



## Chapter 8

### Porcine RPE/Choroidal Explant Cultures

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#### Abstract

The cultivation of retinal pigment epithelium (RPE)-choroid explants gives the opportunity to study the RPE and Bruch's membrane in its natural environment. Porcine eyes are easily available and an excellent model for human RPE. Explants are prepared less than 4 h postmortem from cooled eyes and are transferred in fixation rings. The tissues held between rings are cultured in a perfusion organ culture system for up to a week. Viability of the explants can be investigated by calcein staining.

**Key words** Retinal pigment epithelium, Bruch's membrane, Perfusion culture

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#### 1 Introduction

The retinal pigment epithelium (RPE) is a cell of many functions and a high degree of differentiation and polarization [1]. In order to study the RPE and its interaction in the body, generally cell cultures or animal models are used. The RPE/choroid explant provides an *ex vivo* model, which has advantages over cell culture as well as animal studies, depending on the research question being studied.

The use of animal testing gives the highest degree of differentiation and tissue interaction, however, is not suited for all applications. Inherent to animal research is the high degree of interaction between cells and tissues; therefore, many confounding factors are present in the animal. Concomitantly, in the animal system, single factors are usually not easily accessible to manipulation. Furthermore, financial aspects make the use of animal testing unfeasible for many applications. And finally, ethical reasons need to be considered, as animal testing should be reduced when possible, using replacement, reduction, and refinement [2]. The use of RPE/choroid explants is very recommendable for ethical reasons, as, at least for pigs' eyes, the eyes can be obtained from abattoir as slaughterhouse wastes and can be considered as an active measure to reduce animal experimentation [3]. Apart from its easy

availability, the pig (*Sus scrofa*) is an excellent model for studying the human eye. From all non-primate eyes, the pigs' eye is considered the closest to the humans' [4]. The retina contains ten layers and presents a similar Bruch's membrane and choroid. Both present with a retinal and choroidal circulation. Pigs (like all non-primate mammals) have no fovea, but they do have a high concentration of cones, similar to humans. Contrary to many other farm animals, and like humans, they do not have a tapetum [5–7]. Furthermore, the size of the eyeball is comparable to humans and very convenient for the preparation of RPE/choroid explants [6].

RPE cell culture is widely used in RPE research. While cell culture is a highly valuable tool to study the RPE, it cannot model the three-dimensional interaction between the RPE, Bruch's membrane, and the choroid. RPE cells in culture show heterogeneity in phenotype [8], and dedifferentiation or epithelial-mesenchymal transition can be found [9]. Even if an epithelioid phenotype is reached, melanin pigmentation is generally lost during cultivation [10], especially if adult RPE cells are used and the cells are harvested by digestion and brought into dispensation prior to seeding [11]. Indeed, the capability of RPE cells to mature into a highly epithelialized state has been described as limited [11] with an extended time frame before confluency and an epithelioid phenotype is reached, compared to other epithelial cells [12]. Main causes for the transdifferentiation of RPE cells are the removal of the RPE from the choroid [13] and the loss of the cell-cell contacts of the RPE cells [14].

RPE/choroid explant cultures give the opportunity to study the three-dimensional interaction between the RPE and the choroid without prior dissociation of the cells from the choroid and from its cell-cell contacts, keeping the RPE on its natural substrate and avoiding the induction of mesenchymal transdifferentiation. Furthermore, these explant cultures allow autocrine and paracrine tissue interactions not found in cell cultures, allowing the RPE to dwell in their "natural habitat." As the cells do not have to redevelop their epithelial phenotype, no time is lost in order to reach confluency and for the differentiation procedures.

RPE/choroid explants can be cultured in different systems that can be divided into stationary and perfusion systems [15, 16]. The perfusion system used in this method was developed by Minuth et al. [17–19] and optimized for RPE/choroid explant cultivation [16, 20]. It can be used with neural retina [21] or without [16]. As the neural retina undergoes fast degeneration, especially of the photoreceptors [21] and the ganglion cell layer [22], and this may influence RPE function, here we describe the cultivation of RPE/choroid without neural retina. For cultivation with neural retina, please see Kobuch et al. (2008) [21].

