5-2015

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METHODS FOR GROWING RETINAL PIGMENT EPITHELIAL CELLS: CURRENT PROTOCOLS AND FUTURE RECOMMENDATIONS

By

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A report submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Biological Engineering

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2015
Introduction

The retinal pigment epithelium (RPE) is a layer of tissue found in the vertebrate eye between Bruch’s membrane and the photoreceptor layer of the neural retina [1]. It is derived from the outer layer of the optic cup [2], possesses an innate immune system [3], and consists of a monolayer of highly pigmented cells that fit together in a tight matrix (fig. 1) [4]–[6]. The monolayer is often compared to a mosaic or cobblestones in its configuration, while the shape of the individual cells is usually described as polygonal/hexagonal, columnar (aligned perpendicular to the underlying membrane), or “epithelioid”. The cells are strongly polarized [7], with microvilli on the apical surface [8]. Despite its simplicity as a tissue layer, the RPE plays many complex roles in the vertebrate eye, including regulation of retina development [9], [10], absorbing excess light entering the eye to reduce photo-oxidative stress [11], secreting growth factors such as vascular endothelial growth factor (VEGF) [5], [10], [12], mediating the immune response of the eye [13]–[15], transporting metabolites and fluids [16]–[18], and phagocytosing spent rod and cone outer segments [19]–[26]. The RPE also acts as an intermediate for supplying glucose and other vital nutrients to the retina [27] while maintaining a good environment for the photoreceptors [28] and preventing large molecules from entering the eye from the bloodstream [29]. This last purpose designates the RPE as part of the blood-retinal barrier, which is primarily in place to stop particles from entering the vitreous humor and obscuring vision [30]–[32]. While the RPE plays many roles in the eye, its greatest medical significance comes from its involvement in many ocular disorders [33] that can lead to vision loss or blindness, such as retinitis pigmentosa, diabetic retinopathy, West Nile virus, and macular degeneration [12], [34]–[46].

![Diagram of the outer retina, showing the retinal pigment epithelium and its location between Bruch’s membrane and the neural retina. Pigment granules, tight junctions, and monolayer formation are indicative of natural-type RPE cells.][34]

Macular degeneration in particular is currently of great interest to medical and biological researchers. Age-related macular degeneration (AMD) is a disorder of the retina characterized by loss of sight in the center of the visual field, and is the leading cause of vision loss after age 50 in
developed countries [47]. AMD occurs in two forms: the exudative or “wet” form, and the nonexudative or “dry” form. Wet AMD (the most severe form, also called choroidal neovascularization) is caused by excessive growth of capillaries from the choroid and into Bruch’s membrane and the RPE in response to production of vascular endothelial growth factor (VEGF)[48]. These fragile new blood vessels exude blood, lipid, and protein below the macula (the central region of the retina, necessary for visual acuity), causing scarring and sudden acute vision loss [49]. Dry AMD (the most common type, also called atrophic AMD or geographic atrophy)[50] is a much slower process, characterized by degeneration of the RPE and later the neural retina [49],[51],[52], which can lead in some cases to tearing of the RPE [53]. Its causes are not fully understood [29] [30], although evidence suggests contributing factors include the accumulation of lipofuscin and other substances in the eye [32],[56]–[61], disruption of autophagy and other processes in the RPE [23],[26],[62]–[64], photo-oxidative stress caused by excess light entering the eye [32],[60],[65]–[71], and other retinal disorders like focal choroidal excavation (FCE)[72].

Both AMD types are characterized by the deposition of soft drusen between the RPE and Bruch’s membrane [73]–[76], as well as hypo- and hyperpigmentation of the RPE itself [51][77]. This, along with chemical evidence [27][35]–[39], suggests that the RPE is the site of origin of AMD development, and that research and experimentation with this particular tissue layer is necessary for learning more about this crippling disease [83]–[86].

While AMD research has been conducted for many years, there is still no available chemical or surgical treatment for reversal of vision loss in the dry form of the disease [48],[52],[87],[88]). Many researchers are exploring possibilities for treatment, such as slowing deterioration in the intermediate stage through high doses of vitamin supplements such as lutein and zeaxanthin [89], treatments like laser photocoagulation [47], use of stem cells to replaced degraded cells[90]–[94], and even gene therapy [67],[95]. However, because RPE cells are largely non-proliferative throughout life, regeneration and repair of the already-damaged tissue is normally impossible [35]. Before an effective treatment can be found for AMD, further experimentation must be performed in order to fully understand the role of the RPE in AMD inception and progression. While necessary for visual function, the blood-retinal barrier also makes chemical experimentation with AMD treatment unproductive because it inhibits drug treatments from passing from the blood to the vitreous humor[96]. This, as well as its location at the back of the eye, makes observation of and experimentation with the RPE difficult in vivo[97].

One possible alternative to in vivo RPE research is to culture RPE cells in vitro [98]–[100]. RPE cell culture has been practiced for over forty years at the time of this writing [101] and over the decades many methods for producing viable RPE cells have been demonstrated. While RPE cells are normally non-proliferative in adults, they can be induced to re-enter their glycolytic metabolism stage in which proliferation, migration, and differentiation is possible, allowing them to re-establish a viable monolayer in medium [102]–[104]. Cultured RPE cells can be used in a variety of applications, many of which can be used in AMD research [28]. One, as intimated above, is the use of cultured RPE cells as a testing ground for AMD drug treatments. A researcher searching for a possible cure for AMD could induce atrophy in the RPE monolayer before introducing various chemicals or supplements to observe their effect on the cultured cells. Such an experimenter would most likely desire an RPE culture in which the orientation, configuration, and morphology of the cells are similar to those found in the natural RPE. The culture would fulfill its function most effectively if it produced a dense monolayer of epithelioid cells with dark pigmentation and tight intercellular junctions, similar to the in vivo RPE [105].
However, despite the amount of time and energy that has been spent on improving RPE culturing techniques, difficulties still arise as scientists attempt to produce a pigmented, epithelioid monolayer that best mimics native RPE tissue. RPE cells may exhibit considerable phenotypic variation depending on their growth conditions [106]. Some of the most common problems are loss of pigmentation in the cultured cells, low cell-substrate adhesion rate, alterations in cell morphology (in particular, a tendency for epithelioid cells to transform into long, fibroblast-like, “fusiform” cells) [19], and low yield of viable cells from those harvested [107]. Many of these problems can be attributed to variations in cell seeding density, freshness (for cells grown from living tissue), culture medium composition, incubation conditions, passage number [50], and substrate topography [108], and any researcher attempting to closely replicate native RPE tissue in vitro would be well advised to search published protocols for the right procedure.

