

Methods for culturing retinal pigment epithelial cells: a review of current protocols and future recommendations

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Abstract

The retinal pigment epithelium is an important part of the vertebrate eye, particularly in studying the causes and possible treatment of age-related macular degeneration. The retinal pigment epithelium is difficult to access *in vivo* due to its location at the back of the eye, making experimentation with age-related macular degeneration treatments problematic. An alternative to *in vivo* experimentation is cultivating the retinal pigment epithelium *in vitro*, a practice that has been going on since the 1970s, providing a wide range of retinal pigment epithelial culture protocols, each producing cells and tissue of varying degrees of similarity to natural retinal pigment epithelium. The purpose of this review is to provide researchers with a ready list of retinal pigment epithelial protocols, their effects on cultured tissue, and their specific possible applications. Protocols using human and animal retinal pigment epithelium cells, derived from tissue or cell lines, are discussed, and recommendations for future researchers included.

Keywords

Retinal pigment epithelium, *in vitro*, tissue culture, cell culture, age-related macular degeneration

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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 (Figure 1).^{5–7} 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,⁸ with microvilli on the apical surface.⁹ Despite its simplicity as a tissue layer, the RPE plays many complex roles in the vertebrate eye, including regulation of retina development,^{10,11} absorption of excess light entering the eye to reduce photo-oxidative stress,¹² secretion of growth factors such as vascular endothelial growth factor (VEGF),^{5,11,13} mediation of the immune response of the eye,^{14–16} transportation of metabolites and fluids,^{17–19} and phagocytosis of spent rod and cone outer

segments.^{20–27} The RPE also acts as an intermediate for supplying glucose and other vital nutrients to the retina²⁸ while maintaining a healthy environment for the photoreceptors²⁹ and preventing large molecules from entering the eye from the bloodstream.³⁰ 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.^{31–33} While the RPE plays many roles in the eye, its greatest medical significance comes from its involvement in many ocular disorders³⁴ that can lead to vision loss or blindness, such as retinitis pigmentosa, diabetic retinopathy, West Nile virus, and macular degeneration.^{4,13,35–46}

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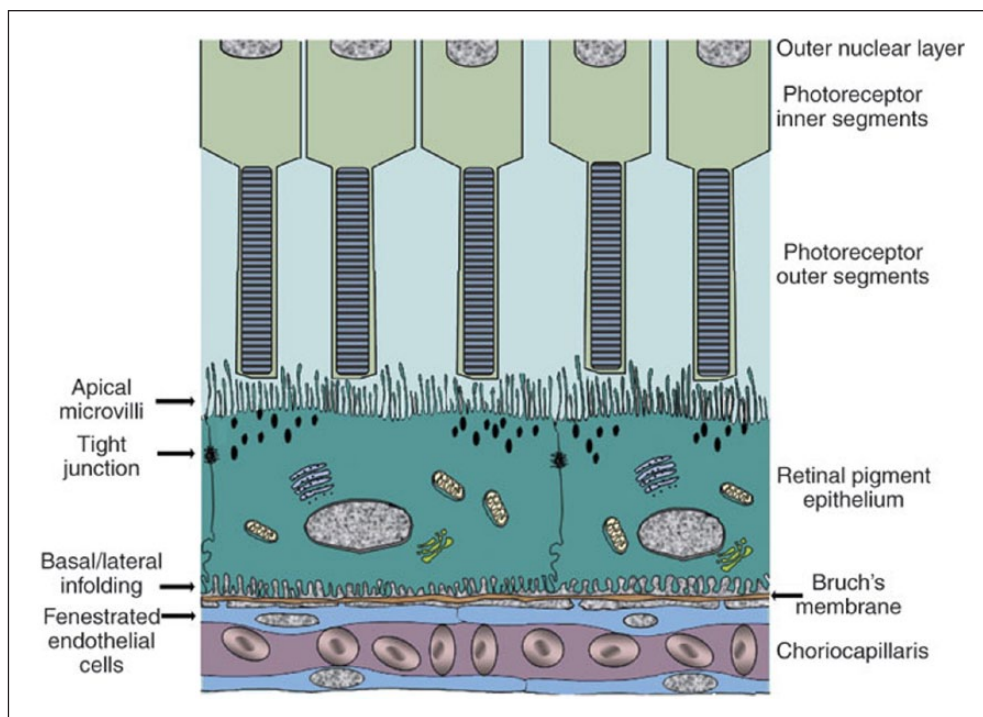


Figure 1. Diagram of the outer retina, showing the retinal pigment epithelium and its location between Bruch's membrane and the neural retina. Pigment granules, tight junctions, and monolayer formation are indicative of natural-type RPE cells.⁴

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 years in developed.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 into Bruch's membrane and the RPE, in correlation with production of VEGF (a soluble factor produced by the RPE).^{48–50} 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.⁵¹ Dry AMD (the most common type, also called atrophic AMD or geographic atrophy)⁵² is a much slower process, characterized by degeneration of the RPE and later the neural retina,^{51,53,54} which can lead in some cases to tearing of the RPE.⁵⁵ Its causes are not fully understood,^{56–58} although evidence suggests contributing factors include the accumulation of lipofuscin and other substances in the eye,^{33,59–64} disruption of autophagy and other processes in the RPE,^{24,27,65–67} photo-oxidative stress caused by excess light entering the eye,^{33,63,68–74} genetic factors,^{36,51} and other retinal disorders like focal choroidal excavation (FCE).⁷⁵

Both AMD types are characterized by the presence of lipid-based deposits, or soft drusen between the RPE

and Bruch's membrane,^{76–79} as well as hypo- and hyperpigmentation of the RPE itself.^{51,80} Other factors involved in AMD, such as iron accumulation, oxidative stress and lipofuscin accumulation,^{33,51,58,81–83} and VEGF production,^{84,85} are processes in which the RPE is directly involved. This suggests that the RPE plays an essential part in AMD progression and perhaps inception. Whether the RPE is directly or indirectly involved with AMD development, evidence suggests that research and experimentation with this particular tissue layer are necessary for learning more about this crippling disease.^{86–89}

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.^{48,54,90,91} 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,⁹² treatments like laser photocoagulation,⁴⁷ use of stem cells to replace degraded cells,^{93–97} and even gene therapy.^{70,98} However, because RPE cells are largely non-proliferative throughout life, regeneration and repair of the already-damaged tissue is normally impossible.³⁵ 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.⁹⁹ This, as well as its location at the back of

the eye, makes observation of and experimentation with the RPE difficult *in vivo*.⁴⁹

One possible alternative to *in vivo* RPE research is to culture RPE cells *in vitro*.^{100–102} RPE cell culture has been practiced for over 40 years at the time of this writing¹⁰³ 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.^{104–106} Cultured RPE cells can be used in a variety of applications, many of which are applicable to AMD research.²⁹ One application, 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 were 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* characteristics of RPE.¹⁰⁷ 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.¹⁰⁸ Some of the most common problems are loss of pigmentation in the cultured cells, low cell–substrate adhesion rates, alterations in cell morphology (in particular, a tendency for epithelioid cells to transform into long, fibroblast-like, “fusiform” cells,^{20,52,105} a natural process that occurs in the eye when the RPE is damaged to produce cells capable of propagating),^{20,103} and low yield of viable cells from those harvested.¹⁰⁹ 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,⁵² and substrate topography,¹¹⁰ and any researcher attempting to closely replicate native RPE tissue *in vitro* would be well advised to search published protocols for the right procedure.

