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Methods in eye research

Ex-vivo-examination of ultrastructural changes in organotypic retina culture using near-infrared imaging and optical coherence tomography

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ABSTRACT

Optical coherence tomography (OCT) dramatically changed the way of diagnostic assessment in retinal diseases during the last years. Using this technique *in-vivo* in-depth analysis of the retina and its layers is possible. Since animal research is changing by intrinsic and extrinsic pressure to animal-(*in-vivo*)-free methods, we adapted OCT-measurements to organotypic cultures. An easy to use protocol was generated to assess standardized OCT assessments in organotypic culture. First, two custom-made devices need to be made to change any commercially available OCT for examinations in humans into a device allowing *ex-vivo* analyses of organotypic culture. The modification is feasible within seconds. After OCT measurement of the *ex-vivo* tissues, quantitative evaluation of the retinas were performed via ImageJ software. OCT pictures of *ex-vivo* retinas were obtained for time periods of seven days and the thickness of retinal tissue was evaluated. The reproducibility of the pictures and measurements was very high (SD < 15%). In conclusion, an easy to use protocol for the investigation of different effects on retinal cultures with commercially available OCT devices was successfully established.

1. Introduction

In-vivo or *ex-vivo* monitoring of physiological and pathological processes in the retina has gained increasing importance in clinical ophthalmology (Adhi and Duker; Kernstock et al., 2012; Schmitz-Valckenberg et al., 2011) as well as in experimental models (Alex et al., 2013; Fischer et al., 2009; Schmitz-Valckenberg et al., 2011). The major advantage of the non-invasive optical coherence tomography (OCT) is that the high-resolution images yield information about the *in-vivo* morphology. By applying the scanning focus to the same location of the retina at different intervals, the technique provides the dynamic change in the course of development and disease progression. Therefore, multiple assessments in form of a time-series can increase the signal-noise ratio also in animal experiments, while the combination with other techniques like immunohistochemistry, protein, and DNA or RNA analysis is still possible. Thereby the here presented adaptation of the OCT can even lead to a reduction of required animals for *in-vivo* studies according to the 3R-concept.

However, as the use of animals gets more and more restricted, researchers are nowadays often switching to organotypic cultures. Organotypic cultures - in contrast to single cell cultures - provide more complex details on the functional integrity of a complex organ such as the retina and are less artificial (Januschowski et al., 2014; Shamir and Ewald, 2014). In comparison to *in-vivo* experiments, organotypic cultures permit a direct and more intense manipulation of the tissue while at the same time having more standardized conditions (Januschowski et al., 2015; Ogilvie, 2001). Furthermore, organotypic cultures are used for investigation of a vast array of retinal diseases and processes, for example retinal ganglion cell degeneration (Johnson et al., 2016; Osborne et al., 2016), photoreceptor degeneration (Paquet-Durand et al., 2010; Sahaboglu et al., 2014), microglia activation (Ferrer-Martin et al., 2014; Karlstetter et al., 2014), ischemia (Januschowski et al., 2015;

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Schultheiss et al., 2016) and retinitis pigmentosa (Arroba et al., 2011; Lipinski et al., 2011). Especially interesting are human organotypic cultures (Fernandez-Bueno et al., 2012; Osborne et al., 2016), although the access to human retinas is -of course-very limited. In the absence of physiological perfusion, the window of observation is limited and cells are likely more vulnerable to certain influences. However, depending on the cells in the focus of investigation, cultures are stable for up to four weeks (Arango-Gonzalez et al., 2010; Pinzon-Duarte et al., 2004).

In addition, the use of dyes might provide some more precise information (Schmitz-Valckenberg et al., 2008). Functional imaging might be possible if the reflectivity behavior of the cellular structures is better understood and the resolution increases (Liu et al., 2014). In near future, the resolution of the imaging might even more improve by the introduction of swept source instruments (Drexler et al., 2014; Liu et al., 2013). This resolution allows a more precise segmentation of the intra- and sub-retinal layers (Antony et al., 2014).

Especially OCT images allow high-resolution *in-vivo* images similar to histological sections (Alex et al., 2013; Toth et al., 1997). A quantitative (e.g. by detecting the thickness) and qualitative (e.g. by assessing the structure and differentiation of the different layers) evaluation of the ultrastructural integrity of the retina can be performed (Curcio et al., 2011). In several experimental induced conditions like ischemia or glaucoma, measuring the thickness or evaluating qualitative inner-retinal changes are very important parameters, which can be hardly accessed through histology without additional artifacts.