Another application for RPE culture is the cultivation of cells intended for direct RPE transplant, a line of research already being explored by several groups [87], [109]–[112]. RPE transplantation experiments have been performed using in vivo harvested cells from the same patient [113], [114] and research oriented toward growing layers of healthy RPE tissue that can be transplanted into the eyes of patients suffering from AMD to replace any damaged or atrophied cells is a growing field [115]. While this course of action may be effective in curing AMD, at least temporarily, it would also require donor/transplant compatibility, invasive ocular surgery, a substrate that can be transplanted with the cells (since experiments using RPE cell suspensions and loose sheets injected directly into the eye have been largely unsuccessful) [87], [116], and an even higher standard of monolayer fidelity and morphological similarity to the human eye than a culture grown for experimentation. Nevertheless, many researchers [47], [87], [117] see RPE transplant as a viable method for treating AMD, and have developed culture protocols accordingly.

One phenomenon that has been observed by RPE researchers is the tendency for cultured RPE cells to spontaneously transform into cell lines [118]. RPE cell lines are often characterized by extended cell life, changes in cell morphology, and sometimes immortality. While this makes them useful for extended experimentation, it renders them unsuitable for transplantation, and in addition may not produce as accurate results in a drug treatment research. Culture protocols that give rise to cell lines, therefore, are best suited for RPE research that allows for morphological differences between the culture and natural RPE.

Another cell transformation phenomenon that has been reported in RPE cell cultures is the activation of ordinary RPE cells into stem cells [35], [119]–[121]). The RPE begins life as a plastic tissue that is originally capable of producing lens and retina in some species, but quickly loses this competency as it differentiates into largely non-proliferative RPE cells [122], [123]. However, either by active induction or spontaneously, RPE cells in culture have shown the ability to transform into self-renewing multipotent cells that can give rise to optical, neural, and mesenchymal tissue under certain conditions [35], [124]. This opens up another avenue of AMD research: producing new RPE cells and perhaps neural retina tissue from retinal pigment epithelial stem cells (RPESCs) to replace those damaged by the disease, even in vivo. Under ordinary circumstances, human RPE cells do not replace themselves when damaged, unlike in some other species, where injury to the eye can result in the RPE not only regenerating itself but also neural retina [35], [125]. The transformation of RPE cells into stem cells is often marked by the appearance of spheroid colonies that detach from the substrate and float, and cells recultured from floating colonies have shown self-renewing properties [120]. Activation of these latent
plastic properties may aid in recovery of AMD patients without the need for entire RPE sheet transplants.

With these four applications (drug experimentation, transplants, cell line and stem cell generation) there is ample opportunity for RPE cell cultures to be used in AMD research. However, not every cell culture method will be right for every application. For example, since self-differentiating multipotent RPE cells are suspected to be the source of mesenchymal cell fates in disorders such as proliferative vitreoretinopathy (PVR) [120], a cell culture that is conducive to the spontaneous production of stem cells may not be suitable for transplants. The same way, a transformed cell line may be useful for some experimentation, but the morphological and growth changes that accompany transformation may make the cells too different from natural RPE to be used in drug treatment tests or transplants. For this reason, a researcher must be careful to select a culturing protocol that will best suit the needs of their project.

The purpose of this literature review is to examine the various protocols that have been developed and used since the earliest days of RPE culture and to compare different methods (including cell source and harvesting procedure, culture medium and substrate, incubation conditions, and passage method) so that researchers attempting to culture RPE cells can have a ready reference to aid in selecting the protocol that is best for them. Research into culturing retinal pigment epithelium tissue is divided into two main branches, based on cell sources: cultures grown from harvested tissue, and those grown from established cell lines (while the generation of RPE cells from stem cells is a third possibility [91], [115], [126], [127], this review will focus on the previous two methods). This review will first examine cultures using donor organisms as a cell source, then the culturing procedures of cell lines.

Cells obtained from tissue

Before the establishment of cell lines, the only possible source for researchers trying to culture RPE cells was from the tissue of living and recently-deceased vertebrates. When cultivating such cells, the basic procedure is to first harvest the cells from the tissue, usually with the help of some sort of digestive enzyme to degrade the structural proteins holding the cells in place. Often this requires removal of the eye from the surrounding tissue (enucleation) and dissection of the eye globe, most commonly by cutting off the anterior portion of the eyeball (see fig. 2) and leaving an “eyecup”, with the RPE at the bottom, into which digestive enzymes (such as trypsin and dispase, two of the most common proteins used) can be poured [128]. An alternative (sometimes used in conjunction with enzymatic digestion) is to remove the RPE from the eye through mechanical means, such as with forceps or dissection blades, often in a single sheet or fragments of a sheet. This can often lead to contamination from choroidal cells, but is beneficial in that it may maintain the epithelial mosaic-like structure of the RPE. After cell harvesting, the next step is usually to transfer the cells into a culture medium and allow them to adhere to a substrate that is conducive to the growth of a cellular monolayer. Incubation follows, usually at a set of standard conditions common to mammalian cell cultures (~37°C, 5% CO₂). If the cells are allowed to grow to confluence, they are usually passaged, again using a mild digestive enzyme to dissolve them from the substrate, with a certain number of passages possible before cell senescence and death.
Generally, the benefit of using tissue as a cell source is that the cells are already in the tiled configuration found in the eye, encouraging the cells to continue to grow in a single monolayer that best matches the natural structure of the RPE. However, as cells are often dissolved from their configuration and resuspended in culture medium, a greater benefit is that the cells may maintain their morphology and pigmentation, which are often lost when using transformed cells from cell lines. In the following sections, established protocols for cultivating cells obtained from humans (living, dead, adult, and fetal) will be examined, followed by cultures of cells taken from nonhuman vertebrates.
Human donors

