EXPERT REVIEWS

Retinal pigment epithelium–choroid organ culture

Expert Rev. Ophthalmol. 6(6), 669–680 (2011)

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The retinal pigment epithelium (RPE) plays a vital role in retinal function, and therefore studies on RPE provide a significant benefit for our visual function. In order to obtain useful information from study results, choosing the suitable experimental model for each purpose of study is of great importance. Although RPE cell cultures are widely used, cells in cell culture have significantly different phenotypical characteristics from *in vivo* cells. The advantage of using native tissue is that cells in the tissue have close biological properties to *in vivo* conditions. This review describes basic characteristics of native RPE–choroid tissues in comparison to RPE cells in cell culture and introduces the possibility of preserving tissues in different culture systems. Advantages and disadvantages of organ culture and suitable studies with recent study results are also introduced.

Keywords: cell culture • laser • native tissue • perfusion culture • physiological study • RPE–choroid organ culture • static culture • tissue degradation • tissue preservation • wound healing

The retinal pigment epithelium (RPE) is a cellular monolayer between the neural retina and Bruch's membrane. It adheres to and interacts with the underlying Bruch's membrane at its basal side and with the photoreceptor outer segments at its apical side. It contributes to a turnover of photoreceptor outer segment, and thus the loss of RPE function may directly cause the loss of visual function. Along with increasing diagnostic and therapeutic methods, we now have the opportunity to explore the functions or the responses of the RPE; for example, the cellular response to the newly introduced intraocularly injected medicines such as anti-VEGF antagonists or the responses following new laser therapies.

Cell culture technique has shown remarkable progress since the 1960s. Basic research with RPE cell culture has been extensively carried out and abundant experimental data have been provided to date. Experiments with monolayer RPE cells on plastic culture dishes or membrane filters have advantages in handling, preservation, stability and experimental repeatability, and is therefore now an essential method for basic scientific research on RPE. For the cell culture study, ARPE-19, a human RPE cell line, and mammalian RPE primary cell cultures are mostly used.

RPE–choroid organ culture is a culture method to preserve RPE–choroid tissue explants for a certain time period in culture medium. RPE adheres to Bruch's membrane, and morphological and functional cell polarity is well preserved in explant tissue compared with cell culture. The role of Bruch's membrane in the pathogenesis of age-related chorioretinal diseases has been paid more attention recently, therefore studying with RPE on natural Bruch's membrane has a big advantage in understanding age-related cellular changes.

RPE–choroid tissue explants have been utilized as an experimental model mostly for RPE morphology and physiology since the 1970s. It has also been used in other studies, such as studies on phagocytosis activity and wound-healing responses of RPE. In addition to the conventional method to preserve the tissue explant in a static culture medium, the perfusion culture system has been recently introduced and provides the tissue with a better organotypical culture environment.

This review article describes basic biological properties of RPE–choroid organ culture in comparison to RPE cell culture. Advantages and disadvantages of RPE–choroid organ culture as well as its future prospects are discussed, along with the discussion of recent studies.

History of RPE–choroid organ culture

Before the cell culture technique was established, many approaches had been attempted in order to investigate RPE functions using RPE–choroid tissue explants. Studies using RPE explants date back to around the 1920s [1,2]. Better

 10.1586/EOP.11.70 ISSN 1746-9899

preservation of the tissue in culture medium was achieved from around the 1960s, after the Eagle's medium was developed [3]. Cellular physiological properties such as active/passive ion transport [4–8] and barrier functions [9] have been studied as short-term (at longest several hours) examinations using RPE–choroid tissue explants. For longer cultivation, morphological assessment [10–12], phagocytosis activity [13–15] and wound healing of RPE [16–18] were reported between the 1970s and 1990s using static organ culture.

At the beginning of the 2000s, RPE–choroid organ culture began a new phase. Using new technology of tissue engineering, Framme *et al.* applied a perfusion culture system established and developed by Minuth [19] for the cultivation of the RPE–choroid tissue explants with flowing medium with controllable flow speed [20]. The introduction of the perfusion system provides a mimicking of the *in vivo* biomechanical environment.

This perfusion system has been subsequently utilized by other researchers in the ophthalmic field [21–27] and some have attempted to study RPE–choroid tissue, including neural retina, in this perfusion system [22–26]. Retinal detachment and faster degenerative changes of the retina are unavoidable in the culture, and this is discussed in more detail later. It is still an open question whether or not it is favorable to culture the retina together with the RPE–choroid sheet, in order to investigate biological cell behaviors of RPE. Hence, this article focuses on the RPE–choroid organ culture without neural retina.

Preparation of the tissue for RPE–choroid organ culture

Donor animal

Frog [28], chick embryo [18], porcine [20–22] or bovine [11,15] eyes have been most commonly used for RPE–choroid organ culture research. Concerning genetic homology, the usage of the eyes from mammals is beneficial in order to obtain data closer to the human eye. In particular, the porcine eye resembles the human eye in anatomic and physiologic characteristics better than any other mammalian eye, except for primates, and therefore has been used most often in recent years.

Due to the difficulty of frequent acquisition and the restriction of the postmortem time, usage of human donor eyes is seldom included in the study with RPE–choroid organ culture.

Preparation of the RPE–choroid tissue

Eyes are required to be freshly enucleated and the postmortem time has to be within 5 h in order to obtain vital RPE cells. Furthermore, the eyes should be kept cold (4°C) until preparation.

