

Mounting of Biomaterials for Use in Ophthalmic Cell Therapies

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Abstract

When used as scaffolds for cell therapies, biomaterials often present basic handling and logistical problems for scientists and surgeons alike. The quest for an appropriate mounting device for biomaterials is therefore a significant and common problem. In this review, we provide a detailed overview of the factors to consider when choosing an appropriate mounting device including those experienced during cell culture, quality assurance, and surgery. By way of example, we draw upon our combined experience in developing epithelial cell therapies for the treatment of eye diseases. We discuss commercially available options for achieving required goals and provide a detailed analysis of 4 experimental designs developed within our respective laboratories in Australia, the United Kingdom, and Belgium.

Keywords

biomaterials, mounting device, amniotic membrane, ophthalmology, stem cell therapy

Introduction

The fields of biomaterials and regenerative medicine (incorporating stem cells and tissue engineering) have developed in a complementary manner over the last 20 y. This research nexus is particularly well demonstrated by the growing exploration of biomaterials as vehicles for cell implantation.¹ Given the breadth of materials and cell types currently under investigation, we have presently chosen to focus on examples drawn from the field of ophthalmology. In doing so, however, we provide a comprehensive discussion of the problems and their potential solutions that we consider will be common to many other surgical fields. More specifically, this article examines the critical issue of how biomaterials should be mounted in preparation for cell culture and implantation.

When designing a cell therapy, the emphasis is generally initially placed on optimizing the culture medium ingredients required to maximize cell yield and purity.^{2,3} During these preliminary studies, it is likely that the experimental cultures are grown on commercially available tissue culture plastics including polystyrene. Tissue culture plastic is nonetheless unsuitable for implantation into the body and so the research team must eventually translate their findings to a more biocompatible substrate. During this translation phase, however, a number of key substrate properties are likely to be altered in ways known to affect the structure and/or function of the bioengineered tissue including substrate rigidity⁴

and surface topography (i.e., 2-dimensional vs. 3-dimensional).⁵ Depending upon how biomaterials are mounted, it may well be possible to optimize these characteristics by applying varying amounts of tension to promote substrate flattening and stretching if required. It may also be advantageous in some cases to mount cultures in a way that supports independent feeding and monitoring of the apical and basal culture surfaces. Moreover, the ability to visualize cell

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cultures using noninvasive techniques (e.g., phase-contrast microscopy) throughout manufacture is highly beneficial for quality assurance purposes. We presently demonstrate how these considerations have been incorporated into methods for mounting biomaterials used in ocular cell therapies.

Overview of Ocular Cell Therapies

Three principal areas of current research focus for ocular cell therapies include the ocular surface, the corneal endothelium (i.e., posterior surface of the cornea), and the retinal pigment epithelium (RPE). The common goal in each case is essentially to restore structure and function to an epithelial tissue. The technical requirements for establishing and validating each epithelial cell function prior to implantation, however, vary considerably between each cell type. These differences are reflected in the choice of techniques for mounting biomaterials used during cell culture and implantation.

Cell Therapies for Ocular Surface Reconstruction

The ocular surface is comprised of 2 distinctly different cell types. The corneal epithelium forms the smooth, transparent corneal surface, and the conjunctival epithelium covers the adjacent sclera and inner lining of the eyelids. Since both epithelia are essential for maintenance of a healthy ocular surface, techniques have been developed for treating diseases of the ocular surface using cultivated sheets of corneal epithelial cells and conjunctival epithelial cells.^{6–8} In the case of the corneal epithelium, the necessary progenitor cells are isolated from the peripheral margin or the so-called corneal limbus.⁹ Progenitor cells for the conjunctival epithelium are typically isolated from the inferior fornix, where the conjunctiva extends onto the inner lining of the lower eyelid.¹⁰ Assessment of culture quality in both cases is essentially limited to confirmation of cell phenotype using microscopy and immunocytochemistry. Although both epithelial tissues display stratification *in vivo*, this is not generally considered essential for culture efficacy following implantation.

