM. CLEM has the profit of two methods, letting scientists study the cell structure with LM and then increase the image with EM (10). LM and EM together provide valuable complementary and often unique information. CLEM is particularly useful when working with specimens where a fluorescent molecule has been incorporated, for example, a chemical tag or transgenically expressed fluorescent protein marker (11-12.).

The ultrastructural analysis of cellular and tissue volume has been realised alternatively using scanning electron microscopy (SEM) by collecting backscattered electron (BSE) signals from serial sections (array tomography) and serial block-face imaging [13]. These volume techniques arising in the 1970s [14], exhibiting high potential for using much larger specimens than possible with TEM [15-16]. However, the first BSE methods were limited first of all by tools, because the BSE imaging resolution was only somewhat better than light microscopy [17-18].

Together with transmission electron microscopy (TEM), other imaging techniques became useful in last decades of the twentieth century. More recently, has been developed new methods to solve the dilemma between the size of a field of view and resolution power [13]. This problem was not new in the biomedical research. Faced with this issue some researchers use serial thin sections for electron microscopy to describe the three-dimensional structure of sub-cellular compounds [19]. Others authors employ thick sections observed with the high-voltage TEM for the same problem [20].

The introduction of the field-emission scanning electron microscope (FESEM) has allowed that can be used to obtain more high-resolution information on the internal characteristics of biological samples processed with classic histological techniques used in TEM studies [21]. The introduction of FESEM equipped with the standard secondary electron (SE) detector to observed thin sections (70–90 nm) of biological tissues has been a significant advance [22]. The use osmium, uranium, and lead salts in TEM, produce an SE signal that allows obtaining imaging of the cells and organelles from the thin sections [21].

The FESEM has also been shown useful for getting images of thin sections processed with cytochemical and immunocytochemical techniques for TEM. This procedure has been helpful for sections are embedded in wax [23], epoxy resins [24], and polyethylene glycol [25]. Embedded sections, in which the matrix not removed, could be examined in an FESEM by using the standard SE detector [21]. The procedure for preparing and staining sections for FESEM examination was identical to that usually used for TEM observation.

The BSE imaging resolution provided today by detectors and the FESEM is a function of several parameters. These include an incident electron beam spot size and the excitation volume within the specimen, which in turn is a function of kV, sample thickness and the nature of contrasting agents employed [17, 26-28].

FE-SEM has been used, as an approach to visualise nanoscale biological structures, like cell organelles and DNA material. However, in the present work, the simultaneous use of FE-SEM for both panoramic view of the laminar distribution of the retina, with a high morphological and ultrastructural resolution detail of different layers' components can make of them a useful tool to study vertebrate retina.

2. Material and Methods

2.1. Biological Preparations

The study was performed on the sturgeon species of Acipenser ruthenus. The animals were kept in an aquarium under 12h:12h light-dark cycles. Previously to the sacrifice, fishes were adapted during two hours in light. Retinas were removed and immersed in the fixative (1% paraformaldehyde, 1.26 % glutaraldehyde, calcium chloride 0.02% in 0.1 M phosphate buffer, pH 7.4, room temperature) at 4ºC overnight. After fixation, the retinas were post-fixed with Osmium tetroxide, 2% for 1 hour, dehydrated in ethanol and embedded in Epon-812. Thick (1µm) and thin (60-90 nm) vertical sections were obtained with a Low-temperature sectioning system (Leica Ultracut UCT® Leica EM FCS). Thin sections were placed on the copper grids, contrasted with lead citrate and observed with electron microscopy (TEM and FE-SEM). Semithin sections were stained with Rosenquist´s method [29]. For this propose, sections were transferred to clean uncoated slides and left overnight in laboratory stove at 80ºC. Epon was removed from retinas by treating slides for 30 min with a solution of sodium ethoxide prepared two days before. Retinas were then stained for 2 hours in 2% silver nitrate at 50ºC, developed in silver gelatin-hydroquinone mixture until the sections turn dark brown, and then toned with 0,3% gold chloride for 3 seconds. Before their observation in a light microscope, silver stained retinas were dipped in 2% oxalic acid for about 4 seconds and treated with 5% sodium thiosulfate for 5min.

2.2. Recording Images

Semithin sections were observed in a Leica Leitz DMRB Fluorescence Microscope (Figure 1 A). Thin sections of biological specimens were observed with a Carl Zeiss Merlin Compact VP-60-43 FE-SEM. The programme used was SmartSEM Version 5.06 with Service Pack 4. Uncoated grids containing the samples were mounted on a grid holder, inserted into the microscope, and onto the stage. The distance between the samples and the detector was 3 mm. Accelerating voltage of 2 kV was used to view the sections, in the ilenseDuo BSE mode (back scattered electrons).

Following observation in the FESEM, the grids containing the sections were transferred to a JEOL JEM-1400 Plus TEM with a beam of electron of 120 KV and a camera Orius SC200 Gatan. The programmes used were TEM centre version 1.5.3.3972 and Gatan Microscopy Suite (DigitalMicrograph) version 2.31.734.0. The voltage used was 120 kV.