RPE–choroid tissue explants are prepared mainly by using these two methods: after the removal of anterior parts of the eye, vitreous and retina, cutting out the RPE–choroid–sclera in pieces using scissors or trepan is then followed by the careful removal of the sclera or the separation of RPE–choroid sheets from the sclera with use of forceps and scissors. Tissue has to be held by good supports in order to avoid its wrinkling or folding. The brief procedure is shown in Figure 1 and a detailed description is found in the appendix.

Retinas lose their adhesion to the RPE layer immediately after death, and the attachment never restores during cultivation. Postmortem retinal detachment is unavoidable, mainly due to a loss of hydrostatic and osmotic pressure difference and a loss of adhesion between RPE and photoreceptor outer segments [29–32]. Degradation of the retina is faster than the RPE [22] and therefore co-culture with the retina might negatively influence RPE–choroid tissue through the secretions coming from the degenerating retina [21]. To date, retinal function, which can be measured by the electroretinogram, can be preserved for up to 10 h in optimal medium [33]. This suggests that retinal function disappears at the first day after preparation, although the morphology is preserved for a few days longer [22].

Cultivation methods

The methodology of organ culture is still developing. There is no gold standard and every researcher uses his/her own method depending on the aim of their study. Here, the different culture methods that have been used are introduced. In terms of the medium supply, the culture method is divided into two; static culture and perfusion culture (Figure 2). Comparisons of the culture conditions of these two different methods are shown in Table 1.

Static culture

RPE–choroid tissue is preserved in a static culture medium in a culture container, such as cell culture dish (Figure 2A) [18,34], Lighton tube [10], Ussing-type chamber (Figure 2B) [4,6–8], or in an eye cup itself [14,15]. As a tissue support, Millipore filters [10,18,34] or custom-made mounting holders [5–8] were used. Glucose consumption of RPE–choroid tissue is so high that the medium needs to be changed more frequently than the RPE cell culture (Table 2), although it is dependent on tissue size and the amount of medium. They are maintained in a CO_2 incubator (37°C, 5% CO_2).

The Ussing chamber is an experimental tool that was invented by Ussing, and applied for the first time by Ussing and Zarahan to measure Na^+ transport in frog epithelium [35]. The Ussing chamber consists of two halves, so that the tissue apical and basolateral sides are cultured under different medium supplies. Many studies using modified Ussing chambers contributed greatly to the understanding of the physiological properties of RPE [4]. In order to keep the medium temperature at around 37°C, some studies were performed with a modified system with a water jacket-surrounded Ussing chamber [7,8].

It is also possible to use a chamber, that differs from the original Ussing-type, where the apical and basal sides are separated, as shown in Figure 2C (prototype in our laboratory). With both Ussing-type chambers and this type of chamber, where apical and basolateral sides are divided, cell biological polarity and transepithelial resistance (TER) can be well preserved. High TER indicates high integrity of RPE tight junctions, which forms the outer blood retinal barrier. For the study of the RPE barrier or transport functions, in which a high TER is required, these chambers are suitable for use.

Perfusion culture

Perfusion culture is a culture method, in which medium flow is controlled by pumping, and therefore the tissue is constantly

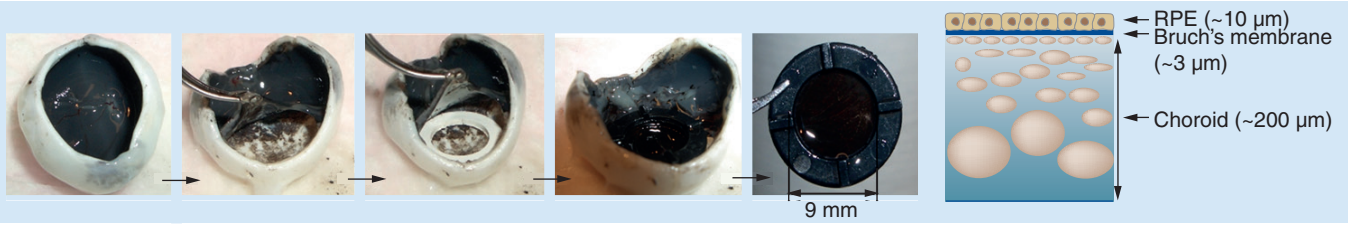

Figure 1. Brief introduction of preparation procedure of retinal pigment epithelium–choroid tissue explants for organ culture. A detailed procedure is given in the appendix.
RPE: Retinal pigment epithelium.

perfused with fresh medium without manual replacement. Therefore, perfusion organ culture systems provide tissues with an environment that more closely mimics the *in vivo* environment.

Perfusion organ culture techniques were introduced in the 1970s [36,37]. Attempts to preserve RPE–choroid tissue in a perfusion culture system were introduced for the first time by Framme *et al.* in 2002 [20]. They used a perfusion culture system that was originally developed and established by Minuth *et al.* for the culture of embryonic renal epithelia [19] (Minucells and Minutissue Vertriebs GmbH). RPE–choroid tissue is held between two rings (Minusheet) and placed in a culture chamber. There are several types of chambers available: a maximum of six tissue-rings can be placed either in parallel (Figure 2D) or horizontally, in which mediums flow at their apical and basal side separately (Figure 2E). The culture chamber is placed on a heating plate, which keeps the temperature of the medium at around 37°C. Flow speed of the medium is controlled by a pump, and reported speeds in past studies range from 1 ml/h [20,22] for one ring to 2.5 ml/h for six rings [21]. For a culture with a flow speed of V ml/min and with a N ml volume of medium in the chamber, the medium dilution process is described with the following equation:

$$A(t) = A(0)e^{-kt}$$

where A(t) = concentration at a given time point, A(0) = initial concentration, k = dilution constant (1/N × V) and t = time (min).