The human RPE is, of course, the best model for testing treatments for human ailments, and is also the acceptable source for human RPE transplants, cell lines, and stem cells. Culturing of human RPE has been going on since the 1970s [101], and many of the practices developed then are still in use today, particularly the method for harvesting cells from the human eye. In the early days, Mannagh et al. (1973) cultured RPE cells in an experiment simply to discover the suitability of human RPE for lab culture, and used a cell-harvesting method still commonly practiced today by RPE researchers. The adult human RPE were extracted from eyes harvested for corneal transplants that had either been found unsuitable for transplantation or had already had the cornea removed. The eyes were first dissected by removing the anterior half of the globe 6 mm behind the limbus (see fig. 2) and the resulting posterior eyecup inverted, removing the vitreous body and retina. The RPE was then digested enzymatically, using 0.03% Pronase (a commercial mixture of proteases derived from Streptomyces griseus) in a calcium and magnesium-free balanced salt solution. The cup was filled with about 5 ml of the Pronase solution and incubated at 37°C for 20 to 30 minutes, freeing the RPE cells from the Bruch’s membrane. The cells were then harvested by repeated aspiration of the Pronase solution through an 18-gauge needle on a 5 ml syringe, and the resulting suspension centrifuged at 1,000 rpm for 3 minutes. During this time, the Bruch’s membranes of the eyes were examined and found to be intact, verifying that no fragments of membrane had been harvested with the cells. After centrifuging, the supernatant solution was pipetted off and the cell pellet resuspended in 1 ml of 15% FBS-Eagle’s minimum essential medium (EMEM), and the new suspension placed in a Rose chamber or T25 flask filled with the same medium. Mannagh et al. noted a negative relationship between the age of the donor and the viability of the culture, with 75% success (57 out of 76 cultures) for donors under 60 years but only 58% (62 of 106 cultures) for donors over 60. Interestingly, in the case of eight pairs of eyes used as cell sources, only one eye in each pair gave rise to a viable culture. The viable cultures were found to form small adherent clusters of round, heavily pigmented cells on the floor of the container 48 hours after inoculation, when mitotic activity was also detected. After cells were observed, the medium was changed to remove the free-floating pigment granules that had escaped into the solution. Mitosis continued for about 30 days, when a confluent monolayer would completely form on the floor of a Rose chamber. Pigmentation loss was noted with each cell division, and attempts to stimulate pigment formation by the addition of adrenocorticotropic hormone (ACTH, 0.5 U/ml) were unsuccessful. The researchers observed spontaneous transformation of primary cultures into cell lines in seven cases, all of which resulted in a change in cell morphology to a smaller, more uniform shape and accelerated growth. These cell lines were successfully passaged and seeded at 1x10⁵ cells/ml. This protocol demonstrated the culturing potential of RPE cells and their ability to spontaneously produce cell lines, and while somewhat less-refined than later protocols is still effective for a researcher merely wishing to culture RPEs for practice or for the purpose of cell line production.

Around the same time, Tso et al. (1973) cultured RPE cells to discover the extent of morphological changes experienced by RPE cells upon removal from Bruch’s membrane. The cells were obtained from both cadavers and from living patients whose eyeballs were enucleated for melanoma and retinoblastoma. The eyes were opened in culture medium RPMI (Roswell Park Memorial Institute medium) 1640, with the RPE and uvea separated together from the sclera, placed on a Millipore filter (EMD Millipore, Billerica, MA, USA) choroid-down, and kept within the medium for the remainder of the procedure. The cultures were placed in Leighton
tubes filled with 2 ml of the same medium and incubated at 37°C, with medium changes twice a week. For the first 3 days, the cells retained epithelial properties and pigmentation, with some less-pigmented cells noted over time. After 3 days to 6 weeks in culture, the tissues were fixed in 2% glutaraldehyde. The adult cultures also had thorotrast added 24 hours after the culture began. After fixation, the tissues were postfixed in Dalton’s chrome osmium fixative for one hour and embedded in Epon. When cells were observed using light and electron microscopy, no difference was found in the growth between cells taken from dead or living donors, and the RPE cell shapes were found to become more irregular after three days, with no spreading observed, with attenuation and necrosis after 6 weeks. The cultures to which thorotrast had been added displayed the ability to phagocytose the thorotrast particles (one of the functions of natural RPE cells), which were engulfed in discrete vesicles after 2 days of incubation. This culture method is recommended for an experimenter attempting to produce a more natural-type RPE monolayer for drug experimentation.

Aronson (1983) attempted to identify a consistent procedure for culturing human RPE cells by using choroid fragments as the seeding vehicle. The choroid fragments were harvested 24 hours after delivery from human aborted fetuses 3-4 months into gestation; they were removed by bisecting the eye through the optic stalk, pulling off the retina with forceps, and then peeling off the RPE and choroid as a single sheet. The sheet was minced with a scalpel and the fragments plated on tissue culture plastic in a small volume of modified MEM medium, and after 1 to 2 days partially-rounded fragments were isolated and placed on non-tissue-culture plastic Petri dishes. The fragments were cultured until they formed spheres. The spheres were then plated on tissue culture plastic in a small volume of medium and allowed to grow for 7 days before the tissue fragment was removed, leaving a new cell line on the plastic. This method of including choroidal fragments in the seeding culture is still used by some researchers [9], and is recommended for researchers looking to start cell lines, although occasionally experimenters report problems from contaminating choroidal cells.