One of the most common methods of measuring RPE confluence and cell density is transepithelial resistance (TER). TER is used to measure the ion flow across the epithelial layer, increasing with time as a culture develops^{7,50} with a higher TER indicating higher confluence, strong polarization of the RPE monolayer, and tight junctions between cells,⁷ all properties that are important to the RPE’s function as a barrier.¹¹¹ Typical TER for the

living human RPE is $150 \Omega \text{ cm}^2$, while cultured RPE monolayers have been measured as low as $25 \Omega \text{ cm}^2$ ³⁴ and as high as $500 \Omega \text{ cm}^2$.⁷ A full range of typical TER values for RPE monolayers in different species both *in vitro* and *in situ* can be found in Rizzolo.³² TER values should be viewed by the RPE researcher as an accurate measure of the cultured monolayer’s similarity to natural RPE, and as the monolayer’s effectiveness as a barrier.

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.^{90,112–115} RPE transplantation experiments have been performed using *in vivo* harvested cells from the same patient,^{116,117} 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.¹¹⁸ 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),^{90,119} and an even higher standard of monolayer fidelity and morphological similarity to the human eye than a culture grown for experimentation. Nevertheless, many researchers^{47,90,120} 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.¹²¹ 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 accurate results in a drug treatment study. 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.^{35,122–124} The RPE begins life as a plastic tissue that is capable of producing lens and retina in some species, but quickly loses this competency as it differentiates into largely non-proliferative RPE cells.^{125,126} 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.^{35,127} 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. 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 the neural retina.^{35,128} 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 re-cultured from floating colonies have shown self-renewing properties.¹²³ 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 generation, 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),¹²³ 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 one of the four purposes listed above. Research into culturing RPE 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,^{94,118,129,130} 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 a 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 Figure 2) and leaving

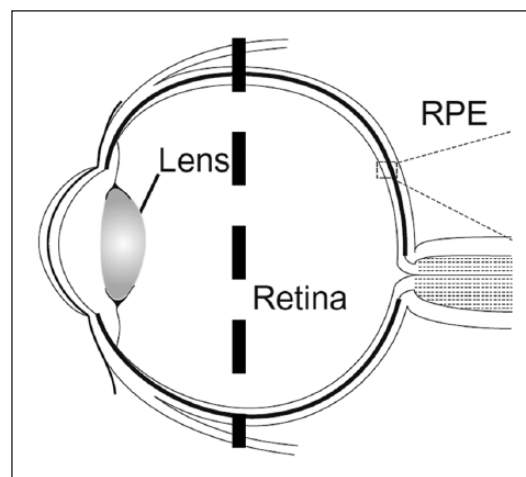


Figure 2. Schematic of the vertebrate eye, with vertical dashed line to illustrate where incision is usually made when creating an “eyecup” from which RPE cells are harvested.³⁵

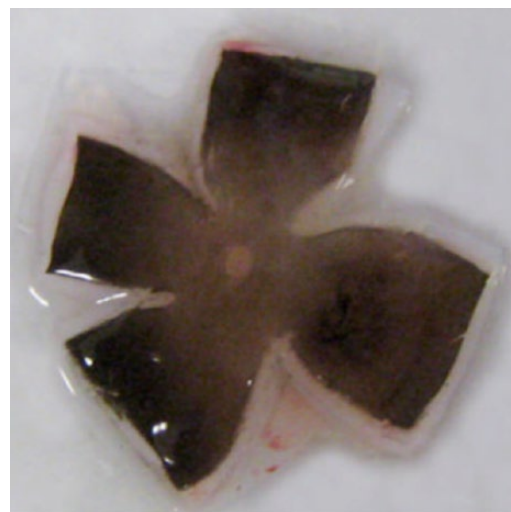


Figure 3. Dissected human fetal eyecup, with neural retina removed and RPE cells visible as dark layer on quartered tissue.⁴

an “eyecup,” with the RPE coating the interior (shown in Figure 3, quartered), into which digestive enzymes (such as trypsin and dispase, two of the most common proteins used) can be poured.¹³¹ 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 ($\sim 37^\circ\text{C}$, 5% CO_2). 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 native mosaic configuration, 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 re-suspended in culture medium, a greater advantage 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 cell sources

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,¹⁰³ 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.¹³² cultured RPE cells in an experiment simply to discover the suitability of human RPE for laboratory culture, and used a cell-harvesting method still commonly practiced today by RPE researchers. The adult human RPE cells 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 (for rough diagram see Figure 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 20–30 min, freeing the RPE cells from 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 in a 5-mL conical tube at 1000 r/min for 3 min. After centrifuging, the supernatant solution was pipetted off and the cell pellet re-suspended in 1 mL of Eagle's minimum essential medium (EMEM), 15% supplemented with fetal bovine serum (FBS),* and the new suspension placed in a Rose chamber or T-15 flask filled with the same medium. 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. 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 years.

The viable cultures were found to form small adherent clusters of round, heavily pigmented cells on the floor of the container 48 h 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 (with the medium being changed twice each week), after which a confluent monolayer would completely form on the floor of a Rose chamber. Confluent cultures were passaged using 0.25% trypsin in calcium and magnesium-free balanced salt solution at 37°C . Pigmentation loss was noted with each cell division, and attempts to stimulate pigment formation by the addition of adrenocorticotrophic hormone (ACTH, 0.5 U/mL) were unsuccessful. The researchers observed spontaneous transformation of primary cultures into cell lines in 7 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 1×10^5 cells/mL. The effectiveness of Pronase as a means of separating the RPE cells from Bruch's membrane was demonstrated by fixing the posterior eyecup in formalin, removing the choroidal tissue and flattening it by radial incision, dehydrating it in alcohol, clearing it with xylene, and mounting it lamina-vitrea-up on a glass slide. The mounted tissue was examined using light and phase microscopy and found to consist of denuded Bruch's membrane, with only RPE cells removed by the process. 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 RPE cells for practice or for the purpose of cell line production.

Around the same time, Tso et al.¹³³ 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 1640 (Roswell Park Memorial Institute medium; Grand Island Biological Co., Grand Island, NY, USA), 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 h after the culture began. After fixation, the tissues were post-fixed in Dalton's chrome osmium fixative for 1 h 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 3 days, with no spreading observed, and attenuation and necrosis after 6 weeks. Different cultures taken from the same patient's eye showed considerable variation in their proliferation and degeneration, while the age of the patient did not affect the growth of the culture. 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.

Aronson¹³⁴ attempted to identify a consistent procedure for culturing human RPE cells using choroid fragments as the seeding vehicle. The choroid fragments were harvested 24 h 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 0.08 mL/cm² of Modified Eagle's Medium (MEM) medium (with Flow Auto Pow No. 11-100 Earle's salts, 1X BME vitamins, 2 mM glutamine, 0.02% sodium bicarbonate, 50 µg/mL streptomycin, 100 U/mL penicillin, and 10% FBS), and after 1–2 days partially rounded fragments were isolated and placed on non-tissue-culture plastic Petri dishes. The fragments were cultured in 0.4 mL/cm² of medium until they formed spheres at around 10 days. The spheres were then plated on tissue culture plastic in 0.08 mL/cm² of medium and allowed to grow for 7 days before the tissue fragment was removed, leaving a new cell line on the plastic. For cell lines maintained in culture for longer periods (up to 10 months), the medium was changed every 10 days to 2 weeks. RPE cells were easily identifiable by their pigmentation at 3–4 months after initial plating. The researchers found that RPE sheet separated from the choroid rarely attached to the plastic and proliferated, while choroid-fragment-associated-RPE cells had a higher attachment and proliferation frequency, often showing fibroblastic outgrowth by day 1. This method involving inclusion of choroidal fragments in the seeding culture is still used by some researchers,¹³⁵ and is recommended for researchers looking to start cell lines, although occasionally experimenters report problems from contaminating choroidal cells.