Culture medium

Mediums used for RPE–choroid organ cultures are similar to the medium for RPE cell cultures. In recent years, Dulbecco's modified eagle medium (DMEM; high glucose) and Ham's F12 are often used. Some researchers culture with 100% DMEM [20,22], and some with a mixture of equal parts DMEM and Ham's F12 [21,25,26]. Regarding serum type and concentration, bovine serum or fetal calf serum with a concentration of between 5 and 15% are mostly used. There is no existing report comparing tissue properties in different medium components. Due to its higher glucose consumption, daily replacement of the medium is recommended in static culture. Perfusion culture does not need manual medium replacement. For example, measured daily glucose consumption of confluent human RPE cell culture in a culture dish (passage 5) was 2.4 µg per unit area, while consumption by a static organ culture was 46.2 µg per unit area [Miura Y; Unpublished Data]. The medium of inlet is kept at 4°C and is warmed up to 37°C while it runs on the heating plate before supplying the tissue. For perfusion culture systems, if the tissues are

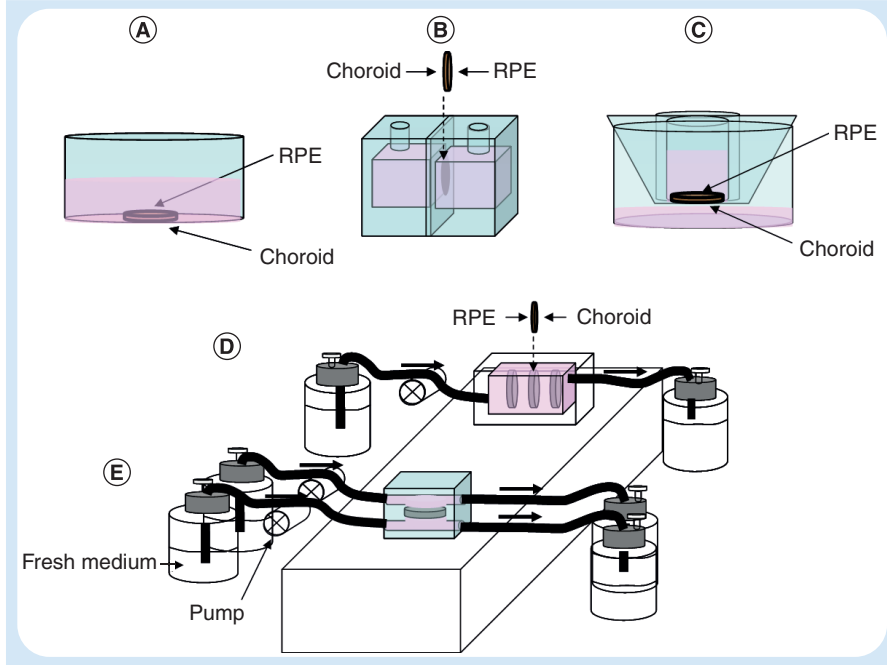

Figure 2. Schematic representation of static and perfusion retinal pigment epithelium–choroid organ culture. (A–C) Static cultures, **(D & E)** perfusion cultures. **(A)** Static culture in a cell culture dish. RPE–choroid tissue is simply placed on the bottom of a culture dish and cultured in the medium. **(B)** Ussing-type chamber. Cell apical and basolateral sides are cultured under different medium supplies. **(C)** Static culture with an insert (laboratory prototype). As with a Ussing-type chamber, tissue explant with its support can be placed between the two different parts containing different medium. Cell apical and basolateral sides are cultured under different medium supplies. **(D)** Bath-type chamber. Tissue can be mounted in organ bath with inlet and outlet of medium flow. **(E)** Chamber for separated medium supply. Medium is supplied to cell apical and basolateral sides separately.
RPE: Retinal pigment epithelium.

Table 1. Basic setup conditions for static and perfusion organ culture.

Culture conditions	Static culture	Perfusion culture
Place of cultivation	Inside incubator	Outside incubator
CO_2	5%	0.03%
Temperature	37°C	37°C
pH stabilizer in medium	Not required	Required
Medium change	Required daily	Not required

cultured outside a CO_2 incubator, a pH stabilizer such as HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) is required. pH and glucose concentration in conditioned culture medium in static and perfusion organ culture after 24 h of cultivation are shown in Table 2 [Miura Y; Unpublished Data]. Due to larger medium supply and its dilution over time, medium glucose concentration does not decline as fast in perfusion culture compared with in static culture. For example, if the perfusion culture medium flows with a speed 1.5 ml/h, with a starting glucose concentration of 2.76 mg/dl (DMEM: Ham's F-12 = 1:1 and 10% serum), the concentration in the conditioned medium of the perfusion culture in 24 h was as high as 2.43 mg/dl (chamber medium volume is 4.5 ml for three tissues, flow volume: 36 ml/24 h = 12 ml/tissue/24 h), whereas it declines to 1.78 mg/dl in a static culture medium (3 ml medium for one tissue). Perfusion culture systems can preserve this glucose concentration as stable over the whole culture period, while tissue in static culture suffers from this concentration fluctuation every day. Medium pH of static organ culture after 24 h of cultivation is kept around 7.3 in a CO_2 incubator, and gradually shifts to acidic if it is cultured after several days. On the other hand, in perfusion culture, which is kept at room temperature, where only 0.03% CO_2 is present, medium pH in the tissue chamber reaches approximately 7.5–7.6. There is a possibility of mimicking the gas atmosphere of the CO_2 incubator with the use of a gaseous exchanger provided by the manufacture of Minucells and Minutissue perfusion culture systems. However, the effect of gas atmosphere on the condition of RPE–choroid tissue has not yet been evaluated.