The perfusion tissue culture system was developed to enable a high degree of cellular differentiation and to optimize the cellular



environment [19]. The explants are placed in a culture container which is constantly supplied with fresh medium, creating a constant environment [19]. This guarantees constant nutrient supply as well as avoids accumulation of harmful metabolic products. In addition, it creates a steady-state equilibrium concerning proteins secreted by the cells [16]. Since this system is mostly run outside of a CO<sub>2</sub> incubator, the medium is supplied with a pH-stabilizing buffer (e.g., HEPES) [19]. The medium is pumped with a peristaltic pump at a rate of 1–2.5 mL/h. In order to supply the culture with oxygen, long silicon tubes with a small diameter with thin wall are used which allow gas exchange with the surrounding atmosphere. The culture container is kept on a thermal plate, and by attaching the silicon tubes to the thermal plate, the medium supplied will have the appropriate temperature when reaching the perfusion container [16, 19]. This perfusion system is especially suited to investigate secreted proteins, e.g., when investigating the efficacy of potential therapeutics to interfere with growth factor secretion [3, 23] or when investigating the pathways of growth factor regulation [24].

Different kinds of containers can be used, notable those for one or six explant rings and those with or without separated apical and basal perfusion (*see* <http://www.minucells.de/index1.html>). The container should be chosen according to experimental preference.

Of note, the life expectancy of the RPE-choroid organ culture in a perfusion culture is limited to about a week. We suggest to start experimentation the second day after preparation. The best time period for experimentation is day 2 until day 5 in culture [16]. Experiments can be conducted over the course of a week to 10 days; however, one has to consider that degenerative changes may influence the results obtained during time periods exceeding 5 days. Calcein staining to investigate cell viability in culture is highly recommended at the end of experimentation. In this assay, calcein AM is readily taken up into the cells and intracellularly converted by esterases into a green fluorescent molecule [25]. Viable RPE cells are easily detected by intense green staining, while the loss of vital RPE cells is visible as non-fluorescent black spots (or areas).

In this chapter, the method to prepare and culture a porcine RPE-choroid organ culture in a perfusion culture system using a system provided by *Minucells and Minutissues*, and the way to evaluate cell viability of the RPE cells with calcein staining, will be introduced.

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## 2 Materials

### 2.1 Equipment

1. Preparation tools, autoclaved (121 °C):
  - (a) Micro Scissors
  - (b) Forceps

- (c) Surgical scissors
2. Perfusion culture chamber, autoclaved (105 °C): Several containers are available. See [www.minucells.de](http://www.minucells.de).
  3. Tissue carrier (e.g., Minusheets), autoclaved (105 °C).
  4. Heating plate: The temperature should be turned on and set around 39 °C (see **Note 1**).
  5. Silicon tubes (narrow diameter, capable of oxygen exchange, autoclaved): (see **Note 2**).
  6. Peristaltic pump with variable settings (e.g., 2.5 mL/h).
  7. Styrofoam boxes with cool packs.
  8. Bottles for medium supply (autoclaved).
  9. Bottles for medium waste (autoclaved).
  10. Optional: T-shape stopcock and liquid fraction collector.
  11. Fluorescence microscope: FITC filter ( $\lambda_{ex}/\lambda_{em} \approx 488 \text{ nm}/520 \text{ nm}$ ) is required for calcein viability test.
  12. Slide glass and cover glass for calcein viability test.

## 2.2 Culture Agents

1. Culture medium: Mixture of equivalent amount of a and b (1:1)
  - (a) Ham's F12 (with L-glutamine)
    - + 10% Porcine Serum
    - + 25 mM HEPES
    - + Antibiotics
  - (b) Dulbecco's Modified Eagle's Medium (DMEM, high glucose, with L-glutamine)
    - + 10% Porcine Serum
    - + 25 mM HEPES
    - + 100 mM Natriumpyruvat
    - + Antibiotics
2. Povidone-iodine solution: For disinfection of the surface of eye ball. Dilute 10% Betaisodona solution 1:3 in water (2.5% povidone iodine). Prepare about 25 mL in a 50 mL conical tube (cooled on ice).
3. Phosphate buffered saline (PBS: without calcium and magnesium) solution in beaker (cooled on ice).

**2.3 Porcine Eyes**

Porcine eyes need to be obtained from a local slaughterhouse on the same day of preparation. Postmortem time after enucleation must not exceed 4 h. Eyes should be preserved in cool dark conditions until use (*see Note 3*).

**2.4 Drugs and Agents for Viability Assay**

1. Calcein AM: Stock solution (1–5 mM in DMSO).
2. PBS (without calcium and magnesium).
3. Culture medium (the same one as used in the perfusion culture).