In most cases, the cultivated corneal and conjunctival epithelial cells have been implanted while attached to sheets of human amniotic membrane (HAM).^{11,12} Standard techniques for processing HAM involve flattening onto nitrocellulose backing membrane and cutting into discs, before being stored frozen in 50% glycerol. Once thawed, the dead remnants of amniotic epithelial cells are usually removed using enzymatic digestion prior to seeding of epithelial cells. Considerable care is required throughout these processes in order to prevent the HAM from becoming detached from the backing paper. Once detached, the HAM readily becomes crumpled when immersed in liquid. Leaving the backing paper on, however, prevents monitoring of cultures by phase-contrast microscopy. The ideal solution is therefore to mount freestanding sheets of denuded HAM within some form of supporting frame that keeps the material taut and flat

during culture and subsequent application to the ocular surface. Similar considerations would also apply using alternatives to HAM such as fibrin glue.^{13,14} Notably, others have reported successful clinical outcomes using contact lenses as a substrate for epithelial cells during their cultivation and implantation.^{15,16} Moreover, others have avoided use of a substrate during implantation via use of temperature-responsive culture plastic that supports release of a free-standing sheet.¹⁷ In the absence of a standardized procedure, however, a variety of experimental substrata including membranes derived from silk proteins^{18,19} are being explored, each with inherent differences in their physical and mechanical properties that can complicate handling during cell culture and surgery.

Cell Therapies for Repairing the Corneal Endothelium

The corneal endothelium is a monolayer of epithelial cells that resides on the posterior surface of the cornea. Corneal transparency is dependent on Na⁺/K⁺-ATPase enzyme activity that resides in the basal lateral surface of corneal endothelial cells. Through regulating the movement of ions between the corneal stroma and anterior chamber of the eye, there is a corresponding movement of water that in turn facilitates the correct spacing of extracellular matrix components required for corneal transparency. Any reduction in corneal endothelial cell density through disease or trauma therefore leads to corneal opacity that can only be rectified via the replacement of lost cells.

Traditionally, corneal endothelial cells are replaced by performing a donor corneal transplant. Owing to the relative immune privilege of the cornea, the majority of corneal transplants last approximately 10 years prior to failure.²⁰ In an effort to extend graft survival time, surgeons have developed a more refined implantation technique where only the most posterior portion of the cornea is implanted using keyhole surgery.²¹ This technique involves implanting the donor endothelium while attached to the Descemet membrane—a thickened basement membrane that separates the endothelium from the posterior corneal stroma. The reliance upon use of donor tissue, however, has prompted researchers to investigate the feasibility of implanting corneal endothelial cells that have been cultivated on some form of prosthetic Descemet membrane.^{22,23} Since the adult corneal endothelium displays limited proliferative capacity, various other progenitor cell types are being considered as an alternative cell source including mesenchymal stromal cells²⁴ and induced pluripotent stem cells (iPSCs).²⁵

Potential substrata for growing corneal endothelial cells include decellularized human corneal tissue,^{26,27} lens capsules,²⁸ sheets of collagen type I,^{29,30} gelatin hydrogels coated with collagen type IV,³¹ chitosan-based membranes,^{32,33} biodegradable polymer membranes,³⁴ polyethylene glycol-based hydrogel films,²³ and hydrogel contact lenses.³⁵ Generally, these materials have been tested with minimal support by simple placement in culture dishes.

One group has generated sheets of corneal endothelial cells after growth on temperature-responsive surfaces.³⁶ In this case, discs of gelatin hydrogel were attached to the apical surfaces of the corneal endothelial cell sheets for handling and transport.³⁶

Examples of materials that have been used in combination with a support structure inside the tissue culture dish to grow corneal endothelial cells are HAM and collagen type I. These materials have been placed on either commercially available tissue culture inserts³⁷ or slim nylon or Teflon rings on the bottoms of tissue culture wells.^{38–40} Corneal endothelial cells can also be grown on membranes of silk fibroin that have been coated with collagen type IV 22 and mounted within Teflon chambers (refer to section “Application to retinal pigment epithelial cells”).

Sheets of corneal endothelium that have been grown in the laboratory are commonly tested for the presence of key proteins that are required for corneal endothelium function by immunocytochemistry. These proteins include zonula occludens-1 (ZO-1), an indicator of barrier function, and Na⁺/K⁺-ATPase, an indicator of ion transport. To check for Na⁺/K⁺-ATPase activity, cell sheets can be tested for their ability to generate ion flow in an Ussing chamber. For this analysis, the cell sheets, supported by their carrier materials, are suspended vertically between 2 chambers, and short circuit current and transendothelial resistance values are recorded. Data from these analyses provide an indication of the potential ability of cells to pump water through the mechanism of electro-osmosis.⁴¹ Although a limited number of research centers have reported success by injecting suspensions of corneal endothelial cells into the anterior chamber of the eye,^{42,43} the majority of efforts are focused on implanting a functioning sheet.