Cellular properties: comparison with cell culture

Morphological properties

Morphological characteristics of RPE in native tissue and in cell culture are schematically shown in Figure 3. RPE cell in native tissue are hexagonal in cross-section and approximately 10–15 µm in height. The apical side of the cell is full of melanosomes and the nucleus is located close to the basal side (Figure 3A). Although cultured RPE develop some of the epithelioid structural features, most of the isolated RPE cells lose their cuboidal shape, apical microvilli and organized cytoskeletal organization (non-polarized, Figure 3B). Native RPE cells have a special feature of including a number of melanosomes, whose density reduces over passage in the cell culture [38,39]. This depigmentation is due to the dilution of pigment granules among daughter cells, although there is some evidence of melanogenesis *in vitro* RPE cells, which occurs however, only under special conditions that are different from normal cell culture conditions [40]. Cells on membrane filters, whose apical and basolateral sides can be cultured by separated medium supply, present a better biological polarity compared with cells on the culture dish (polarized, Figure 3B) [41–43], although the depigmentation over passage is unavoidable due to the reason described above. Native RPE in mammalian eyes has its apical microvilli [11,22]. Although the RPE in the explants has initially no apical microvilli, they recover during the first 2–3 h of cultivation [11].

For an integrated physiological function, cytoskeletal and junctional properties play significant roles. A tight junction protein occludin in a confluent RPE cell culture is distributed at RPE cell–cell junctions similar to those in native tissue (Figure 4B & E). On the other hand, there is a significant difference in the distribution of F-actin filament. In cell culture after several weeks of confluence, most F-actin fibers are distributed in stress fibers, which run along the long axis of the cells (Figure 4C) and do not form clear circumferential bands as seen in tissue explants (Figure 4F). A zonular pattern of the actin cytoskeleton is an important measure of epithelialization since the actin network is one of the determining prominent features of an epithelial cell. This cytoskeletal immaturity of confluent RPE cell culture has also been reported in previous studies [44].

Table 2. Medium properties after 24 h of cultivation in static and perfusion organ culture.

Medium properties	Static organ culture†	Perfusion organ culture‡
pH	Pre: 7.29 Post: 7.29	Inlet§: 7.29 Outlet§: 7.57
Glucose concentration (mg/dl)	Pre: 2.76¶ Post: 1.78	Inlet: 2.76 Outlet: 2.43

†1×RPE–choroid tissue (diameter 9 mm) was placed on the dish bottom and cultured for 24 h in 3-ml static culture medium in incubator.
‡3×RPE–choroid tissue (diameter 9 mm) were placed in the chamber (rest volume: 4.6 ml) and cultured for 24 h in perfusion culture system, with medium flow 2.5 µl/min.
§Inlet indicates the medium before flowing into the chamber and outlet indicates the medium flowing out of the chamber.
¶Medium: Dulbecco's modified eagle medium (high glucose 4.5 g/dl): Ham's F-12 (glucose 1.8 g/dl) = 1:1 with 10% porcine serum, 1% penicillin–streptomycin and with 2.5% HEPES (4-[2-hydroxyethyl]piperazine-1-ethanesulfonic acid).

Gene-expression properties

Interaction of the cell basement membrane with transmembrane molecules modulates cellular gene expression. Once isolated from its natural basement membrane, RPE cells, which are attached and grown on a new different scaffold, express significantly different gene profiles from native tissue.

Tian *et al.* reported a comparison of mRNA phenotypes between native RPE cells and ARPE-19 cells grown on different materials. Their results suggest that the overall expression profile of cultured cells is significantly different from that of native RPE. Surprisingly, ARPE-19 cells

grown on a plastic bottom were the closest to native RPE compared with cells grown on extracellular matrix [45]. They suggest that this *in vitro*-induced alteration of the expression of the genes related to cell cycle and morphology would make it difficult to interpret age-related changes obtained in an *in vitro* culture system.

A transcriptome analysis presented a global expression profile of 'signature genes' encoding proteins involved in important visual functions of human RPEs: native human RPE tissue from fetal and adult donor eyes and cultured RPE cells (fetal cultured RPE cells and ARPE-19 cells). Approximately half of the RPE signature genes are expressed at lower levels in ARPE-19 compared with native tissue, while fetal RPE culture approximately one-fifth [46].

Since RPE in organ culture maintains its genetic phenotype, it might be suitable to utilize in the study of age-related cellular changes. The RPE cells in 8–10-day-old perfusion organ culture have been shown undergoing apoptosis and morphological deterioration [21], which is similar to the changes of RPE in the macula from the donor eyes [47].