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**3 Methods****3.1 Preparation of Explants (Fig. 1)**

1. Clean the eyes of all connective tissue, fat, and muscles, and incubate them in Betaisodona solution for 5–10 min. Transfer eyeballs in sterilized PBS solution.
2. Make a clean circle dissection at the sclera 5–6 mm behind the corneal limbus with scalpel or razor blade and scissors to remove the anterior part of the eye (cornea, lens, iris) and the vitreous. Do not touch RPE sheet with forceps! The retina may or may not remain on the RPE after this step. If it remains, it is better to go to the next step without peeling it here.
3. For convenience, the nasal part of the eyecup can be resected (Fig. 1, *asterisk*) (*see Note 4*).
4. Lift just (retina-) RPE/choroid sheet carefully with fine forceps at the incision site, and separate it from the sclera using forceps and very fine and sharp surgical scissors. Always lift the tissue sheet carefully during this procedure, since it may easily tear if too much is pulled. Do not use teared tissues or tissues with holes.
5. When a tissue sheet of about 1 cm<sup>2</sup> is released from the sclera, carefully insert a white plastic ring (Minusheet) under the tissue, so that the whole ring is covered. If the retina has remained on the tissue until now, now carefully remove the retina from the tissue by picking up the retina outside the ring area and slowly pull it off.
6. Put the black ring on top of the white ring (with the tissue sheet in between). Let it “click” with soft pressure. Now pick up the ring with forceps and resect the tissue around the rings. (Note: the more accurate the surrounding tissue is removed, the less background “noise” will be interfering with the measurements).

Transfer the ring carriers with tissue in a petri dish and have it covered with medium at 37° (on a heating plate) until moved to the perfusion chamber.





**Fig. 1** Procedure to prepare the RPE-choroid explant, referring to the statements in Subheading 3.1

### 3.2 Tissue Culture

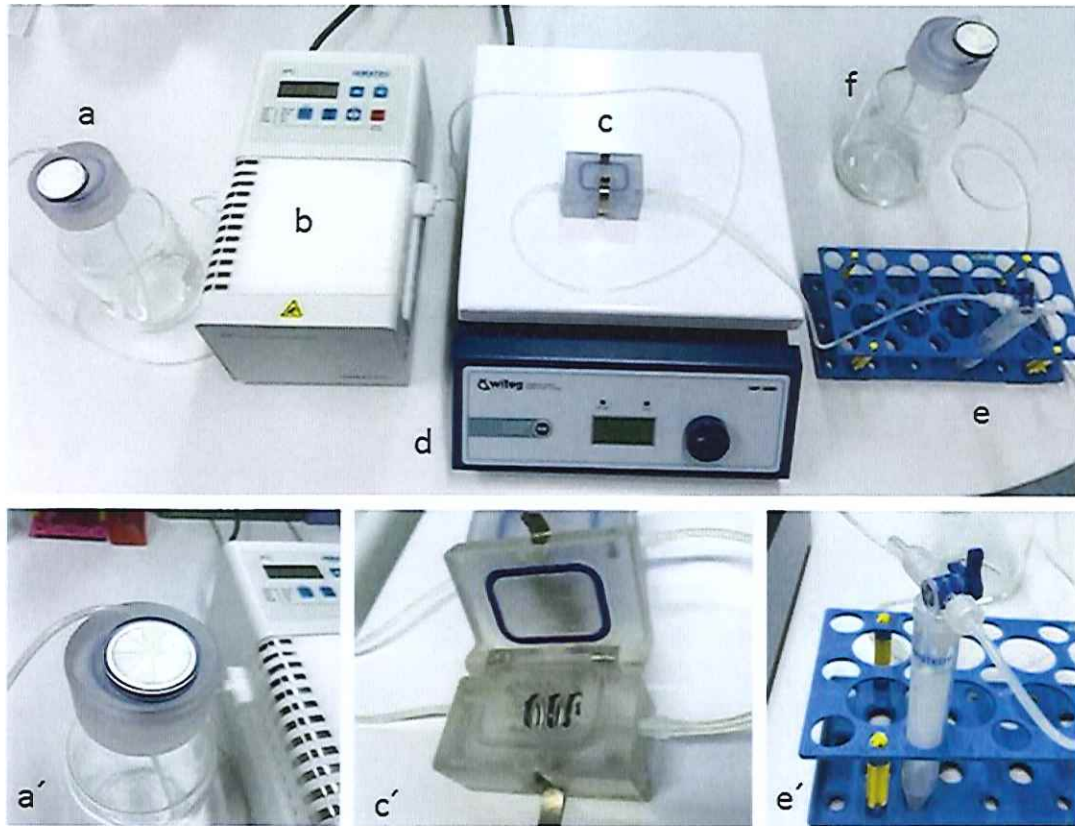
For perfusion organ cultures, several containers are available. There are horizontal chambers where media can be differentially supplied to the apical and basal compartment, and there are vertical chambers, in which the tissue is surrounded by a single medium. Also, chambers can be loaded with single tissue rings or multiple tissue rings. The chamber used needs to be chosen according to the experimental design.