Oka et al.¹³⁶ compared the effects of traditional serum-supplemented medium and serum-free defined medium

(DM) 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.¹³² The eyecup was rinsed with warm Sal FM three times and the neural retina removed, after which each eyecup was filled with 3–5 ml chicken-trypsin-hyaluronidase solution (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 in equal volumes of a culture medium (CM) composed of Dulbecco's Modified Eagle's medium (DMEM)-F12 supplemented with 20% (v/v) FBS. After centrifuging, the supernatant was removed and the cell pellet re-suspended in CM. The process of dispersal, centrifuging, and re-suspension was repeated several times until all suspensions were pooled in warm CM. For the final culture, one of the two media were used: either CM or DM (serum-free DMEM-F12 supplemented with 5.0 µg insulin/mL, 5.0 µg transferrin/mL, 8.0 ng epidermal growth factor (EGF)/mL, 0.5 mU follicle stimulating hormone/mL, and 50 ng all-*trans* retinoic acid/mL). As with most methods listed, the cells were incubated in a 5% CO₂ atmosphere at 37°C, and the medium 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 DMEM:F12 mixture than in either DMEM 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 DMEM 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 h, which diminished in later passages to 20–25 h and in very late passages increased to 100 h. 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),¹³⁵ serum-containing medium (CM) was found to be necessary for cell attachment and spreading although using serum-free medium (DM) after the initial 24-h plating period in CM resulted in exponential growth. Conversely, cells grown in DM retained epithelioid morphology, while CM-grown cells were larger, non-epithelioid, and irregular. 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.⁹ were able to form viable cultures using cells extracted from eyes donated for corneal transplant, all from humans aged <40 years. The eyes were first dissected by removing the anterior portion of the eye globe, vitreous, lens, and neural retina to expose the RPE (again, the method outlined by Mannagh et al.¹³²), which they then rinsed with Hank's basal salt solution (HBSS). They then filled the eyecup with 0.5 g trypsin/0.2 g ethylenediaminetetraacetic acid (EDTA)/mL and incubated it at 37°C for 15 min. 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 re-suspended in this same medium, and seeded onto a variety of surfaces, among which are listed multi-well tissue culture dishes, Millicell (EMD Millipore) or Costar (Sigma-Aldrich, St. Louis, MO, USA) culture well inserts, and polycarbonate fibers. All culture surfaces received a coating to test cell adhesion, with different coatings tested including laminin, fibronectin, type IV collagen, and Matrigel (an extracellular matrix (ECM) exudate from a tumor cell line). The extraction process yielded high concentrations of pigmented cells, with some erythrocytes present 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 h. The cells were maintained in the same 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 concerned in cell adhesion. The cells were also found to have transferrin receptors, a component of natural RPE cells. 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 concerned with circumventing the blood-retinal barrier.

Tezel and Del Priore¹³⁷ 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 Oka et al.¹³⁶ (see above). They used cadaver eyes incubated in 25 U/mL dispase for 30 min after the same dissection used by Mannagh et al.¹³² 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 15 mM of HEPES buffer, supplemented with insulin, transferrin, EGF, follicle stimulating

growth hormone, retinoic acid, sodium selenite, hydrocortisone, triiodothyronine, 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 r/min for 5 min and the pellet re-suspended in CDSFM, 15% FBS DMEM, or serum-free DMEM, before inoculation on bare or bovine corneal endothelial (BCE)-ECM-coated tissue culture plastic wells. The cells were incubated in 5% CO₂ at 37°C and the respective media of the three treatments changed every other day. Researchers found the cells to be confluent after 12 ± 3 days on BCE-ECM and 21 ± 5 days on bare tissue culture plastic. 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 bare tissue culture plastic, and that the cells formed fewer non-hexagonal (fusiform) morphologies. Researchers also observed higher proliferation rates for the RPE cells in 15% FBS DMEM than in CDSFM (although their respective seeding efficiencies were not significantly different), demonstrating the benefit of the serum-supplemented medium (in addition, DMEM without serum was found to promote lower cell proliferation rates than either of the other media). Both serum-supplemented DMEM and CDSFM produced hexagonal epithelioid monolayers, while serum-free DMEM resulted in large non-mitotic fusiform cells within a week. This culture technique (using 15% FBS DMEM or CDSFM) is recommended for drug experimentation, due to the generally epithelioid structure of the resulting cells and the effects of the various media.

Singh et al.⁹⁰ 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, Canada) 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 h 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 ECM factors by being

placed in a well plate with 1 mL of poly-D-lysine (molecular weight (MW): 540,000, 20 $\mu\text{g/L}$) and HBSS for 5 min, after which the solution was aspirated off and the hydrogels were incubated overnight at 37°C in 2 mL fibronectin solution (20 $\mu\text{g/mL}$) in HBSS. After a day, this solution was aspirated off as well and the hydrogel allowed to dry for 30 min under ultraviolet (UV) light (256.7 nm) in a tissue culture hood. The RPE cells themselves were extracted from eyes of cadavers aged 65–70 years, obtained 8–24 h after death, using the dissection method found in Mannagh et al.,¹³² after which the eyecup was washed with HBSS (Ca^{2+} and Mg^{2+} free) and treated with 0.25% trypsin for 1 h at 37°C. After incubation, the trypsin was aspirated off and replaced with 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% CO_2 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 6×10^5 cells/mL and incubated in the same conditions as before.

Results from the study show that cells adhere to and grow well on both surfaces tested, attaching within 24 h and forming confluent epithelioid monolayers over a period of 4–5 days. Viability analysis (Trypan blue exclusion) found $94\% \pm 0.15\%$ viability on the lens capsules and $90\% \pm 0.16\%$ on hydrogel, while immunohistochemical staining showed the presence of ZO-1, a protein associated with tight junctions. 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.¹⁰³ 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 (1 mm^2), which were plated in a 35-mm Falcon dish with 0.5 mL 20% FBS F12 medium. After 1–2 h, an additional 0.5 mL of the same medium was added to the culture; this action was repeated with 1 mL of medium after 24 h. 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 min at 37°C to detach the RPE from the stroma. The RPE cells were then centrifuged and re-suspended in 10% FBS F12 medium. Pigmented cells attached to the culture dish overnight after seeding and began spreading in 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, while pigmentation continued to decrease. The cells became senescent after 6 passages. In both cases, the cells were grown at 37°C in a 5% CO_2 atmosphere, and medium was changed every 3 days until confluence. Immunocytochemical analysis showed the case 2 cells to have characteristics of epithelial cells, including the presence of cytokeratin. This protocol is recommended for drug experimentation, due to the successful growth of RPE cells with a 6-passage Hayflick limit but with loss of pigmentation.