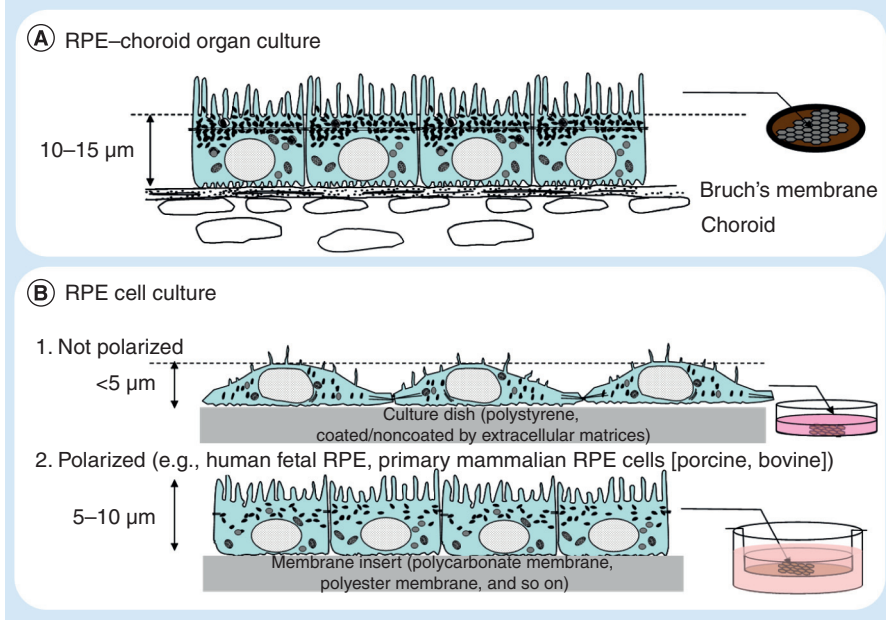

Figure. 3. Schematic illustration of morphological typical characteristics of retinal pigment epithelium cells in retinal pigment epithelium–choroid organ culture (native tissue extract) and in cell cultures of pigmented mammalian retinal pigment epithelium. (A) RPE cells in native tissue extract have their original cuboidal epithelial morphology with melanosomes at cell apical side and nucleus at basal side. **(B)** RPE cells in passaged RPE cell culture on cell culture dish. Cells in passaged cell culture typically lose their native morphological characteristics (lower cell height, loss of polarity, lower microvilli and fewer basal folding). **(C)** RPE cells cultured on the membrane filter preserve their epithelioid morphological characteristics with better polarity.
RPE: Retinal pigment epithelium.

Physiological properties

Physiological properties of native RPE were studied using RPE–choroid tissue explants in the 1980s and 1990s. Extensive bodies of works by many researchers contributed to the understanding of RPE physiology and electrophysiology; membrane transport mechanisms of ion and fluid, potassium, sodium, chloride, bicarbonate, calcium and other organic molecules [4,6,48–50], and basic electrophysiological properties such as TER, transepithelial potentials, apical and basal basement membrane potentials, and apical-to-basolateral membrane resistances ratio [5,7,8,51] were also studied.

The function of RPE as an outer blood retinal barrier is determined by its structural and physiological polarities. For studying RPE physiology or pathophysiology, an RPE cell monolayer with high barrier function is required. High TER reflects high junctional integrity of epithelium. ARPE-19 cell culture has significantly lower TER compared with native RPE [52–54]. Most cell cultures need approximately 1 month after confluence for TER to become stable [41,51]. Ablonczy *et al.* showed that confluent monolayer of ARPE-19 does not exhibit TER above 100 $\Omega \bullet m^2$ even after 90 days [52]. In contrast to ARPE-19, human fetal RPE primary cells cultured on a membrane filter exhibits epithelial morphology and higher junctional integrity with higher TER compared with ARPE-19 [42,43], and is utilized as an alternative model for a RPE physiological study [43,55–57]. Due to the possibility of more stable and longer lasting culture conditions and the direct accessibility to RPE basolateral membrane without choroid, studies with human fetal RPE cell culture on the membrane filter seemed to be the preferred method last decade. However, there are disadvantages of cell culture, such as loss of polarized expression of the Na^+/K^+ pump [56,58] and different expression of ion channels than in native RPE [59,60]. These matters have to be taken into account.

Table 3 shows the electrophysiological properties of native RPE tissue from animals, ARPE-19 cell culture and human fetal RPE cells.

Preservation of tissue explants

Morphological alteration

During the first several days of cultivation, RPE cells in RPE–choroid organ culture preserve their epithelial characteristics: apical-aggregated melanin granules as seen *in vivo* [10,12,21]. Morphological alteration occurs faster than in cell culture, which was assessed with histological study. RPE in perfusion organ culture begins to change its morphology, with a loss of polarity of melanin distribution irregularly in 4–6 days [21]. In 8–10 days, this polarity loss appears more significant and some cells become enlarged, rounded and dome-shaped. More apoptotic changes other than necrotic changes take place [21]. Tight junctions of RPE still exist after 1 week [12,22], but the integrity is disrupted over time [21]. Bruch's membrane does not show any decomposition during the cultivation, in which the central elastic layer lies between two collagen layers [21].