Oka et al. (1984) compared the effects of traditional serum-supplemented medium and serum-free defined medium on both human and bovine RPE cells grown in culture. In this section, the human cells will be considered. The cells were isolated by taking enucleated eyes and rinsing them in 95% ethanol and Sal FM, then removing the anterior section of the eyeball and vitreous, as previously described in Mannagh et al. (1973). The eye cup was rinsed with warm Sal FM three times and the neural retina removed, after which each eye cup was filled with 3-5 ml CTH (a mixture of 2% (v/v) chicken serum, 0.25% (w/v) trypsin, and 0.1 (w/v) hyaluronidase in calcium- and magnesium-free Puck’s Saline F) and allowed to incubate for 30 min at 37°C. This incubation period allowed the RPE cells to disperse into the CTH in a suspension, which was then removed and centrifuged lightly in equal volumes of a medium composed of DME-F12 medium supplemented with 20% (v/v) FBS (designated CM medium). After centrifuging, the supernatant was removed and the cell pellet resuspended in CM. The process of dispersal, centrifuging, and resuspension was repeated several times until all suspensions were pooled in warm CM. For the final culture, one of two media were used: either CM or defined medium (DME-F12 supplemented with 5.0 µg insulin/ml, 5.0 µg transferrin/ml, 8.0 ng epidermal growth factor/ml, 0.5 mU follicle stimulating hormone/ml, and 50 ng all-trans retinoic acid/ml) (DM). As with most methods listed, the cells were incubated in a 5% CO₂ atmosphere at 37°C, and the media was changed every 2-3 days. The cells were cultured on 60 mm culture dishes and passaged using 0.25% trypsin. The effects of the serum (CM) versus serum-free medium (DM) were observed and recorded. Results showed that plating efficiency
was consistently higher in a 1:1 DME:F12 mixture than in either DME or F12 alone. It was also observed that the highest plating efficiency was achieved when the 1:1 mixture was supplemented with 20% FBS (CM, as designated above). Pure DME medium was found to result in larger, less numerous colonies of RPE cells, while pure F12 resulted in smaller, more numerous colonies. The CM mixture resulted in a doubling time of approximately 50 hours, which diminished in later passages to 20-25 hours and in very late passages increased to 100 hours. Fourth passage cells were found in many instances to stop dividing before confluence. Despite the attempt to completely eliminate serum from the culturing process (due to the introduction of hormones and other factors that may affect cell development) [9], serum-containing medium (CM) was found to be necessary for cell attachment and spreading, though using serum-free DM after the initial 24-hour plating period in CM resulted in exponential growth. This procedure is recommended for cultivation of RPE cells for drug experimentation, since it produces a viable cell culture that is similar to natural-type RPE.

Hunt et al. (1989) were able to form viable cultures using cells extracted from eyes donated for corneal transplant, all from humans less than 40 years old. The eyes were first dissected by removal the anterior portion of the eye globe, vitreous, lens, and neural retina to expose the RPE, which they then rinsed with Hank’s basal salt solution (HBSS). They then filled the eyecup with 0.5 g trypsin/0.2 g EDTA/ml and incubated it at 37º C for 15 minutes. The detached cells were then aspirated off and trypsin digestion repeated. All removed cells were then washed in Ham’s F-10 medium supplemented with 20% FBS, ITS plus (Collaborative Research), antibiotics, and a retina extract made by incubating human retina and vitreous in growth medium followed by filtration. The cells were resuspended in this same medium, and seeded onto a variety of surfaces, among which are listed multi-well tissue culture dishes, Millicell (EMD Millipore, Billerica, MA, USA) or Costar (Sigma-Aldrich, St. Louis, MO, USA) culture well inserts, and polycarbonate fibers. All culture surfaces were received a coating to test cell adhesion, with different coatings tested including laminin, fibronectin, type IV collagen, and Matrigel (an extracellular matrix exudate from a tumor cell line). The extraction process yielded high concentrations of pigmented cells, with some erythrocytes mixed in in some cases, and it was found that when seeded onto the plating surfaces the RPE cells adhered rapidly, with non-adhering cells being removed and the medium changed after 48 hours. The cells were maintained in medium until they grew to confluence, the time required for which depended on both the seeding concentration and the donor. Results showed that the laminin-coated substrates (which were coated in 20 µg/ml laminin in Ham’s F-10 medium) yielded the greatest cell growth, with cells forming highly pigmented epithelioid monolayers with intercellular junction complexes as seen in the natural RPE. This was determined to be due to the fact that laminin is a component of basal RPE lamina and is thought to be involved in cell adhesion. This protocol is recommended for drug experimentation studies, particularly due to the natural-type intercellular junctions produced by the procedure, which may be useful in experiments involved with circumventing the blood-retinal barrier.

Tezel and Priore (1998) attempted to develop a chemically-defined serum-free medium (CDSFM) to avoid the problems associated with the use of serum in RPE culture, similar to [132] (see above). They used cadaver eyes incubated in 25 U/ml dispase for 30 minutes after the same dissection used by Managh et al. (1973). The RPE and choroid were removed together using forceps, and the loosened RPE sheets pipetted off and placed in CDSFM, a 1:1 mixture of DMEM:F12 with pyridoxine HCl, L-glutamine, and 15mM of HEPES buffer, supplemented with insulin, transferring, epidermal growth factor, follicle stimulating growth hormone, retinoic acid,
sodium selenite, hydrocortisone, tri-iodothyronine, streptomycin, penicillin, gentamycin, amphotericin, linoleic acid (10 µg/ml), and putrescine (0.3 µg/ml) (see publication for further preparation details). The RPE cells were then centrifuged at 1000 RPM for 5 minutes and the pellet resuspended in CDSFM or DMEM before inoculation on bare or bovine corneal endothelial extracellular matrix (BCE-ECM)-coated tissue culture plastic wells. The cells were incubated in 5% CO₂ at 37°C and the medium changed every other day. Researchers found the cells to be confluent after 9-15 days on BCE-ECM and 16-26 days on bare tissue culture plastic (fig. 4). Passaging of confluent cultures took place by trypsinization and subsequent trypsin deactivation by addition of aprotinin. Results showed higher seeding efficiency on BCE-ECM than on ordinary tissue culture plastic, and that the cells formed fewer non-hexagonal (fusiform) morphologies. Researchers observed higher proliferation rates for the RPE cells in DMEM with serum than in CDSFM. This culture technique is recommended for drug experimentation, due to the generally epithelioid structure of the resulting cells.