Akrami et al.¹²³ cultivated human RPE cells in vitro in order to produce retinal stem cells. The RPE cells were obtained by opening the eyes (obtained from cadavers 24 h after death), removing the neural retina with forceps, and 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 min at 37°C in 2 U/mL dispase. The resulting cell suspension was centrifuged for 5 min at 300g and 4°C, and the pelleted cells placed in T25 flasks coated with FBS. The culture medium used was DMEM:F12 supplemented with FBS (20% at the beginning of the culture, 10% with all medium changes afterward), penicillin (120 $\mu\text{g/mL}$), streptomycin (220 $\mu\text{g/mL}$), gentamycin (50 $\mu\text{g/mL}$), and amphotericin B (2.5 $\mu\text{g/mL}$). The medium 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 3×10^5 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, with more spheroids forming from cells harvested from fetal or newborn cadavers. The spheroids increased in number when confluent cultures were deprived of FBS and were collected and re-cultured in 10% FBS DMEM:F12 (either having been dissociated using trypsin or left intact). The dissociated spheroids produced new, secondary spheroids, while the untrypsinized primary spheres reattached to the substrate and produced a new monolayer that in turn led to more floating spheroids. After eight passages, photoreceptors and neural-like cells were detected in the cultures, suggesting they developed either from retinal stem cells produced by the colonies or as the result of trans-differentiating or de-differentiating RPE cells. When evaluated immunocytochemically, the cells were found to express RPE-specific markers RPE 65 (100%) and cytokeratin 8/18 (30%), and retinal stem/progenitor cell markers Pax-6 (8.6%), Chx-10 (11.1%), and Nestin (almost all). This protocol is best suited for growth of stem cells for the production of self-renewing RPE tissue, and to trace the origin of the non-RPE tissue.

Salero et al.³⁵ found human RPE 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.¹³² After dissection, the RPE cells were dissociated by incubation using enzyme-free Hanks'-based cell dissociation buffer (Gibco, Grand Island, NY, USA) at 37°C. After 10 min, the dissociation buffer was gently removed and the eyecup filled with 10% FBS DMEM:F12. RPE sheets of 1 mm² were removed using gentle scraping with a double bevel spoon blade (3.0 mm). The sheets were plated in Matrigel (BD Biosciences, Franklin Lakes, NJ, USA)-pretreated tissue culture plates, then grown using "RPE medium" (MEM- α modified medium, 2 mM L-glutamine, penicillin/streptomycin (1:100), 1% Na-pyruvate, 10% FBS) supplemented with THT (taurine, hydrocortisone, and triiodothyronine) and N1. The cells were cultured in a 37°C 5% CO₂ humidified incubator, and the medium changed every 3 days. 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 non-adherent conditions, supplemented with knockout serum replacement (KSR) medium. This resulted in the development of spheroid colonies, which were found to demonstrate self-renewing properties when re-cultured. 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 young and elderly donors. This protocol would be useful for growing both natural-type RPE cells (for drug treatment experiments) and stem cells for AMD treatment.

Maminishkis et al.¹³⁸ used fetal eyes in an attempt to establish a reproducible protocol for culturing human fetal RPE cells to resemble native tissue, with specific requirements including a TER > 100 Ω cm². The eyes were obtained from random donors, all used less than 26 h after enucleation. The eyes were rinsed in 10 \times diluted antibiotic-antimycotic solution for 3–5 min, and then rinsed twice with medium (described below) or PBS to remove the antibiotic. The anterior portion of the eye was removed (as in Mannagh et al.¹³²) and the posterior portion incubated in dispase-I solution for 30 min. The posterior poles were then placed in silicon-padded Petri dishes containing 5% FBS "RPE medium," a modified medium, the specifics of which are outlined in Maminishkis et al.¹³⁸ (the mixture is similar to Salero et al.,³⁵ in the previous paragraph). The eyecups were dissected in quadrants, and the retina and RPE monolayer removed with forceps. The RPE sheets were then placed in 5% FBS 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

Primaria flasks (Fisher Scientific, Pittsburgh, PA, USA) with 15% FBS RPE medium. After 1 day, the medium was replaced with 5% FBS RPE medium, which was changed every 3–4 days afterward. They displayed confluence and uniform pigmentation after 3–4 weeks, after which they were trypsinized in 0.25% trypsin for 10–15 min, re-suspended in 15% FBS RPE medium and re-seeded onto 12-mm 0.4- μ m-pore polyester membranes (Fisher Scientific) in Transwell cell culture inserts (Corning Costar, Corning, NY, USA) coated in human ECM (10 μ g in 150 μ L HBSS per well) cured with UV light for 2 h in the hood. The cells were seeded at a density of 200 \times 10³ cells/well, and a similar procedure (excluding the ECM coating) was used to seed flasks as well. The resulting cells formed confluent monolayers with epithelial morphologies and heavy pigmentation, with the apical membrane microvilli found in natural RPE. The cells were experimented with once the TER exceeded 200 Ω cm². The final mean TER of 35 monolayers experimented with was found to be 501 \pm 138 Ω cm². This protocol would be most suitable for drug experimentation and transplantation studies, based on the successful production of epithelioid monolayers with high TER.

Gamm et al.¹³⁵ 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, and shipped overnight at 4°C. The eyes were dissected in ice-cold DM (dissection medium, consisting of 70% DMEM containing 4.5 g/L D-glucose, and 30% F12 containing L-glutamine, and 1% antibiotic-antimycotic solution; Invitrogen, Carlsbad, CA, USA), 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 the three ways: in the first treatment, the RPE-choroid sheets were incubated in a 2% dispase-DM solution for 30 min at 37°C and washed in DM twice, then the choroid peeled off with forceps and the resulting isolated RPE sheets chopped into 200- μ m sections with a McIlwain automated tissue chopper, then placed in laminin-coated tissue culture plastic in serum-free RPE DM medium (SFRM, DM supplemented with either 2% B27 (SFRM-B27; Invitrogen) or 1% or 2% N2 (SFRM-N2; Invitrogen)). In the second treatment, the RPE-choroid sheets were immediately chopped into 200- μ m sections without dispase digestion, and only those fragments containing large pieces of RPE were placed in laminin-coated tissue culture plastic flasks or wells, where they were also 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 2 was modified in that the chopped RPE-choroid fragments were

first placed in suspension culture in SFRM-B27, and then placed in tissue culture dishes. Spherical tissue aggregates formed within hours and became uniformly pigmented after 2–4 weeks in culture. After each treatment, the cultures were incubated at 37°C and 5% CO₂, and 50%–75% of the medium was changed every 1–2 days. In all treatments, both cells supplemented with B27 and cells supplemented with N2 showed outgrowth in the first passage although 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 in serum-free medium, due to its successful production of viable RPE cultures.

In 2009, Sonoda et al.⁷ established a protocol for culturing and differentiating human fetal RPE cells. Their cells were obtained from preserved tissues taken from 18- to 20-week-old fetuses, shipped within a day to the experimenters. The anterior portion of the eye (referred to as the cornea–iris complex) was cut off and the vitreous removed. The posterior eyecup was dissected into four quadrants using a razor blade before the RPE-choroid layer was peeled from the retina with forceps (Figure 3). The sheets were then dipped in a holding buffer and placed in 2% dispase solution for 30 min at 37°C and 5% CO₂. After the 30-min 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. After 24 h, the medium was replaced with 5% FBS RPE medium to prevent cell overgrowth. Experimenters observed cell adhesion within those 24 h. The medium was changed twice a week until confluence. Within 14 days, the cells formed a dense film, with hexagonal cell morphology which were then passaged and transplanted onto ECM-coated Transwell membranes. TER was measured regularly and found to resemble *in vivo* values within 4 weeks after passaging, indicating confluence and tight intercellular junctions. 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 make culturing RPE cells from nonhuman animal donors a preferred alternative. RPE cells have been studied in nonhuman mammals,^{33,62,139–141} birds,^{1,142} and amphibians^{44,115,128} and have been successfully

harvested and cultured from several species of animals.¹⁴³ They have been grown *in vitro* for about as long as people have been culturing human RPE,¹⁴⁴ and many of the protocols established for human cells are also suitable for the cultivation of animal RPE. Oka et al.,¹³⁶ in addition to culturing human RPE cells in serum-free DM (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 that were grown and passaged successfully, making this protocol also useful for drug treatment experimentation in nonhuman subjects.