The workplace should be equipped with bottles in cooled styrofoam boxes with medium supply, a peristaltic pump, a heating plate, and bottles for waste collection or liquid fraction collector (Fig. 2). The medium supply should be connected to the tissue container with silicon tubes which connect to the container. Depending on the kind of container, one or two supply bottles may be needed. The silicon tubes are positioned and fixed on the heating plate to ensure the heating of the medium to 37 °C. The medium should be pumped through the system by the peristaltic pump.

The procedures below need to be performed in the clean bench, as long as the system is not closed.

1. Connect all parts of the perfusion system (the bottles for the fresh medium, containers, the bottles for the wastes) with tubes in the clean bench.
2. Attach the cassettes for the peristaltic pump to the silicone tubes between the medium bottle and the culture container.
3. Fit the cassettes at the pump device, and set the rotation speed of the peristaltic pump, so that the medium flow reaches as desired, generally 1–2.5 mL/h (*see Note 5*).
4. Start the pump, let the medium flow into the tissue container, and fill the container with the medium.
5. Place the tissues carriers in the container (in the clean bench). Close the lid (*see Note 6*).
6. Start the pump.

We highly recommend leaving the container undisturbed until the second day after preparation, in order to allow a steady-state equilibrium to build up. We do not recommend exceeding culture time for more than 1 week.



**Fig. 2** Setup of perfusion culture system. (a) (a' for a large image) the glass bottle for the fresh medium with a filter for the air filtering, (b) peristaltic pump, (c) culture container (c' shows, how the tissues can be aligned), (d) heating plate, (e) (e' for a large image) T-shape stopcock with a collecting tube, (f) the glass bottle for the waste

### 3.3 Calcein-AM Viability Staining

1. Prepare 1–10 mM calcein-AM solution with culture medium.
2. Transfer the tissue carriers from the perfusion culture container to a 12-well plate filled with the calcein-AM-containing medium.
3. Incubate the tissues for 30 min in a 37° incubator.
4. Wash the explant twice with PBS.
5. Carefully release the tissue from the tissue carriers by using a pair of forceps; place the RPE-choroid sheet on the slide glass with the RPE side up. Put the cover glass onto the RPE without any extra mounting solution. The tissue is wet enough to keep the cover glass attached.
6. Inspect it with the fluorescence microscopy. Use the filter for FITC ( $\lambda_{ex}/\lambda_{em} \approx 480 \text{ nm}/520 \text{ nm}$ ). Living cells are detected due to green fluorescence of calcein (dye is distributed both in cytoplasm and in nucleus), whereas the loss of vital RPE cells is observed as a dark spot or area (Fig. 3) (see Note 7).