Cell Therapies for Restoring the RPE

The RPE is a monolayer of epithelial cells which separates the photoreceptor layer of the retina from the vascularized choroidal tunic of the eye. While performing a range of barrier and transport functions typical of other epithelia, the RPE is essential for the function and survival of adjacent photoreceptor cells.⁴⁴ RPE cells also share properties with white blood cells since they are engaged in phagocytosis of photoreceptor outer segments shed daily from the tips of adjacent rods and cones. In addition, RPE cells facilitate maintenance of a healthy blood supply through the balanced release of proangiogenic and anti-angiogenic growth factors from their basal and apical surface, respectively.

There is presently wide interest in developing cellular therapies based upon the implantation of cultured RPE owing to the increasing prevalence of age-related macular degeneration (AMD).⁴⁵ Two main forms of AMD have been identified. In so-called “wet AMD,” the degeneration of RPE cells and adjacent photoreceptors is accompanied by ectopic growth of leaky blood vessels into the subretinal space, whereas the “dry” form of AMD is limited to

localized or geographic atrophy of the RPE and adjacent photoreceptor cells. In wet AMD, the growth of blood vessels beyond the choroid is facilitated by disruption to Bruch membrane, a thin layer of extracellular matrix components contiguous with the basement membranes of the RPE and adjacent capillary layer of the choroid (choriocapillaris). Strategies for reconstructing both Bruch membrane and the RPE may therefore be equally important for the treatment of AMD patients.

Although it is possible to generate cultures of RPE cells from adult donor eye tissue,⁴⁶ the resulting cultures can vary greatly due to a number of factors (e.g., age of donor tissue, availability of tissue within a certain time frame after death, and the presence of retinal disease). Current strategies for RPE cell therapy are therefore predominantly focused on the creation of RPE cells from pluripotent stem cells, including embryonic stem cell and induced (adult) stem cell (iPSC) sources. A number of clinical trials have been working with autologous iPSC-RPE cells derived from individual patients as a type of personalized medicine.⁴⁷ However, due to the cost and timescale of production, if a cellular therapy was to be available on a large scale, the utilization of cell banking will likely be the future of iPSC technology.^{48,49} This will ideally involve matching the human leukocyte antigen (HLA) type between donor and patient. This is a routine procedure with most organ transplants. Banking multiple HLA types will increase the availability of the therapy option and avoid immune rejection of the donor cells by the patient’s immune system. This approach has been trialed using human RPE cells derived from donor retinal tissue⁵⁰ and more recently using primate RPE cells derived from donor iPSCs.⁴⁹

A biomaterial membrane that could be used as a substitute for Bruch membrane would ideally also serve as a cell carrier during transplantation and act as a template to guide reconstruction of the subretinal architecture. This poses several issues with how to handle the biomaterial during cell culture and then during surgery. Out of the multitude of studies that have evaluated potential substitutes for Bruch membrane (comprehensively tabulated by Jha and Bharti),⁵¹ only a few have progressed toward preclinical and clinical studies. One of these biomaterials, a permeable polyester substrate (Clinical Trial #NCT01691261),⁵² has been reportedly transplanted into a human patient; however, the trial is currently suspended.

The cultured RPE monolayer on an appropriate Bruch membrane substitute will most likely need to be fully functional prior to implantation in order to achieve best clinical outcomes. A number of functional tests have therefore been developed to test RPE cultures *in vitro*. Electron microscopy can be used to confirm the correct morphology of RPE monolayers at the ultrastructural level including the formation of dense apical microvilli and tight junction complexes between neighboring cells. The culture should be pigmented with melanin granules polarized to the apical cytoplasm. The basal surface of the cells should show membrane infoldings, with native extracellular matrix deposition evident along the

cell–biomaterial interface. The transepithelial resistance of cultures can be measured over time as a way to study the development of barrier function, and this test has been used as a sign of readiness for further functional testing and implantation studies. The expression of important RPE proteins can be localized using immunocytochemistry to demonstrate polarization and indicate functionality. These proteins include bestrophin, claudin-19, cellular retinaldehyde binding protein, cytokeratin 8/18, ezrin, Na⁺/K⁺-ATPase, RPE-65, and ZO-1. The polarized secretion of growth factors can be quantified using proteomic analysis of conditioned apical and basal culture media by immunoassay. Finally, the phagocytosis of fluorescently labeled extracellular particles (e.g., labeled photoreceptor outer segments or synthetic surrogates) is often used as a critical measure of RPE function. This test can be quantified using laser scanning confocal microscopy as well as flow cytometry. More recently, the National Eye Institute (Bethesda, MD, USA), has developed a comprehensive suite of physiologically relevant tests to analyze the ATP-mediated, purinergic signaling pathway within their iPS-RPE cell cultures.⁵³ This pathway is critical for controlling the composition and volume of the subretinal space, and the tests are able to evaluate the intactness of the monolayer.