Ho et al.¹²⁰ 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 h of death in HBSS (Gibco). 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-Aldrich)), where they were dissected by removing the anterior part of the eye. The vitreous and neural retina were removed and the eyecup 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 min at 800 r/min. The cells were re-suspended in 10% FBS MEM PSF and placed in 60 mm tissue culture dishes for culturing, where they were grown to confluence using 20% FBS MEM PSF. The cultures were incubated at 37°C and 5% CO₂ until they reached confluence, being fed 10% FBS MEM PSF with supplements of 50 mg/mL gentamycin (Sigma-Aldrich) and 1 ng/mL basic fibroblast growth factor (bFGF, Gibco) twice a week. Cells reached confluence in 3–4 weeks for primary cultures, after which they were split 1:5 and passaged into six-well plates or 60 mm culture dishes. First passage cells grown this way were then harvested using 0.25% edetic acid (Figure 4) in HBSS and transferred to other culture dishes with high viability (96.7% ± 2.7% by Trypan blue exclusion). However, it was found that cell polarization affected reattachment when replated: cells plated apex down formed aggregates rather than reattaching in a monolayer, while cells plated base down reattached and proliferated within 24 h. This protocol is recommended for experimentation with RPE transplants that do not use a solid substrate during transplantation.

Singh et al.⁹⁰ also used pig RPE cells (in addition to human cells, as detailed in the previous section). The cells were harvested 2–4 h after death from pig eyes that had been soaked in sterile prepolydine solution 10 min 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 6×10^5 cell/mL seeding densities. RPE cells attached and proliferated well on both lens capsule (95% \pm 0.56% viability) and hydrogel surfaces (94% \pm 0.15% viability), forming confluent monolayers of polygonal pigmented cells (Figure 5). The pig cells demonstrated the presence of ZO-1 as in the human cells, suggesting tight junctions. This protocol is recommended for anyone wishing to perform drug treatment tests or RPE transplantation experiments, based on the highly epithelial structure of the cell culture.

Hartnett et al.⁵⁰ grew bovine RPE cells in an experiment to discover the effects of endothelial cells on the barrier

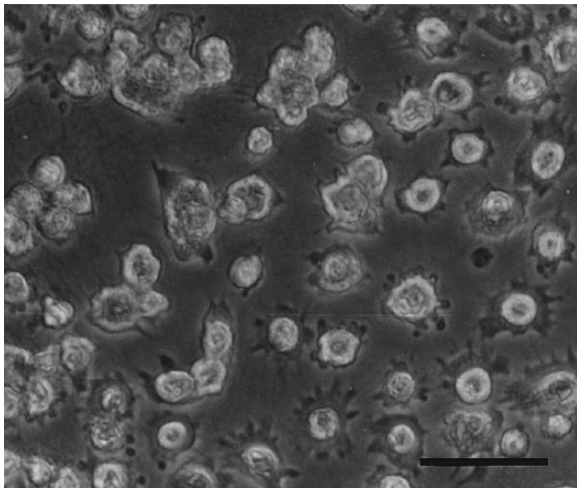


Figure 4. Porcine RPE cells in suspension after 12 min in 0.25% edetic acid during passaging. Scale bar = 50 μ m.¹²⁰

function of the RPE. Prior to experiments with endothelial cells, however, the RPE cells were grown in solo culture to find the best medium to produce optimum monolayers with epithelial and barrier characteristics. The fresh bovine eyes were cleaned of extraneous tissues (including Tenon's capsule) and the globes soaked in 20% povidone-iodine PBS. The eyes were then dissected according to the Mannagh et al.¹³² method, and the eyecups filled with 0.125% trypsin warmed to 37°C. After 60 min, the RPE cells were gently triturated and placed in 10% FBS DMEM, where they were pelleted. After being re-suspended in medium, the cells were seeded into T25 tissue culture plastic flasks and grown at 37°C in 5% CO₂ using 10% FBS DMEM supplemented with GPS (0.234 mg/mL glutamine, 80 U/mL penicillin, and 80 μ g/mL streptomycin C). Experimentation with medium type and its effect on cell growth followed, using one of three types (all supplemented with GPS): a hormonally DM (HDM) with insulin, triiodothyronine, and hydrocortisone), insulin–transferrin–selenium (ITS DMEM supplemented with 1% ITS), or calf serum (CS; DMEM with 1% heat-inactivated CS). The cells were plated at $0.8\text{--}2 \times 10^5$ cells/cm² on 0.4- μ m-pore inserts of Transwell plates in GPS-supplemented 10% FBS DMEM and grown until confluence (which took 14 days for primary cells and 2–3 days for passaged cultures). After that, the medium was replaced with one of the above experimental media (HDM, ITS, or CS) and grown in a 37°C, 5% CO₂ environment. Medium was changed twice a week. Results of this initial experimentation showed that, while TER rose with time in all media tested, cultures fed with HDM reached the highest TER (at 10 days), which remained stable for 20 days. This led to HDM being selected as the culture medium for the remaining mixed epithelial-endothelial cell experiments. This protocol is recommended (using HDM as the culture medium) for drug treatment testing and barrier property studies on the RPE.

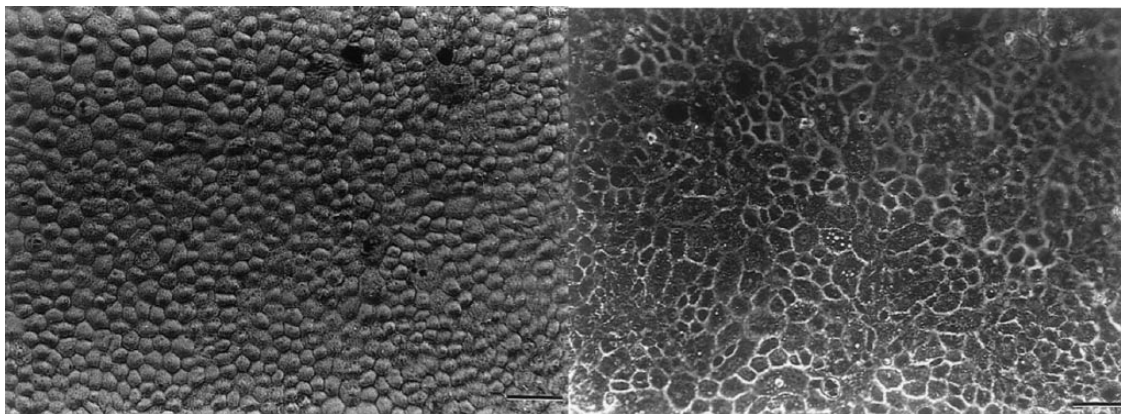


Figure 5. Cultured porcine RPE cells, grown on a pig lens capsule (top) and a hydrogel (bottom) respectively, as found in Singh et al.⁹⁰ Scale bar = 50 μ m.

Israel et al.¹⁴⁵ cultured embryonic RPE cells from domestic chickens by dissecting embryos at stages 29–31 of development. The RPE cell sheets were dissected from the eyes and dissociated in Coon's collagenase-trypsin-chick serum-EDTA enzyme solution (a mixture of 6 U/mL collagenase, 0.1% trypsin, 2% chick serum, with 4 mM EDTA added). The resulting suspension was plated in 3 mL of 5% FBS MEM or F12 medium (supplemented with 0.06 mg/mL penicillin and 0.3 mg/mL glutamine) 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 epithelioid colonies of heavily pigmented cells that grew in mosaic-like monolayers, while cells grown in F12 were large, fibroblastic, and had little pigmentation (although the centers of the F12 cells become pigmented at 5–6 weeks). Electronic microscopy revealed further morphological differences between the two cell cultures, with the MEM culture demonstrating the greatest similarity to natural RPE. Furthermore, the MEM cells were found to be capable of phagocytizing photoreceptor outer segments and laying down an ECM similar to Bruch's membrane. 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. 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 h after death.¹¹¹ 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.^{15,42,78,80,89,146–148} 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, resulting in a decreased TER when compared to tissue-derived primary cultures;⁷ the original derivers of the ARPE-19 cell line reported that they were unable to produce sublines that had a greater TER than the parent cells.¹¹¹ 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.¹³² 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 that 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 70- and 21-year-old donors, respectively. This tendency of RPE cells to spontaneously form cell lines has been observed in multiple instances,^{111,121} and the cells produced by such cell lines are recommended for drug treatment experiments.