Figure 4. Morphology of RPE cells grown in CDSFM by Tezel and Priore (1998). (A) Cells at the edge of primary RPE sheets began to flatten on day one (arrows). (B) Densely-packed RPE cells grown to confluence on BCE-ECM, with edge of primary RPE sheet shown by arrow. (C) RPE cells grown on tissue-culture plastic, also densely-packed. (D) Second-passage RPE cells grown to confluence on BCE-ECM, with fusiform cells indicated by arrows. (E) Second passage RPE cells on tissue-culture plastic, with greater number of fusiform cells than BCE-ECM. (F) Third-passage RPE cells on tissue-culture plastic, with a greater number of fusiform cells. Magnification x 100 for all photographs. [133]
Singh et al. (2001) [87] cultured human RPE cells to be specifically used for transplantation to treat AMD, experimenting with two separate substrates (human lens capsule tissue and hydrogel) to use in the transplants. The lens capsules were taken from 55-year-old-and-older patients undergoing cataract surgery and stored in HBSS at 4°C until used, with the lens epithelial cells scraped off from the capsule surface using soft-tipped silicon tubing. After cell removal was confirmed by examination under an inverted phase microscope, the capsules were laid epithelial-side up in 24-well tissue culture dishes. The hydrogels (manufactured by Organogel Canada L. Tee, Quebec) were prepared by free radical co-polymerization of methacrylate and (meth)acrylamide precursor monomers (ratio 1/0.41 by weight), with ethylene glycol dimethacrylate as a crosslinking agent. Polymerization occurred between two pre-cleaned glass microscope slides spaced by a coverslip for 4 hours at 35°C, after which the polymer membranes were peeled and washed in distilled water until equilibrium swelling. The swollen gels were cut into circular disks by a cork borer and then sterilized by autoclaving and stored in distilled water. The final water content of the hydrogels was 60%, for a thickness of approximately 25 µm. The hydrogels were then coated with extracellular matrix factors by being placed in a well plate with 1 ml of poly-D-lysine (MW 540,000, 20 µg/L) and HBSS for 5 minutes, after which the solution was aspirated off and the hydrogels were incubated overnight at 37°C in 2 ml fibronectin solution (20 µg/ml) in HBSS. The day after this solution was aspirated off as well and the hydrogel allowed to dry for 30 minutes under UV light (256.7 nm) in a tissue culture hood. The RPE cells themselves were extracted from eyes of cadavers 65-70 years old, obtained 8-24 hours after death, using the dissection method found in [129], after which the eyecup was washed with HBSS (Ca²⁺ and Mg²⁺ free) and treated with 0.25% trypsin for 1 hour at 37°C. After incubation the trypsin was aspirated off and replaced with Dulbecco’s minimum essential Eagle medium (DMEM) with 10% phosphate buffered solution (PBS) and 100 U/ml penicillin-streptomycin. The cells were loosened with gentle pipetting and seeded onto six-well tissue culture dishes, where they were incubated at 37°C in 5% CO₂ in DMEM with 10% FBS and 100 U/ml penicillin-streptomycin and the media changed every 3-4 days. When the cells became confluent, they were dissociated from the culture plate using 0.05% trypsin-EDTA, washed, and seeded onto the lens capsules and hydrogels in 24-well tissue culture dishes at a density of 6 x 10⁵ cells/ml and incubated in the same conditions as before. The cells were found to adhere to and grow well on both surfaces tested, attaching within 24 hours and forming confluent epithelioid monolayers over a period of 4-5 days. This protocol is recommended for anyone wishing to perform drug treatment tests or RPE transplantation experiments, based on the highly epithelioid structure of the cell culture.

Tseng et al. (2004) reported an RPE culture grown from cells obtained by the evisceration of two living humans during surgery. In both cases, the choroidal membrane was removed and stored in F12 medium. In the first case, the choroid was then cut into small pieces, which were plated in a 35 mm Falcon dish with 20% FBS F12 medium. Within 7 days of incubation, cells had migrated from the tissue and formed colonies with heterogeneous morphology, including pigmented epithelioid and fibroblast-like (fusiform) cells. The fibroblast-like cells were capable of propagating and outnumbered the RPE cells 10 days after culture. In the second case, the choroid was digested in 0.25% trypsin for 30 minutes at 37°C to detach the RPE from the stroma, centrifuged, and resuspended in 20% FBS F12 medium. Pigmented cells attached to the culture dish overnight after seeding and began spreading in both polygonal and epithelioid morphologies after 3 days. The adherent cells began dividing 5 days after incubation,
losing pigmentation with growth and reaching confluence after 15 days. Subculturing resulted in further growth and continued epithelioid morphology. The cells became senescent after 6 passages. In both cases, media was added and renewed every 3 days until confluence. This protocol is recommended for drug experimentation, due to the successful growth of RPE cells with a 6-passage Hayflick limit.

Akrami et al. (2009) cultivated human RPE cells in vitro in order to produce retinal stem cells. The RPEs were obtained by opening the eyes (obtained from cadavers 24 hours after death) removing the neural retina with forceps, then washing the interior of the eyeball globe with PBS. The RPE layer was pulled out with forceps and cut into small sections, which were then incubated for 90 minutes at 37°C in 2 u/ml dispase. The resulting cell suspension was centrifuged for 5 minutes at 300 g and 4°C, and the pelleted cells placed in T25 flasks coated with FCS. The culture media used was DMEM:F12 supplemented with FCS (20% at the beginning of the culture, 10% with all medium changes afterward), penicillin, streptomycin, gentamicin, and amphotericin. The media was changed twice a week until the cells reached confluence (usually within 2-3 weeks), after which they were passaged into new T25 flasks using 3x10^5 cells as the standard concentration per flask. The confluent cells formed an epithelioid monolayer but showed some loss of pigmentation, and also formed spheroid colonies indicative of the presence of stem/progenitor cells. This protocol would be best suited for growth of such stem cells for the production of self-renewing RPE tissue.

Salero et al. (2012) found human retinal pigment epithelial cells to contain stem-like cells, which were found after RPE was isolated from adult donor eyes (some from cadavers as much as 99 years old) obtained from eye banks using the method found in Mannagh et al. (1973). After dissection, the RPE cells were dissociated and plated in adherent conditions, then grown using serum. The cells grew well, doubling once every 2 days, and could be passaged at least 6-8 times. The cultures formed pigmented epithelioid monolayers similar to those found in the living eye. From some of these monolayers, cells were then isolated, dissociated, and grown again at clonal density in nonadherent conditions, supplemented with knockout serum replacement medium (KSR). This resulted in the development of spheroid colonies, which were found to demonstrate self-renewing properties when recultured. Other cultured epithelioid monolayers received treatment for 4 weeks with differentiation media resulted in cell markers being detected for neural, adipocyte, chondrocyte, and osteogenic cells. Similarity was found between the growth of cells from both young and elderly donors. This protocol would be useful for growing both natural-type RPE cells and stem cells for AMD treatment.