Current Options for Mounting of Biomaterials

Strategies Used by Developers of Technology

The first option to consider when working with a biomaterial is to simply place the material flat in the bottom of a standard cell culture dish or suspended within a hanging porous cell culture insert (e.g., Transwell cell culture insert).^{54,55} This approach is particularly well suited to more rigid materials such as scaffolds prepared from synthetic polymers. In the case of buoyancy, some form of ring-shaped weight can be applied to maintain submersion in culture medium. Silicone rubber O-rings are particularly useful as weights since they are inexpensive and can be readily sterilized via multiple methods including use of an autoclave. The O-ring size can also be selected to fit snugly to the inner wall of culture wells, thus helping to prevent floating of biomaterials. Nevertheless, the use of less rigid materials including HAM and fibrin glue has led to a variety of strategies being developed.

In the case of HAM, a number of initial studies described placing this material flat in the bottom of either a conventional culture dish^{56,57} or within a cell culture well insert.⁵⁸ Alternatively, the HAM was draped across a platform of stainless steel mesh with a large central hole to facilitate visualization of the cultures.^{7,59} Cell culture inserts subsequently emerged as the preferred support platform for HAM since this culture system supports the use of techniques designed to optimize epithelial cell culture, namely, cultivation at the air–liquid interface and use of mesenchymal cell feeder layers.^{60–62} In later studies, HAM was secured to the

base of cell culture inserts, with or without removal of the porous membrane base.^{61,62} Tethering of HAM was presumably performed for the purpose of flattening the membrane and thus achieving a more even confluency of cells. These authors may also have intended to improve cell growth on HAM by increasing substrate tension, but this goal to the best of our knowledge has never been stated. More recently, others have mounted sheets of HAM within a frame constructed from 2 stainless steel interlocking rings.⁶³ This simple and effective design is similar in concept to that developed independently by 2 of our own groups, which we will discuss later (see sections “The ‘Ludowici’ chamber” and “The Zakaria chamber (Amnion ring)”).

Although fewer researchers have utilized fibrin glue as a substrate for ocular cells, this material has been routinely adopted by at least 1 center, based upon many years of research by one of the field’s pioneers, Graziella Pellegrini.⁶ The method reported by this group describes casting a commercial preparation of fibrin glue designed for hemostasis (Tissucol, Baxter-Immuno, Wien, Austria) within a 3-cm-diameter plastic ring.^{13,14} Presumably, the plastic ring facilitates handling of the fibrin gel during cell culture and application to the ocular surface, although little reference is made to this in the literature.

One of the more elaborate methods used to support biomaterials for RPE implants, developed by a team within the California Institute for Regenerative Medicine, has been to fabricate a material known as a mesh-supported parylene membrane (MSPM).⁶⁴ Essentially, a mesh frame is formed using a multistep approach that includes lithography, wet etching, and the creation of circular holes. A submicron thick membrane of parylene-C is then layered on top of this mesh support and used as a biomaterial membrane (after coating with Matrigel or vitronectin) for the culture of RPE cells. It is assumed that the MSPM is used as an anchored structure inside tissue culture plastic flasks that are flooded with culture medium.⁶⁵

Commercially Available Options

Although numerous patents have been written describing different cell culture systems, surprisingly few commercial products are readily available to researchers looking to mount their test biomaterials. Two widely available product ranges are Snapwell cell culture inserts manufactured by Corning/Costar (New York, NY, USA) and CellCrown cell culture inserts manufactured by Scaffoldex (Tampere, Finland). Both products are distributed internationally by Sigma-Aldrich (St Louis, MO, USA) Specific details for each product range including approximate prices are listed in Table 1. A third option developed by researchers from the University of Regensburg (Regensburg, Germany) and marketed as MINUSHEET is available as part of a more comprehensive cell culture system.⁶⁶ A further commercial product for discussion has been specifically designed for the