Tezcaner et al.¹⁴⁹ cultured cell-line-derived 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 at 37°C, and passaged at confluence using 0.05% trypsin-EDTA. They were then used in experiments to test for the cells' ability to reattach to surfaces after dissociation and proliferate on polyhydroxybutyrate (PHB) films (specifically, PHBV8, either untreated or treated with oxygen plasma to increase hydrophilicity). During the experiments, the cell culture medium was changed once every day. PHBV8 films treated with 100 W oxygen plasma for 10 min (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. The cell seeding densities were also tested, with an optimal concentration of 25×10^3 cells/cm² resulting in 49.6% reattachment. Cell seeding densities above and below this value were found to result in a decrease in cell reattachment percentages, due to overcrowding or lowered cell interactions, respectively. 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.¹⁵⁰ used the ARPE-19 cell line for 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 100,000 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 (no frequency of medium change given) in 10% FBS DMEM:F12 medium, and then in either serum (DS) or 1% bovine serum

albumin (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.¹²² cultured cells of the ARPE-19 and H80HrPE-6 (a cell line derived from an 80-year-old human eye) cell lines in an experiment to evaluate their ability to trans-differentiate into neurons. Initially, the cells were cultured in 8% heat-inactivated FBS 1% penicillin–streptomycin MEM at 37°C with 5% CO₂ (designated the epithelial culture). The medium was changed every 3 days, and the cells were found to form a viable epithelioid monolayer with flat, polygonal morphology. They were able to be passaged using trypsin–EDTA solution and remained healthy after freezing and thawing. Neither cell line showed pigmentation in short-term culture, although the ARPE-19 cells developed visible pigmentation after 5 months in culture. After 2 weeks, when examined immunocytochemically, the cultured cells showed immunoreactivity for the epithelial cell marker pancytokeratin, ZO-1, and β -III tubulin (normally only associated with neurons in the retina). After several weeks, the cells were transferred to a neural stem cell maintenance culture composed of serum-free DMEM:F12 supplemented with 20 ng/mL N₂ (Gibco), 20 ng/mL bFGF (Genzyme, Cambridge, MA, USA), and REC human EGF (Genzyme) at 37°C with 5% CO₂. While maintained in this medium, the cells were grown on laminin/poly-L-ornithine-coated dishes and the medium was changed every 3 days. During this time (dubbed the stem-cell-culture period), the H80HrPE-6 cells became spherical or fusiform after 2 months, and ARPE-19 cells became first elongated after several days and then spherical or fusiform. Immunocytochemical analysis showed no pancytokeratin, ZO-1, or neural markers in the cells. After more than 2 weeks in stem cell culture, the cells were induced to differentiate by being placed in DMEM:F12 with N₂, 0.5% FBS, and 0.5 mM retinoic acid for 10 days, resulting in their putting out multiple processes into the medium. Immunocytochemical analysis showed positive results for β -III tubulin in all cells and for the neural markers MAP5 and NF200 in some cells of both lines. The presence of mature neural markers and loss of RPE-associated markers suggest trans-differentiation into neural tissue, while the higher ratio of MAP5-positive cells in the ARPE-19 cell line suggests a negative relationship between the age of the donor and the differentiability of the cells. Due to these results, this protocol is recommended for stem cell differentiation experiments involving cell lines.

Results and conclusion

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 section “Conclusion.”

Summary of methods

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

Cell sources and extraction methods

According to the literature, the use of cells from all sources listed resulted in viable cultures. For human cells, both adult and fetal cells grew well, and removal from eyes 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 initiate, due to the lack of need for dissection or dissociation, but often led to alterations in cell morphology, including loss of pigmentation although this may be overcome. There is no indication that one cell source is better than another for increasing chances of adhesion and growth, although with dead specimens, freshness is highly desired for a viable culture.

Culture media

In general, the medium used was either a form of MEM, usually supplemented with bovine-derived 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 to help prevent contamination. Despite the problems associated with using sera as supplements to cell culture media, only four publications reviewed attempted use of non-serum medium in RPE culture.^{133,135–137} Of these, the two that compared the non-serum medium to serum-supplemented medium found the serum-supplemented medium to produce superior results in terms of cell attachment and proliferation, suggesting

serum-supplemented medium as the best choice for RPE culture.

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 study¹⁴⁹ found the smoothest substrate to be the most effective in inducing cell attachment. Others^{90,137} 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 surfaces resembling Bruch's membrane (which natural RPE cells adhere to *in vivo*) more or less closely. Incubation was performed almost universally at 37°C and 5% CO₂, even for nonhuman cells, the exception being Israel et al.,¹⁴⁵ who incubated avian RPE cells at 37.5°C. Medium change intervals ranged from 2 to 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 and 30 days to reach confluence, depending on the size of the surface they were grown. Seeding concentrations ranged from 8.5×10^3 to 6×10^5 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, although DMEM and its variations were the most commonly used medium, and serum-based supplements were found to lead to greater cell attachment and proliferation than non-serum media, although serum may affect cell morphology.^{136,137} Cultured RPE cells 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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Note

* Fetal bovine serum (FBS) is used throughout the review as synonymous with fetal calf serum (FCS), despite which name is used in the source publication.

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Appendix

Table 1. Summary of RPE culture methods.