Maminishkis et al. (2006) used fetal eyes in an attempt to establish a reproducible protocol for culturing human fetal RPE cells to resemble native tissue. The eyes were obtained from random donors, all used less than 26 hours after enucleation. The eyes were rinsed in antibiotic-antimycotic solution for 3-5 minutes, then rinsed twice with medium or PBS to remove the antibiotic. The anterior portion of the eye was removed (as in Mannagh et al. (1973)) and the posterior portion incubated in dispase-I solution for 30 minutes. The posterior poles were then placed in 5%-serum-containing “RPE medium” (a specific medium described in (Maminishkis et al. (2006)) and dissected in quadrants, the retina and RPE monolayer removed with forceps. The RPE sheets were then placed in 5%-serum-containing RPE medium, and centrifuged at 100 g, pipetted gently to separate the cells from the syncytium with or without trypsin treatment), and then placed in flasks with 15%-serum-containing RPE medium. After one day, the medium was replaced with 5%-serum-containing RPE medium, which was changed every 3 to 4 days afterward. They reported confluence and uniform pigmentation after 3-4 weeks, after which they
were trypsinized and passaged. The resulting cells formed confluent monolayers with epithelial morphologies and heavy pigmentation, with the apical membrane microvilli found in natural RPE. This protocol would be most suitable for drug experimentation and transplantation studies, based on the successful production of epithelioid monolayers.

Gamm et al. (2008) also worked with human fetal RPE cells, searching for a serum-free system for growing human RPE. The eyes were taken from fetuses between 10 and 16 weeks of gestation, obtained from The University of Washington Birth Defects Laboratory and shipped overnight at 4°C. The eyes were dissected in ice-cold DMEM:F12 and 1% antibiotic-antimycotic solution, removing the anterior section and vitreous as previously detailed. The eyecups were washed with DM twice and the neural retina removed by forceps, using irrigation with DM to loosen retinas that did not detach spontaneously during vitreous removal. The RPE and choroid were then removed with forceps, and treated in one of three ways: in the first treatment, the RPE-choroid sheets were incubated in a 2% dispase-DM solution for 30 minutes at 37°C and washed in DM twice, then the choroid peeled off with forceps and the resulting isolated RPE sheets chopped into 200-µm sections with a McIlwain automated tissue chopper, then placed in laminin-coated tissue culture plastic in serum-free RPE DM medium (SFRM) supplemented with either 2% B27 (SFRM-B27) or 1% or 2% N2 (SFRM-N2) (both B27 and N2 are commercially-available supplements). In the second treatment, the RPE-choroid sheets were immediately chopped into 200-µm sections without dispase digestion, and only those fragments containing large pieces of RPE were placed in laminin-coated tissue culture plastic in serum-free RPE DM medium (SFRM) supplemented with one of the two medium supplements mentioned previously. In the third treatment (which was devised after observing the reactions of the cells to the previous two procedures) treatment two was modified in that the chopped RPE-choroid fragments were first placed in suspension culture in SFRM-B27, then placed in tissue culture dishes. Spherical tissue aggregates formed within hours and became uniformly pigmented after 2 to 4 weeks in culture. After each treatment, the cultures were incubated at 37°C and 5% CO₂, similar to other procedures used by other researchers. In all treatments, both cells supplemented with B27 and cells supplemented with N2 showed outgrowth in the first passage, though in all subsequent passages only B27-supplemented cultures showed substantial continued expansion, and within 5 days the cells became confluent and adopted a characteristic RPE morphology. Treatments 1 and 2 were found to have strong disadvantages (adherence failure and contaminating choroidal cell colonies, respectively), while treatment 3 was found to overcome both these disadvantages. This protocol is recommended for drug treatment experimentation, due to its successful production of viable RPE cultures.

In 2009, Sonoda et al. established a protocol for culturing and differentiating human fetal RPE cells. Their cells were obtained from corporation with preserved tissues taken from 18-20-week-old fetuses, shipped within a day to the experimenters. The anterior portion of the eye (referred to as the cornea-iris complex) is cut off and the vitreous removed. The posterior eye cup is dissected into four quadrants using a razor blade before RPE-choroid layer is peeled from the retina with forceps (fig. 3). The sheets are then dipped in a holding buffer and placed in 2% dispase solution for 30 min at 37°C and 5% CO₂. After the 30-minute period, the sheets were placed back in the holding buffer to deactivate the dispase, and the RPE peeled off from the choroid using forceps. The cells were suspended in 10% FBS RPE medium (RPMI medium) and placed in a T75 flask. 24 hours later, the medium is replaced with 5% FBS RPE medium to prevent cell overgrowth. Experimenters observed cell adhesion within those 24 hours. Within 14 days, the cells formed a dense film, with hexagonal cell morphology. This protocol was found to
produce well-polarized RPE cells in a discrete monolayer, similar to that found in the human eye, useful for drug treatment and transplant experimentation.

Nonhuman cell sources

While human cells are most desirable for forming anthropogenic RPE monolayers, the ethical issues and limited availability of human donors makes culturing retinal pigment epithelial cells from nonhuman animal donors a preferred alternative. RPE cells have been studied in nonhuman mammals[32, 59, [135]–[137], birds[1], [138], and amphibians[44, [112], [125], and have been successfully harvested and cultured from several species of animal [139]. They have been grown in vitro for about as long as people have been culturing human RPEs [140], and many of the protocols established for human cells are also suitable for the cultivation of animal RPEs. Oka et al. (1984) in addition to culturing human RPE cells in serum-free defined medium (see above section), also worked with bovine cells, with similar results. The dissection and cell-harvesting process was the same as in human eyes, as were the culture conditions. Results showed bovine cells formed confluent monolayers which were grown and passaged accordingly, making this protocol also useful for drug treatment experimentation in nonhuman subjects.