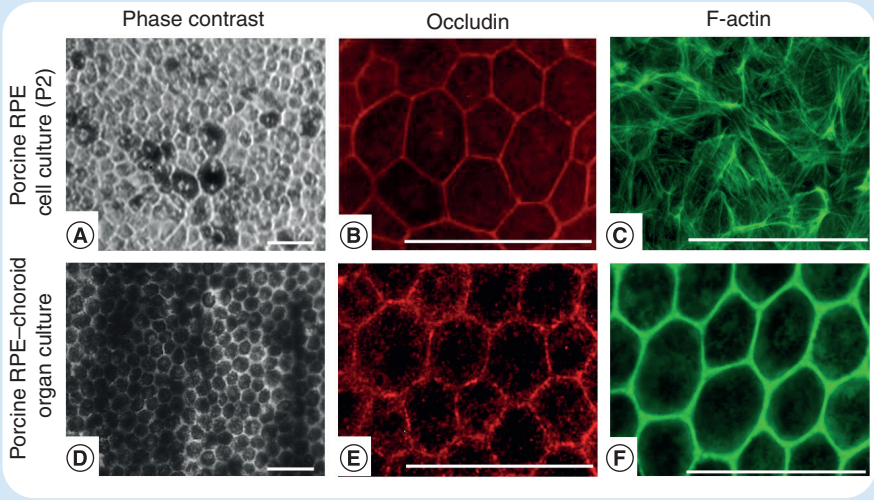

Figure 4. Comparison of (A) phase contrast imaging, (B) occludin staining (red) and (C) F-actin staining (green) between porcine retinal pigment epithelium cells in confluent cell culture and retinal pigment epithelium–choroid organ culture. Although cells in cell culture show cobble-stone appearance **(A)** and occludin is distributed at the cell–cell junction **(B)** as in organ culture **(D & E)**, many F-actin in cell culture is distributed in stress fibers **(C)** rather than circumferential bands **(F)**.
Bar = 50 µm.
RPE: Retinal pigment epithelium.

Functional alterations

According to the results of Calcein-AM cell viability test, RPE cells in perfusion organ culture are vital at least 8–10 days of cultivation. However, some apoptotic changes are observed in culture older than 5 days old [21].

VEGF in the culture medium is one of the indicators of cell functionality [61]. On the other hand, VEGF is also an indicator of cellular stress. If the cells suffer from stresses; for example, oxidative stress or ischemic stress, expression and secretion of VEGF from RPE cells increases [62,63]. We reported that VEGF secretion from RPE in perfusion organ culture is stable until the fifth day of cultivation and starts to increase gradually after that [21]. This suggests that the RPE–choroid in perfusion organ culture can be functionally stable until around the fifth day of cultivation before the functional deterioration starts.

Considering the morphological and functional aspects described above, it seems that the RPE in perfusion culture is vital and has normal functionality until the fifth day of cultivation, and experimental results obtained from perfusion organ culture after more than 1 week should be considered as not reflecting normal tissue response [21]. Although information about comprehensive analysis on viability and morphological/functional properties of RPE–choroid static culture does not exist, a previous study with neural retina showed a significantly higher degenerative rate of RPE in static culture compared with perfusion culture [22].

Studies with RPE–choroid organ culture

RPE wound healing

One of the suitable applications for RPE–choroid organ culture is the investigation of RPE wound healing [16–18,20,34,64]. Wound healing requires cell spreading, migration and proliferation. A small wound can be covered by cell spreading and migration, whereas the bigger wounds require cell proliferation. Hergott *et al.* demonstrated that migrating cells at a wound wider than 125 µm of its diameter expressed the proliferating cell nuclear antigen, which indicates that cells are in the S-phase of the cell cycle and are proliferating [18]. Since cell substrate is essential for cell spreading, migration and proliferation of the cell, it plays a significant role in cellular behavior during wound healing. The RPE cells at the wound edge in organ culture have adhesion to Bruch's membrane, on which they spread. On the other hand, the cells in the cell culture need at first to come into contact with the suitable substrates for spreading. An interesting contrast has been demonstrated in the previous studies. Integrin antibody 2A10 inhibited cell spreading, migration and proliferation at the wound of the RPE cells grown on fibronectin and on laminin substrates [65], whereas it inhibited cell migration and proliferation, but not cell spreading in the RPE–choroid organ culture [18].

Moreover, structurally integrated RPE basement membrane is essential for sufficient wound healing and differentiated RPE function [64,66], which is an important matter, especially for RPE transplantation.

Study for laser effect on RPE

RPE–choroid organ cultures are also very suited for the study of laser effects with respect to heating cells. RPE cells in cell culture are not suited for laser studies due to their limited number of melanosomes, the absorber of laser radiation within the range of therapeutically used wavelengths. The depigmentation of cells in cell culture was briefly mentioned above in the morphological properties section. The 532-nm laser radiation, which is mostly used in retinal photocoagulation, is absorbed more than 99% in strongly pigmented porcine RPE–choroid explants, and only 50–60% in RPE primary cell culture isolated from pigmented porcine eye, and 10–30% in passaged cultures [Miura Y; Unpublished data]. In order to overcome this limitation, some studies were conducted with RPE cell cultures using black paper below the culture dish [67,68] or by adding external melanosomes to the cells [69] for energy absorption. Experimental methods using black paper underneath the cell culture dish needs some considerations: normal polystyrene culture dish bottom is approximately 1 mm thick, and this material has quite low thermal conductivity (0.03 W/m•K); therefore, a significantly higher temperature increase at the level of absorbing black paper is required in order to achieve sufficient temperature rise at the cells across the plastic. For example, if a study needs to damage the cells within a time duration approximately 0.1 s, as is used in photocoagulation, the

temperature at targeted RPE cells should be at least 53°C. In order to achieve sufficient temperature increase to mimic 'laser spots' on the RPE, laser irradiation on the black absorber must be performed with relatively high power, which might reach over 100°C. Polystyrene has a glass transition temperature of 100°C, thus melting of the dish bottom might occur.