METHOD	SOURCE TYPE	CELL SOURCE	PREPARATION METHOD	EXTRACTION METHOD	CULTURE MEDIUM	SUBSTRATE	INCUBATION AND CULTURE	PASSAGE	SPECIAL NOTES
Akrami et al. (2009)	Human	Cadavers	Eye opened and neural retina removed, interior irrigated with PBS	RPE layer removed with forceps and diced, then incubated in dispase	20% FBS DMEM:F12 (later 10% FBS), supplemented with penicillin, streptomycin, gentamicin, amphotericin	FBS-coated T25 flasks	37°C and 5% CO ₂ , medium changed twice a week until confluency	At confluency, using 3x10 ⁵ cell concentration	
Amemiya et al. (2004)	Cell line	ARPE-19 and H80-HPE cell lines	n/a	n/a	8% heat-inactivated FBS 1% penicillin-streptomycin MEM	Collagen- or laminin/poly-L-ornithine-coated dishes	(in epithelial culture) 37°C and 5% CO ₂ , medium changed every 3 days	Trypsin EDTA	
Aronson (1983)	Human	Aborted fetuses	Bisecting the eye through optic stalk and removing the retina with forceps	Peeling off RPE and choroid as single sheet with forceps, then mincing with scalpel	10% FBS modified MEM	First tissue culture plastic, later non-tissue-culture plastic dishes	37°C and 5% CO ₂ , left to grow for 7 days before forming cell line	Not passaged	
Gamm et al. (2008)	Human	Fetal eyes	Dissected in ice-cold DMEM:F12 and 1% antibiotic-antimycotic solution, anterior portion removed, vitreous and retina removed	Wash eyecup with DM twice, RPE and choroid removed with forceps, and either 1) incubated in 2% dispase-DM solution for 30 min, the RPE peeled off the choroid and the chopped into 200-um sections, 2) chopped without dispase digestion, or 3) chopped, then put in suspension culture of SFRM-B27	SFRM-B27 or SFRM-NZ	Laminin-coated tissue culture plastic	Incubated at 37°C and 5% CO ₂	Not passaged	
Hartnett et al. (2003)	Nonhuman (bovine)	Fresh bovine eyes	Eye globe cleaned of extraneous tissue, then soaked in 20% povidone-iodine in PBS, anterior segments removed and neurosensory retinas scraped free	37°C 0.125% trypsin placed in eyecups for 60 minutes, RPE cells triturated and placed in 10% FBS DMEM	Initially 10% FBS GPS-supplemented DMEM; later, HDM medium selected as optimal for producing wild-type RPE monolayer	Initially T25 plastic tissue culture flasks, then porous inserts of Transwell culture plates (0.4 µm pore size)	37°C and 5% CO ₂ , medium changed twice a week	Not passaged	
Ho et al. (1997)	Nonhuman (pig)	6-month old pigs	Eyes removed and transported to lab within 4 hours of death, carried in HBSS, eyes enucleated and sterilized by dipping in 70% ethanol, air-dried and transferred to MEM PSF, dissected by removing anterior part of eye, vitreous and retina are removed	Eyecup washed with 10% FBS MEM PSF, cells harvested using pipette	10% FBS MEM PSF	60 mm tissue culture dishes	37°C and 5% CO ₂	Split 1:5 and passaged into 6-well plates or 60 mm dishes after 3–4 weeks	

Table 1. (Continued)

METHOD	SOURCE TYPE	CELL SOURCE	PREPARATION METHOD	EXTRACTION METHOD	CULTURE MEDIUM	SUBSTRATE	INCUBATION AND CULTURE	PASSAGE	SPECIAL NOTES
Hunt et al. (1989)	Human	Cadaver eyes	Anterior portion removed, vitreous expelled, retina removed	Eyecup washed with HBSS, then filled with 0.5 g/0.2 g EDTA/ml and incubated for 15 minutes, dislodged cells aspirated off, then incubation repeated	Ham's F-10 medium supplemented with 20% FBS, ITS plus, antibiotics, and a retina extract made by incubating human retina and vitreous in growth medium	Multi-well tissue culture dishes, Millicell well inserts, or polycarbonate fibers, coated with laminin, fibronectin, type IV collagen, or Matrigel	Unspecified temperature and atmospheric conditions, medium changed first after 48 hours to allow for cell adhesion	Not passaged	Laminin is component in natural RPE basal lamina
Israel et al. (1980)	Nonhuman (chicken)	Embryos at stages 29-31	Dissection	RPE removed in sheets and dissociated in Coon's collagenase-trypsin-chick serum-EDTA enzyme solution	5% FBS MEM or F12	60 mm plastic tissue culture dishes	37.5°C and 5% CO ₂ , medium replaced every 3 days	Not passaged	
Maminishkis et al. (2007)	Human	Fetal eyes	Rinsed with antibiotic antimycotic solution for 3-5 minutes, then twice with medium or PBS, anterior portion of eye removed	Posterior eyecup incubated in dispase-I solution for 30 min, then placed in medium and dissected in quadrants, removing retina and RPE with forceps	5% or 15% FBS "RPE medium" (see publication for details)	Flasks and porous polyester membranes coated with ECM	37°C and 5% CO ₂ , medium changed every 3-4 days	At 3-4 weeks with 0.25% trypsin	
Mannagh et al. (1973)	Human	Fresh human eyes (removed for corneal transplants)	Anterior portion removed, eyecup inverted, vitreous and retina removed	Eye cup filled with 0.03% pronase digestion in Ca ²⁺ - and Mg ²⁺ -free salt solution, 20-30 minutes	15% FBS EMEM, with 0.5 U/ml ACTH (to stimulate pigment formation)	Rose chamber or T25 flask	37°C and 5% CO ₂	After 30 days, with 0.25% trypsin	
Oka et al. (1984)	Human	Enucleated eyes	Anterior portion removed, vitreous expelled, eyecup rinsed with warm Sal FM and retina removed	Eyecup filled with 3-5 ml CTH for 30 min	Two separate media: each powdered DMEM and F12, 90% H2O with sodium bicarbonate (53.6 mM) and HEPES (15 mM), one with 20% FBS, one with insulin, transferrin, EGF, FSH, and RA	60 mm culture dishes	37°C and 5% CO ₂ , medium change every 2-3 days	0.25% trypsin	
Oka et al. (1984)	Nonhuman (bovine)	Cow eyes	Anterior portion removed, vitreous expelled, eyecup rinsed with warm Sal FM and retina removed	Eyecup filled with 3-5 ml CTH for 30 min	Two separate media: each powdered DMEM and F12, 90% H2O with sodium bicarbonate (53.6 mM) and HEPES (15 mM), one with 20% FBS, one with insulin, transferrin, EGF, FSH, and RA	60 mm culture dishes	37°C and 5% CO ₂ , medium change every 2-3 days	0.25% trypsin	

(Continued)

Table 1. (Continued)

METHOD	SOURCE TYPE	CELL SOURCE	PREPARATION METHOD	EXTRACTION METHOD	CULTURE MEDIUM	SUBSTRATE	INCUBATION AND CULTURE	PASSAGE	SPECIAL NOTES
Salero et al. (2012)	Human	Eyes obtained from eye banks	Anterior portion removed, eyecup inverted, vitreous and retina removed	RPE isolated and dissociated and plated in adherent conditions	"RPE medium" (MEM- α modified medium, 2 mM L-glutamine, penicillin/streptomycin (1:100), 1% Na-Pyruvate, 10% FBS) 10% FBS DMEM with penicillin/streptomycin	Matrigel-pretreated tissue culture plates	37°C and 5% CO ₂ , medium changed every 3 days	6-8 times	
Singh et al. (2001)	Nonhuman (pig)	Dead pigs	Harvested 2-4 hours after death and soaked in sterile prepodyne for 10 minutes	Anterior portion removed, vitreous and neural retina removed, eyecup washed with Hank's balance salt solution and trypsinized with 0.25% trypsin for an hour at 37°C, cells removed by pipetting		Six-well plates, later lens capsules and hydrogels	37°C and 5% CO ₂ , media changed every 3-4 days	Using 0.05% trypsin-EDTA, seed at 6 x 10 ⁵ cells/ml	
Sonoda et al. (2009)	Human	Fetal eyes	Anterior eye portion removed, vitreous removed, eyecup cut in quadrants	RPE-choroid layer peeled off and dipped in holding buffer, placed in 2% dispase for 30 min, then back in holding buffer; RPE then peeled from choroid with forceps	10% FBS RPE medium (RPMI medium); after 24 hours, 5% FBS RPE medium	T75 flasks and ECM-coated Transwell membranes	37°C and 5% CO ₂	At confluence	
Tezcaner et al. (2003)	Cell line	D407 cell line	n/a	n/a	5% FBS DMEM	Thin surface-modified and unmodified PHBY8 films in 24-well plates with teflon o-rings, all sterilized by 20 minutes of UV	37°C and 5% CO ₂	At confluence with 0.05% trypsin-EDTA	
Tezel and Del Priore (1997)	Human	Cadaver eyes	Anterior portion removed, vitreous expelled, retina removed	Eyecup filled with 25 U/ml dispase for 30 min, RPE and choroid removed using forceps	CDSFM (see text for details)	Bare or BCE-ECM-coated tissue culture plastic wells	37°C and 5% CO ₂ , medium change every other day	Trypsin, deactivated with aprotinin	
Tian et al. (2005)	Cell line	ARPE-19 cell line	n/a	n/a	10% FBS DMEM:F12	T75 flask	37°C and 10% CO ₂	Passed 6 times before senescence	Cells were grown in different conditions
Tseng et al. (2004)	Human	Living humans	Eyes eviscerated during surgery	Choroidal membrane removed and stored in F12 medium, then cut into small pieces	20% FBS F12 medium	35 mm Falcon dish	37°C and 5% CO ₂ , medium changed every 3 days	Not passaged	
Tso et al. (1973)	Human	Cadavers and eyes from living melanoma/retinoblastoma patients	Eyes opened in RPMI 1640	RPE and uvea separated together from sclera and placed on Millipore filters choroid-down	RPMI 1640	Leighton tube	37°C and 5% CO ₂ , medium changes 2 times a week	Not passaged	

Table 2. Summary of culture method results.