Ho et al. (1997) cultured cells taken from pig eyes for use in experiments with RPE transplantation. The eyes were removed from 6-month old adult pigs and transported to the laboratory within 4 hours of death in Hank’s balanced salt solution (HBSS, Gibco, Grand Island, NY, USA). The eyeballs were enucleated and sterilized by rapid dipping in 70% ethanol, then air-dried and transferred to MEM PSF (Eagle’s minimum essential medium (Gibco) with 110 U/ml penicillin G sodium, 0.1 mg/ml streptomycin sulfate, and 2.5 µg/ml fungizone (Sigma, St. Louis, MO, USA)), where they were dissected by removing the anterior part of the eye. The vitreous and neural retina were removed and the eye cup was washed with 10% FBS MEM PSF with gentle pipetting. RPE cells were harvested using a fire-polished pipette and the resulting suspension centrifuged for 5 minutes at 800 RPM. The cells were resuspended in MEM/10 PSF and placed in 60 mm tissue culture dishes for culturing. The cultures were incubated at 37°C and 5% CO₂ until they reached confluence, using MEM/10 PSF as medium, with supplements of 50 mg/ml gentamicin (Sigma) and 1 ng/ml basic fibroblast growth factor (bFGF, Gibco). Cells reached confluence in 3-4 weeks for primary cultures, after which they were split 1:5 and passaged into 6 well plates or 60 mm culture dishes. First passage cells grown this way were then able to be harvested using 0.25% edetic acid (fig. 5) in Hank’s balanced salt solution (HBSS) and transferred to other culture dishes with high viability (96.7 ± 2.7% by Trypan blue exclusion). This protocol is recommended for experimentation with RPE transplants that do not use a solid substrate during transplantation.

Singh et al. (2001) also used pig RPE cells (in addition to human cells, as detailed in the previous section). The cells were harvested 2-4 hours after death from pig eyes that had been soaked in sterile prepodyne solution 10 minutes before dissection. The cells were obtained by removing the anterior portion of the eye, followed by the vitreous and neural retina. The eyecup was then washed with HBSS and trypsinized with 0.25% trypsin for an hour at 37°C. The trypsin was then aspirated off and replaced with 10% FBS DMEM, which was then pipetted gently to remove the cells from the Bruch’s membrane. The RPE cells were seeded onto six-well tissue culture dishes and incubated at 37°C in 5% CO₂ in 10% FBS DMEM, and the culture changed every 3-4 days. Passaging was performed using 0.05% trypsin-EDTA, and cells later plated onto human and porcine lens capsules and hydrogels at 6 x 10⁵ cell/ml seeding densities. RPE cells
attached and proliferated well on both lens capsule and hydrogel surfaces, forming confluent monolayers of polygonal pigmented cells (fig. 6). This protocol is recommended for anyone wishing to perform drug treatment tests or RPE transplantation experiments, based on the highly epithelioid structure of the cell culture.

Figure 5. Porcine RPE cells in suspension after 12 minutes in 0.25% edetic acid during passaging. Scale bar = 50 µm. [117]

Figure 6. Cultured porcine RPE cells, grown on a pig lens capsule (top) and a hydrogel (bottom) respectively, as found in Singh et al. (2001), Scale bar = 50 µm. [87]

Israel et al. (1980) cultured embryonic RPE cells from domestic chickens by dissecting embryos at stages 29-31 of development. The RPE cell sheets were removed from the other cells and dissociated in Coon’s collagenase-trypsin-chick serum-EDTA enzyme solution. The resulting suspension was plated in 3 ml of 5% FBS MEM or F12 medium on 60 mm plastic tissue culture dishes. The cells were incubated at 37.5°C in 5% CO₂ and the medium replaced every 3 days. Cells grown in MEM were shown to form colonies of heavily pigmented cells that grew in epithelioid monolayers, while cells grown in F-12 were large, fibroblastic, and had little pigmentation. The protocol that resulted in the first type of cell is recommended for drug treatment experimentation, although its application to humans may be limited due to the different nature of the avian RPE cells.
Cell lines

An alternative to using cells from living or dead specimens is to culture cells that have already been transformed into cell lines. Immortalized RPE cells are available commercially, and often come with a standardized culturing protocol attached. One of the most commonly-used RPE cell lines is the ARPE-19 cell line, which was established from cells isolated from the enucleated globes of a 19-year-old male human donor 2 hours after death [142]. While ARPE-19 cells have been used in many studies since the cell line was first established, they show some morphological and developmental differences from natural-type RPE cells [14], [42], [75], [77], [86], [143]–[145]. In general, immortalized cell lines have some physiological differences from natural cells, and may have different culturing requirements as well; however, variations in culturing technique are less prevalent than in human- and animal-derived cells, due to standardization of culture protocol for established cell lines. One of the most common problems with cell-line-derived RPE cells is a lack of pigmentation and different morphological characteristics than those found in natural RPE; however, it is possible to induce immortalized cells to form pigmented epithelioid monolayers, as shown in the examples below.

RPE cells can also form cell lines spontaneously, as found by Mannagh et al. (1973) who observed that of the 119 primary RPE cultures they managed to establish from human donors, 7 spontaneously transformed into cell lines. The researchers found the cells became smaller and more uniform upon transformation, as well as more closely spaced, and began demonstrating accelerated growth, approximately halving their generation time. They also discovered that the transformed cells remained in a monolayer and gradually replaced the remaining primary culture cells. Further study showed that the transformed cells had changed from diploid to heteroploid, a common indication of transformation of a primary culture into a cell line. Two of the cell lines established by the study survived into later years, and came from a 70-year-old and 21-year-old donor respectively. This tendency of RPE cells to spontaneously form cell lines has been observed in multiple instances [118], [142], and the cells produced by such cell lines are recommended for drug treatment experiments.

Tezcaner et al. (2003) cultured cell-line-grown RPE cells with the goal of providing transplants for RPE disorder patients, using cells from the D407 cell line. The cells were cultured in 5% FBS DMEM in a 5% CO₂ atmosphere, and passaged using 0.05% trypsin-EDTA. The cultures were grown on thin surface-modified and un-modified PHBV8 films that were kept in 24 well plates, with Teflon O-rings placed on top to prevent the films from floating after medium was added. The cells were seeded at a concentration range of 8.5 x10^3-45x10^3 cells/cm². Both the films and the O-rings were sterilized using 20 minutes of UV radiation. When the cells reached confluence, they were passaged using 0.05% trypsin-EDTA, with a reattachment rate of 25 x 10^3 cells/cm² after 8 hours of incubation. This increase in reattachment rate with time was only found to occur within the previously-mentioned seeding density range, and the PHBV8 films treated with 100 W oxygen plasma for 10 minutes (the smoothest substrate used in this experiment) were found to have the greatest success in cell reattachment and growth, forming confluent monolayers within 7 days. This protocol is recommended for experiments with drug treatments and transplantation using cell lines, due to the successful formation of confluent monolayers.