Regarding the addition of extra energy absorbers to the cells, it is not easy to obtain equally pigmented RPE cell culture in this method, and foreign pigments might cause alterations of cellular physiological properties.

Recent developments of new retinal laser techniques, for example, micropulse laser [70] and selective retina therapy laser [71], require more basic data for their biological therapeutic mechanisms, and studies using RPE–choroid organ culture might be helpful to investigate early tissue responses. One of the most innovative laser technologies, real-time retinal temperature determination and temperature control during laser photocoagulation, has been recently introduced [72]. Beside the technology development, studies on temperature-dependent biological tissue responses are investigated in our laboratory. With this technique it is possible to study RPE response dependent on the temperature and the time of elevated temperature. We have already presented the changes of VEGF and PEDF secretion over time following sublethal laser irradiation using RPE–choroid organ culture [27].

Table 3. Electrophysiological properties of retinal pigment epithelium in tissue explant, human ARPE-19 and human fetal retinal pigment epithelium cell culture.

Electrophysiological properties	RPE–choroid explant†	ARPE-19 cell culture‡	hfRPE cell culture§
Transmembrane potential (mV)	5.0–12.8	No existing information	2.6 ± 0.8
Transmembrane resistance (ohm-cm^2)	138–350	45 ± 5	501 ± 138
Apical membrane potential (mV)	-65~-79.0	No existing information	-52.3 ± 5.4
Basolateral membrane potential (mV)	-59~-70.3	No existing information	-49.8 ± 3.7
Ration apical-to-basolateral membrane resistance	0.22–0.56	No existing information	0.25 ± 0.2

†Data taken from [7,8,48–51].
‡Data taken from [52].
§Data taken from [43].
hfRPE: Human fetal retinal pigment epithelium; RPE: Retinal pigment epithelium.

RPE phagocytosis

Since engulfment of photoreceptor outer segment by the RPE cells occurs only at the apical surface of the cell, it seems obvious that organ culture, whose polarity is better preserved than cell culture, might be suitable for a study to understand phagocytotic activity reflecting the *in vivo* condition. In the 1960s, the participation of the RPE in the renewal of photoreceptor outer segment was first reported [28]. Studies with small segments of bovine or human RPE–choroid tissue explants presented RPE phagocytotic activity of latex beads [13–15,73]. According to their reports, phagocytosis of latex beads by bovine and human RPE cells in organ culture begins after a latent time period of 4–17 h. There seems to be a time-requiring interaction between the cell membrane and latex beads. On the other hand, engulfment of latex beads in human RPE cell culture was already observed after 1–2 h [74,75]. Rat RPE cells showed no latent time period and phagocytosis of latex beads or photoreceptor outer segment occurs as early as 1 h after the suspension of the particles [73,76]. An *in vivo* study using monkeys revealed that the phagocytosis of subretinal space-injected polystyrene spheres begins after a 6-h latent time period [77]. Considering these differences in latent time period, there seems to be a significant difference in the properties of phagocytosis activity between RPE in native tissue and in cell culture. However, most of the recent studies assessing RPE's phagocytosis activity are habitually performed with RPE cell culture. If it is suited to the study purpose, studies on phagocytosis activity for a time period of several days could be performed with more organ culture, which has similar phagocytotic properties as native tissue.

Phamacological assessment

RPE–choroid organ cultures are used in studies of toxicological and pharmacological assessment [23,25,78]. In particular, perfusion systems can provide an *in vitro*-mimicking cell environment regarding fluid flow. With retina–RPE–choroid perfusion culture, Klettner and Roider compared the efficacy of different anti-VEGF antagonists [25]. The big advantage of perfusion culture systems for this kind of study is that conditioned medium can be collected at any point in time, from the medium flowing out of the culture chamber. It is possible just by disconnecting one part of the outlet tube temporarily. By the increasing number of new pharmacological applications for retinal diseases, the significance of perfusion culture is expected to rise.

Limitations of RPE–choroid organ culture

In comparison to cell culture, cellular characteristics of RPE cells in RPE–choroid organ culture vary greatly depending on the postmortem time and state of preservation of the eyes before preparation. Marked variation in initial tissue condition causes a big variability of the experimental results. Therefore, organ culture needs a strict control in preserving eyes in order to obtain constant and reliable experimental results. Differences in the preparation techniques also greatly influence the quality of isolated tissue; thus considerable skill is required for tissue preparation.

Unfortunately, many of former studies on RPE's cytological functions have been conducted without investigating cellular viability. Thus, it is unknown whether these studies were performed with living or dying cells. Therefore, it is strongly recommended

that every study with organ culture in any culture system should only be conducted after proving cellular viability and functionality beforehand. At present, approximately 5–7 days is the longest-reported time limit for normal RPE cell characteristics in perfusion organ culture. If the experiments are performed over a longer time period, cellular viability and functionality is not yet guaranteed and the results can be very different from those occurring in normal tissue *in vivo*. As described above, however, cellular morphological and functional changes in 8-day-old perfusion organ culture were similar to the changes of RPE in the macula from donor eyes; thus old tissue organ culture might be a useful model to study age-related RPE changes. However, this thesis needs further evaluation.