While there are more and more reports about experiments in living animals (Otani et al., 2011), here we wanted to test the feasibility and reliability of a commercially available imaging device for the analysis of tissue culture. Using the OCT, one retina and its structural integrity can be monitored at different time points in the same fashion as *in-vivo*. Since *ex-vivo* retinas are used there is no interference of anesthesia or stress on the animal due to the measurements. This therefore results in refinement of animal experiments according to the 3R-concept. Thus promoting animal welfare, less bureaucracy regarding animal regulations, lower costs and more standardized results, while still being closely related to the *in-vivo* situation. Finally, the presented OCT-measurements are not limited to healthy retinas or a specific retinal disease. Hence, every retinal disease that can be evaluated via histology or *in-vivo* OCT can also be monitored *ex-vivo* using this technique. Therefore, it is our goal to describe and evaluate in detail the transfer including difficulties and troubleshooting from *in-vivo* experimental settings to *ex-vivo* or retinal organotypic culture imaging.

2. Materials and supplies

2.1. Retinal organ culture preparation

Postnatal day 14–15, 2 Lister-Hooded (Charles River, Germany) rats were killed with carbon dioxide inhalation, yielding n = 4 eyes for each condition. Eyes were enucleated immediately after death, washed in ethanol, PBS and R16-basal medium. Then the eyes were transferred under a sterile hood and the retinas were prepared in R16-basal medium as described previously (Arango-Gonzalez et al., 2010). After preparation, the retinas were transferred on culture plate with inserts (Corning Incorporated, NY, USA) with the ganglion cell layer facing up. Next, R16-complete medium was added into the wells (Fig. 1) and all probes were incubated in an incubator at 37 °C in an environment containing 5% CO_2.

The medium was changed the first day in culture and every second day thereafter. Eyes were transferred immediately after preparation to the incubator. To proof the possibility of investigating damage models, glutamate (1 mM) in R16-complete medium was used to induce toxicity. After 24 h of incubation the media was exchanged to R16-complete media without glutamate. For the glutamate experiments another three retinas (n = 3) were investigated.

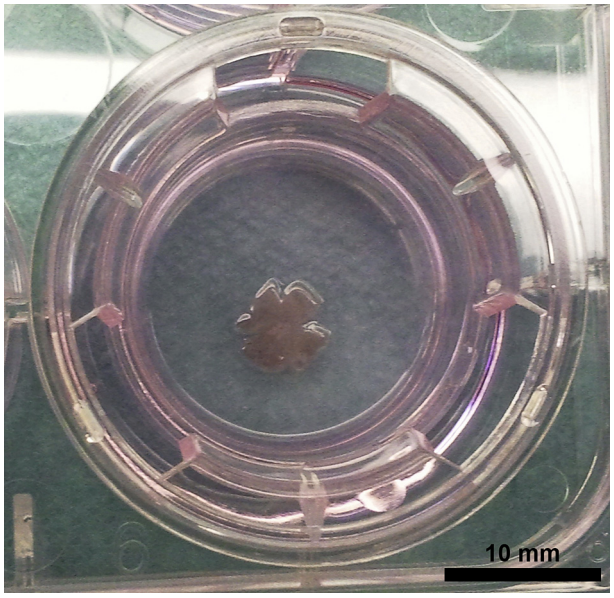

Fig. 1. Representative picture of a retinal whole-mount in the insert and 6-well plate.

2.2. OCT

High-resolution optical coherence tomography (OCT) was performed with a commercially available SD-OCT and HRA + OCT platform device (Heidelberg Engineering, Heidelberg, Germany). In order to enable standardized orthogonal repositioning of the sample, a customized mounting device was used (Fig. 2A). The mounting device was custom made to exactly fit the 24 mm cell culture inserts used for the experiments. The holder could easily be adapted to the needs of different cell culture inserts. To mimic the missing lens and cornea, we mounted an additional 78 Diopter lens (78D double aspheric, Volk Optical Inc., Mentor, United States of America) onto the OCT also using a custom made device (Fig. 2B). The devices were not permanently fixed to the OCT device. The devices were constructed to be removed from the OCT within seconds, without leaving any permanent changes to the OCT, so it can be still used on other animals or patients.

3. Detailed methods

3.1. OCT- and infrared-pictures

Cultures were investigated immediately (4 h), 24 h, 48 h and 7 days after preparation. Flat-mounts were mounted in a custom-made mounting device in a <90° angle in front of a Spectral-Domain-OCT (Fig. 2C).

According to the infrared-overview, 30°-line scans (ART: 100) were performed (Fig. 3). Additional volume scans were performed for better orientation. Volume scans consisted of 37 horizontal cross-sectional images of each retina with a distance of 154 μm between B-scans. By selecting images with sufficient quality (score > 30), a similar optical resolution was achieved. The maximum scan-depth allowed capturing the whole tissue. The average image sizes were 1536 × 1536 pixels for the infrared