Tian et al. (2005) used the ARPE-19 cell line, cultivating the cells in order to check for transcriptional differences between the cell line and native RPE from fresh cadaver eyes. All ARPE-19 cells were seeded at 10,000 cells/cm² or 1000,000 cells/cm² for 3 days in T75 flasks in
10% FBS DMEM:F12 and incubated at 37°C in 10% CO₂. Confluent cultures were then grown for 7 days, and then their medium was replaced with fresh medium containing either serum (CS treatment) or 1% bovine serum albumin (CSW) for 3 days. Other cells were grown for 2.5 months in 10% FBS DMEM:F12 medium, and then in either serum (DS) or 1% bovine serum albumin (BSA) (DSW) for 3 days. Results showed ARPE-19 cells grown on plastic to have fewer transcriptional differences with native RPE than ARPE-19s grown on other surfaces, and that the two treatments with the fewest transcriptional differences with native RPE were CSW and DSW. However, the DS culture showed the greatest morphological similarities to native RPE, displaying a tight mosaic-like monolayer configuration with polygonal, columnar, highly pigmented cells. Due to these results, the protocol is recommended for drug treatment experiments, particularly where transcriptional differences are an issue.

Amemiya et al. (2004) cultured cells of the ARPE-19 and H80HrPE cell lines in 8% heat-inactivated FBS 1% penicillin-streptomycin MEM at 37°C with 5% CO₂. The medium was changed every 3 days, and the cells were found to form a viable epithelioid monolayer and remained healthy after freezing and thawing. They developed pigmentation after 5 months of culture and, when examined immunocytochemically, showed immunoreactivity for some epithelial cell markers. This protocol is recommended for drug treatment experiments involving cell markers.

Results and Conclusions

The various RPE culture protocols examined in this review are summarized in the following section. While each method was successful in producing a culture of viable RPE cells, differences in resulting cell life, morphology, function, and structure make different methods preferable for a researcher desiring a specific type of culture. The effects of cell source, preparation and extraction method, culture medium, substrate, incubation, and passaging are evaluated in the conclusions section.

Summary of methods

Each method for culturing RPE cells reviewed is summarized in Table 1 (Appendix). Results of each method are summarized in Table 2 (Appendix).

Cell sources and extraction methods

According to the literature, use of cells from all sources listed resulted in viable cultures. For human cells, both adult and fetal cells grew well, and removal from eyeballs was generally accomplished by first peeling off the RPE and choroid as a single sheet, then dicing/mincing the tissue with a scalpel, with or without digestion by some digestive enzyme first (trypsin or dispase). Nonhuman cells, on the other hand, were more often digested and then dissociated with pipetting, either in the eyecup or after the RPE-choroid sheet had been removed. Cultures from cell lines were generally less complicated to begin, due to the lack of need for dissection or dissociation, but often led to alterations in cell morphology, including loss of pigmentation, though this may be overcome. There is no indication that one cell source is better than another for increasing chances of adhesion and growth, though with dead specimens freshness is highly desired for a viable culture.
Culture media

In general, the medium used was either a form of Modified Eagle’s Medium (MEM) often supplemented with FBS or some other serum, or a mix specific to the publication without a standardized name. Concentrations of supplemental sera varied from 5% to 20%, and other supplements included streptomycin, penicillin, and other antibiotics that helped prevent contamination. No general trend was seen indicating the success of one medium over another in cell growth, though differences in cell morphology and growth rate were seen in relation with different medium mixtures, and the same media may react differently to human and nonhuman RPE.

Substrates and incubation

Well-plates, culture dishes, and flasks (either T25 or T75) were the most common growth substrates used. Each demonstrated successful cell attachment and growth. At least one study [146] found the smoothest substrate to be the most effective in inducing cell attachment. Others [87], [133] found a more irregular surface more conducive to cell adhesion and the development of polygonal, epithelioid cell morphologies. This variation in adhesion potential may be due to the surface resembling Bruch’s membrane that natural RPE cells adhere to. Incubation was performed almost universally at 37°C and 5% CO₂, even for nonhuman cells, the exception being Israel et al. (1980), who incubated avian RPE cells at 37.5°C. Medium change intervals ranged from 2-4 days, with no difference in cell growth detected between variations.

Passaging

Of the cells successfully passaged, the most commonly-used dissolving agent was trypsin, ranging in concentration (in EDTA) from 0.05% to 0.25%. Most cultures were passaged at confluence, with successful cell reattachment and growth on the new surface. Cultures took between 5-30 days to reach confluence, depending on the size of the surface they were grown. Seeding concentrations ranged from 8.5x10³ to 6x10⁵ cells/ml, with successful adhesion and cell growth within that range. On average, non-immortalized cells were able to go through 4-7 passages before growth ceased and cells became senescent, often altering their morphology at this point.

Conclusion

The science of RPE cell culture, despite having been practiced for over four decades at the time of this writing, remains an experimental process. Researchers are still attempting to discover the correct combination of conditions, chemicals, and procedures to produce an artificial RPE best suited for macular degeneration testing. While there is still territory left to explore, a few standards have been established that can be used as guidelines for a cell scientist wishing to recreate the RPE in vitro.

First, while pigmentation loss and degradation of the epithelioid formation are common problems for cells harvested from living or cadaverous tissue, both can be minimized with proper medium mixes and plating techniques. While cells from cell lines often differ from their parent
tissue, some differences (such as pigmentation and cell shape) can be fixed by manipulating culture conditions. RPE cells can be grown to form confluent epithelioid monolayers on a variety of surfaces and in a variety of media, though DMEM and its variations were the most commonly-used medium. Cultured RPEs can be grown to confluence and successfully passaged, and have been shown to be able to produce cell lines and retinal stem cells, and while no cultured RPE monolayer has been successfully transplanted into a human eye yet, as culturing techniques improve the cells come closer to the state found in the natural RPE, making such future surgical endeavors a possibility. Ultimately, the path to devising the ideal culture protocol for approximating the natural human RPE can only be found through further experimentation. Using this review as a guide will greatly aid researchers attempting to further research into the retina and AMD treatment.

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