METHOD	SOURCE	RESULTS	APPLICATION RECOMMENDATION
Akrami et al. (2009)	Human - cadavers	Cells formed viable epithelioid monolayer that reached confluence within 2–3 weeks, with some pigmentation loss. Spheroid colonies pertaining to stem/progenitor cells formed, forming earlier and in greater numbers from cells from fetal or newborn sources. Spheroid colonies could be recultured, either as intact spheres or dissociated by trypsin. Cultures produced photoreceptor and neural-like cells. Cultured cells showed markers for RPE and retinal stem/progenitor cells.	Generating stem cells
Amemiya et al. (2004)	Cell line - ARPE-19, H80HrPE	Cells of both lines formed flat, polygonal epithelioid monolayer without pigmentation. Some ARPE-19 cells developed visible pigmentation after 5 months in culture. Cells remained healthy after freezing and thawing. Immunocytochemical analysis showed markers for epithelial cells and tight junctions. Further experimentation with other media produced transdifferentiated cells with neural markers.	Generating stem or differentiated cells from cell lines
Aronson (1983)	Human - fetuses	Choroid-RPE fragments formed spheres and formed cell lines when attached to substrate. RPE cells on choroidal fragments showed greater attachment and proliferation than mechanically-separated RPE cells.	Starting cell lines
Gamm et al. (2008)	Human - fetuses	Three different medium treatments were tested, with B27-supplemented medium found to be superior, producing culture expansion after first passage.	Drug treatment experimentation in serum-free medium
Hartnett et al. (2003)	Nonhuman – bovine	Use of HDM medium produced viable RPE monolayers similar to wild-type RPE, with higher TER than those produced by other media tested. High TER remained stable 20 days.	Drug treatment experimentation and barrier property studies
Ho et al. (1997)	Nonhuman - porcine	Cells reached confluence in 3–4 weeks, formed hexagonal monolayer, survived treatment with edetic acid. Reattachment after edetic acid harvesting treatment was found to be polarization dependent.	Transplant experiments
Hunt et al. (1989)	Human - cadavers	Extraction process yielded high concentration of pigmented cells with some erythrocytes mixed in. RPE cells adhered rapidly, laminin-coated surfaces yielded highest cell growth and showed epithelioid pigmented monolayer with intact junction complexes. Cells were found to have transferrin receptors.	Drug treatment experimentation
Israel et al. (1980)	Nonhuman - avian	Cells grown in MEM formed monolayers of heavily-pigmented polygonal cells, while cells grown in F-12 were larger, fibrocytic, and had less pigmentation. MEM cells demonstrated ability to phagocytize outer segments and lay down ECM.	Drug treatment experimentation
Maminishkis et al. (2007)	Human - fetuses	Confluency and heavy uniform pigmentation after 3-4 weeks. Cells formed epithelioid monolayers with average TER of $501 \pm 138 \Omega \cdot \text{cm}^2$, showing tight junctions. Cells had apical microvilli similar to natural RPE.	Drug treatment experimentation and transplantation studies
Mannagh et al. (1973)	Human - cadavers	Found negative relationship between age of donor and culture viability. Cells formed small adherent clusters of round, heavily-pigmented cells on floor 48 hours after inoculation. While cells remained epithelioid with growth, pigmentation decreased, and ACTH was unsuccessful at stimulating further pigment formation. Cultures spontaneously produced cell lines.	RPE culture practice or cell line production
Oka et al. (1984)	Human (cadavers) and non-human (bovine)	Highest plating efficiency found using CM mixture with FBS. DME resulted in larger, less numerous colonies than pure F12. CM medium was found to be necessary for cell attachment and spreading, though serum-free DM increased growth after plating and led to epithelioid morphology.	Drug treatment experimentation
Salero et al. (2012)	Human - cadavers	Cultures formed pigmented epithelioid monolayers within four weeks and formed stem-like cells that had markers for neural, adipocyte, chondrocyte, and osteogenic cells. Similar results found for cells from young and elderly donors.	Drug treatment experiments and stem cell generation
Singh et al. (2001)	Human (cadavers) and nonhuman (porcine)	Human and pig cells on both hydrogels and lens capsules attached and proliferated, forming polygonal epithelioid monolayers with high viability. Immunohistochemical staining revealed ZO-1, showing tight junctions.	Drug treatment and transplant experiments
Sonoda et al. (2009)	Human - fetuses	Cells adhered within first 24 hours and formed a dense monolayer film of hexagonal cells within 14 days, highly polarized, similar to natural RPE. TER found to resemble <i>in vivo</i> values at 4 weeks after passaging.	Drug treatment and transplant experiments
Tezcaner et al. (2003)	Cell line - D407	Cells formed confluent epithelioid monolayers within 7 days when seeded at 8.5×10^3 - 45×10^3 cells/cm ² on PHBV8 films treated with 100 W oxygen plasma for 10 minutes. Optimal cell seeding density found to be 25×10^3 cells/cm ² resulting in 49.6% reattachment.	Drug treatment and transplant experiments
Tezel and Del Priore (1997)	Human - cadavers	Produced confluent epithelioid cells with some decreased pigmentation and a few mixed fusiform cells. Highest seeding efficiency with DMEM with serum on BCE-ECM. BCE-ECM led to fewer fusiform cells. 15% FBS DMEM produced highest proliferation rates. Both 15% FBS DMEM and CDSFM produced epithelioid monolayers, while serum-free DMEM resulted in large fusiform cells.	Drug treatment experiments
Tian et al. (2005)	Cell line - ARPE-19	Cells grown for either 7 days or 2.5 months in 10% FBS DMEM:F12 medium, then three days in 1% bovine serum albumin, showed fewest transcriptional differences with native RPE. The DS cells showed the greatest similarity to native RPE, forming a pigmented epithelioid monolayer with tight junctions.	Drug treatment experiments
Tseng et al. (2004)	Human - living evisceration specimens	Case 1: Cells migrated from choroid fragment and formed colonies within 7 days, mostly fibroblast-like cells. Case 2: Pigmented cells began growth 5 days after incubation began, lost pigmentation, reached confluency in 15 days, showing immunocytochemical indicators of epithelial cells.	Drug treatment experiments
Tso et al. (1973)	Human - cadavers and living patients	No difference found between cadavers and live patients, cell shapes became more irregular after 3 days, no spreading, attenuation and necrosis after 6 weeks. Cells retained epithelial morphology and relatively uniform pigmentation the first 3 days. Thorotrast-supplemented cultures demonstrated ability to phagocytize thorotrast particles. No variation demonstrated due age of the patient.	Drug treatment experiments