3. Results

Figure 1 shows the most significant results of our study collected synthetically. Figure 1A present the image of a semithin section (1 µm) of sturgeon retina stained with the technique of Rosenquist. It clearly shows the different layers of the retina from the retinal pigment epithelium (PE), at the upper edge, to the innermost layer of optic nerve fibres (OFL), passing through the layer of photoreceptors (Ph), the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL) and ganglion cell layer (GCL). Similar or even better results are obtained when we look thin sections using the FE-SEM (Figure 1 B) at low magnification.

However, the crucial in the data achieved with the FE-SEM is the capacity of this microscope to combine the ability to observe, from thin sections of the retina, both its structure laminar, at lower magnification, as the smallest details to large ones. Indeed, as seen in Figures 1A and 1B, both the image stained with the method Rosenquist (1A), obtained at lower magnification, with FESEM (Figure 1B), show a high quality and capacity descriptive of the structure of the retina. Especially good quality has the image obtained with FE-SEM.

Moreover, when we compare the images obtained with the TEM (Figure 1C) with the images obtained with the FE-SEM to high magnification, it shows the high resolution and quality of the picture obtained with this latter microscope. These data show the potential capacity of the FESEM for jointly study the structure and ultrastructure of the vertebrate retina. At intermediate magnifications of 4,000 to 8,000 X, cells and their major organelles (Figure 1D) are easily distinguished in the images of FESEM obtained from the thin sections. The nuclei, membranes, synaptic vesicles, synaptic ribbons, microtubules, etc., can also be readily identified in retinal cells at higher magnifications.

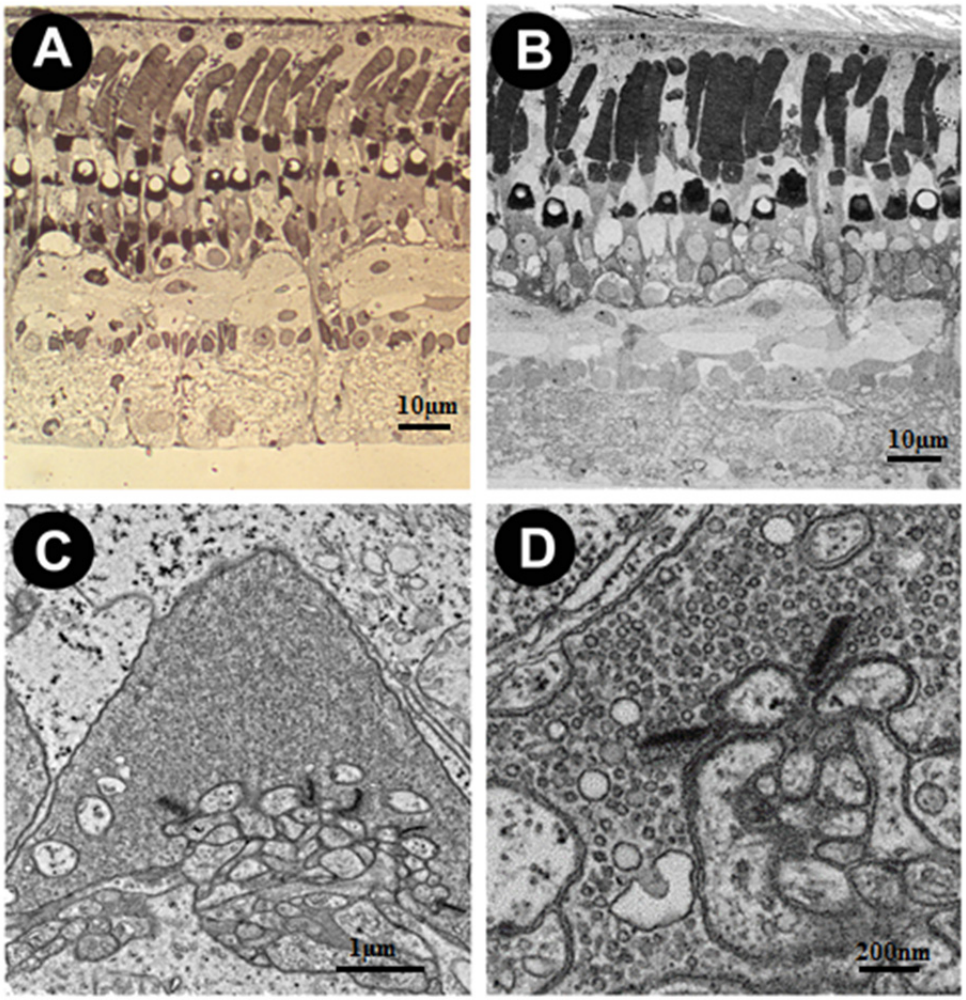

Figure 1: A and B represent retinal layer organisation visualised by, A: light microscopy using the Rosenquist method and B: FE-SEM. C and D represent retinal ultrastructural details using TEM and FE-SEM respectively.

4. Conclusions

Here, we have studied sturgeon retinas, processed with techniques for transmission electron microscopy (TEM). The ultra-thin sections prepared for TEM, when observed with FESEM, provide excellent images of the vertebrate retinas. This method allows a panoramic screening scanning to search and study in detail retinal specific cells.

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