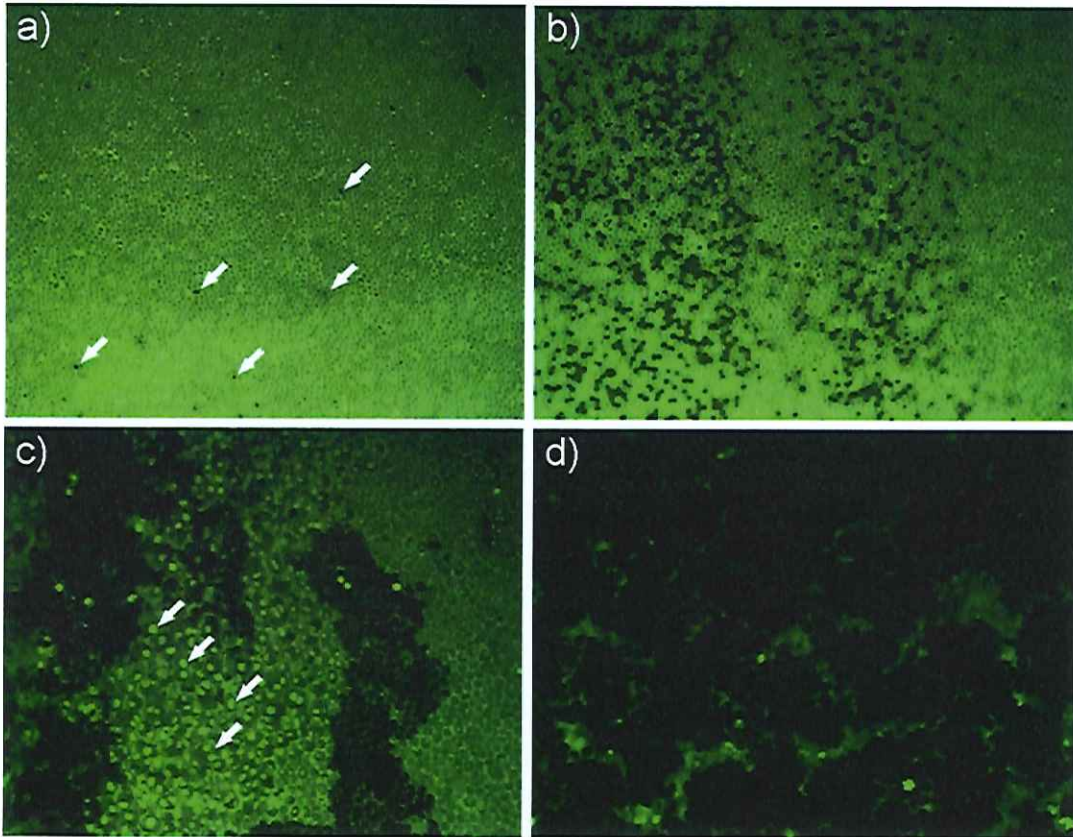


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## 4 Notes

1. It is necessary beforehand to adjust the setting temperature of the heating plate, such that the medium temperature in a culture container can be preserved around 37 °C. It depends on the material and thickness of the container wall and the atmosphere temperature.
2. The medium should be exposed to the atmosphere, which allows for gas exchange and the partial equilibration of the perfusate O<sub>2</sub>/CO<sub>2</sub> levels with the atmosphere. In most studies with RPE explants in perfusion culture system to date have been performed under normal air conditions with silicone rubber tubes having a high oxygen permeability rate. However, in case aeration is performed and the medium with higher oxygen concentration than in the air is to be provided to the tissues, loss of oxygen from perfusate flowing through the tubing wall during perfusion may be a crucial matter. Previous study reported that oxygen-aerated medium lost oxygen the most through the silicone rubber tube, compared to other materials as polyvinyl chloride (PVS), polyethylene, or polyamide (nylon) [26].
3. In order to obtain viable cultures and reproducible results, a high quality of explants is vital! Eyes need to be obtained 4 h after death and immediately cooled. Quality of eyes and cultures may decrease in summer. If neural retina is to be used, postmortem times should be reduced to 1–2 h, and eyes should be kept in the dark [21].
4. For this procedure the position of the optic nerve head can be used for a guide. The narrower side next to the optic nerve head is the nasal side.
5. Medium flow speed may vary with the tube diameter. Moreover, there are several different types of pumps with different lower/higher speed limit and the range of the suitable size of tubes. These points are to be considered in choosing the pump and tubes.
6. In perfusion culture, gas bubbles attaching to the explant may be a problem. Forty-five degrees or vertical positioning of the culture may be helpful.
7. Since apical part of the RPE cells are mostly occupied by a number of melanosomes, the fluorescence signals in the cytoplasm of vital cells can be blocked to some extent, and stronger signal comes from the cell-cell border. Nevertheless it is still no problem to distinguish the vital cells from the dead cells (Fig. 3).





**Fig. 3** Examples of calcein stainings. Dead RPE cells are visible as dark areas. Depicted are (a) healthy tissue with minimal dead RPE cells (arrows), (b) tissue with extended cell death, (c) tissue with extended dead area and condensed nuclei (arrows), (d) tissue with almost complete RPE cell death. Only tissue (a) is suitable for experimentation

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