Cell visualization in RPE–choroid organ culture is difficult during cultivation. RPE is strongly pigmented and therefore observing cells in detail with phase-contrast microscopes is difficult. For immunostaining studies, fluorescence-conjugated secondary antibodies are required in most cases if the RPE is strongly pigmented. Moreover, in fluorescence immunocytochemistry it should be noted that both excitation and emission wavelength are significantly blocked by melanin pigments, which are distributed on the cellular apical side. Even with the use of fluorescence dyes with longer wavelength, where absorption by the melanosomes is smaller, fine examination inside of the cells is quite difficult.

Although the quality of RPE–choroid organ culture has been very much improved by the development of the culture medium and culture system, further improvement is desired for a better and longer cultivation. Modification of culture conditions such as medium components, medium amount, medium flow rate and medium pH is desired. Moreover, retinal co-cultivation is also one of the issues. Many problems have to be overcome; for example, immediate retinal detachment and fast retinal degradation. The retina is a neural tissue and needs a different culture medium from the medium for RPE–choroid tissue in order to preserve its functionality [33].

Expert commentary

Tissue for organ culture has big advantages of holding its native biological properties and of having the same basement membrane as *in vivo*, which is very important for phenotypical preservation. In order to allow researchers to reconsider a suitable experimental model for their study, these advantages of organ culture were described. It is a welcome development that perfusion culture system was introduced, which provides more stable culture condition to the tissue for extended periods of time. For laser studies with respect to heating cells, the RPE–choroid organ culture model is the only suitable model apart from *in vivo* studies, due to its abundant melanosomes. The application of organ culture seems to be especially increased in this field.

On of the major challenges with RPE–choroid organ culture as opposed to cell culture is tissue degeneration. Nevertheless, most of the previous studies using organ culture have been performed without the proof of cellular vitality during cultivation. Moreover, except for very short-term studies on RPE cell physiology, interpretations of different experimental results were given in an uncoordinated fashion. This led to confusion or misunderstanding about the practicality of RPE–choroid organ culture.

Thus, more widely dispersed basic fundamental understanding of RPE–choroid organ culture will pave the way for its further development and use in wide fields of basic study.

Five-year view

With an increasing number of treatment options for retinal disease, such as new pharmacological agents or new-type laser therapies, we have an increased need for elucidating the mechanism of cellular responses to these therapies. Due to the loss of polarity and significant phenotypical differences in cell culture, study with organotypical culture, which is closer to *in vivo* tissue, is of great value. Recent development of perfusion culture systems has made it possible to prolong the stable maintenance of RPE–choroid tissue, and may cause an increasing number of researchers looking to use RPE–choroid organ cultures as a model for their studies in the near future. We are now actively working with organ culture, in order to elucidate laser-induced temperature-dependent RPE responses using many different analytical tools. Along with experimental studies, innovative modifications to the organ culture system are required for better tissue preservation. Eventually, investigations to realize co-culture with neural retina with longer vital condition are also desired.

Key issues

- Retinal pigment epithelium (RPE) cells in cell culture are phenotypically different from RPE cells in native tissue.
- Morphological and physiological polarities of native RPE cells are lost in cell cultures on plastic dishes.
- Melanosomes in RPE cells are not generated in normal cell culture conditions; therefore, cell cultures after several passages are much less pigmented.
- RPE in RPE–choroid organ culture has its native phenotypes, and thus experimental results using organ culture are considered to reflect normal tissue responses *in vivo*.
- There are two different types of RPE–choroid organ culture in terms of medium supply; static culture and perfusion culture.
- RPE–choroid organ culture is suitable to be used for studies on RPE morphology, physiology, phagocytosis activities, pharmacological responses and biochemical responses to laser irradiation.
- Perfusion culture enabled longer and stable tissue preservation, and to date, the time period of tissue preservation with normal morphology and functionality is up to 5 days. RPE cells in longer cultivation show ongoing apoptotic changes.
- Further attempts for longer tissue preservation are also desired.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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Appendix

Preparation procedure of retinal pigment epithelium–choroid organ culture

Porcine eyes, tissue are supported by the MINUSHEETs provided by the manufacturer Minucells and Minutissues GmbH.

1. Keep freshly enucleated eyes at 4°C. The postmortem time should be no longer than 5 h.
2. Remove the adjacent tissues.
3. Immerse the eyes for 1 min in antiseptic solution then in sterilized PBS(-) (phosphate-buffered saline without calcium and magnesium).
4. Cut the sclera at approximately 5 mm from the limbs, followed by circumferential dissection.
5. Remove the anterior part of the eye, as well as the lens and vitreous (eye cup is made).
6. Discard the nasal part of the eye cup.
7. Separate the retina–retinal pigment epithelium–choroid sheet from the sclera with forceps and scissors.
8. Place one of the MINUSHEETs (white ring) below the separated tissue and cover the ring by the tissue sheet.
9. Remove the retina carefully by pulling it from periphery (if it is difficult, soak the tissue with sterilized PBS[-]).
10. Place another MINUSHEET (black ring) over the RPE and carefully make two MINUSHEETs engaged across the tissue, so that the two MINUSHEETs can fix the tissue.
11. Remove the excess tissue.
12. Place the tissue ring in the culture container filled with the culture medium.