Table 1. Commercially Available Options for Mounting Membranes.

| Product/Description | Manufacturer/Distributor | Cat. No. (Plate Size/Quantity) | Price ^a |
|--|--|---|--------------------|
| Snapwell: Cell culture inserts with removable collar containing a porous (0.4 μm) membrane composed of either PC (10^8 pores/ cm^2) or PE (4×10^6 pores/ cm^2). | Corning/Costar | 3407 (PC: 24-well/24) | US\$223.57 |
| | | 3801 (PE: 24-well/24) | US\$219.31 |
| | Sigma-Aldrich | CLS3407 (PC: 24-well/24) | A\$534.76 |
| | | CLS3801 (PE: 24-well/24) | A\$212.00 |
| CellCrown: Cell culture inserts with removable collar for securing materials of choice. Collars also available with preattached nylon or polycarbonate porous membranes | Scaffdex | C00003S (6-well/3) | €22 |
| | | C00002S (12-well/6) | €38 |
| | | C00001S (24-well/12) | €70 |
| | | C00004S (48-well/6 \times 4) ^b | €99 |
| | | C00005S (96-well/8 \times 6) ^b | €141 |
| | Sigma-Aldrich | Z681806 (6-well/3) | A\$49.60 |
| | | Z681849 (12-well/6) | A\$85.50 |
| | | Z681903 (24-well/12) | A\$157.60 |
| | | Z681962 (48-well/6 \times 4) ^b | A\$222.90 |
| | | Z682004 (96-well/8 \times 6) ^b | A\$317.40 |
| MINUSHEET | Minucell and Minutissue Vertriebs GmbH | 1300 (24-well/6) | €60 |
| | | 1301 6-well plate | €560 |
| | | 1302 24-well plate | €680 |
| ProKera: Cryopreserved amniotic membrane clamped between 2 PMMA symblypharon rings. | Bio-Tissue; TissueTech Inc | PK-16 | US\$949 |

Abbreviations: PC, polycarbonate; PE, polyester; PMMA, poly(methyl methacrylate).

^aPrices exclude delivery charges and refer to sterile product option where available. All prices are subject to change.

^bManufacturing to cease in 2017.

purpose of mounting and applying HAM to the ocular surface (e.g., ProKera/Bio-Tissue, Doral, FL, USA).

Snapwell culture inserts are essentially similar to standard cell culture inserts with the notable exception being that the insert base housing the porous membrane can be removed, thus facilitating tests of cell function in other devices (e.g., Ussing chamber). The added advantage of this design, however, is that test membranes, if sufficiently thin and strong, can be clamped between the upper and lower insert components. Theoretically, the porosity of the fitted membranes should enable adequate movement of both nutrients and culture products to facilitate coculture experiments and measurements of growth factor secretion through the basal culture surface. CellCrown cell culture inserts are likewise supplied with detachable bases, thus enabling other membranes of choice to be mounted. The bases, resembling collars, can also be purchased without fitted membranes, thus providing free access to the basal culture surface. A wider range of different insert sizes is also available compared to Snapwell inserts, although the manufacturing of 48-well and 96-well format inserts will be discontinued from 2017.

MINUSHEET is sold commercially by Minucell and Minutissue Vertriebs GmbH (Bad Abbach, Germany) as part of a comprehensive cell culture system designed for in vitro studies of tissue engineering and pharmacological research. Numerous publications are based upon successful use of these products, and a detailed discussion of the factors to consider in performing such work are provided in a freely available e-book by Professor Will W. Minuth and Lucia Denk.⁶⁶ The MINUSHEET (Part No. 1300) consists of a base ring designed to accommodate a 13-mm-diameter

membrane of choice and held in place with a tension ring. Both ring components can reportedly be sterilized using an autoclave, ethanol or formaldehyde. The assembled components are designed to fit within wells of a 12-well style cell culture plate but can also be inserted into a variety of different perfusion chambers supplied by the same company.

In the case of researchers already committed to using HAM, this material can be purchased from TissueTech Inc. (Doral, FL, USA) as a premounted membrane clamped between 2 poly(methyl methacrylate) rings. While this product range (ProKera/Bio-Tissue) is specifically sold as a therapeutic bandage for the treatment of various ocular surface disorders, it may theoretically also be used as a substrate for the cultivation and implantation of cells. The mounting ring resembles that used for the prevention of symblypharon formation following trauma to the ocular surface. Standard symblypharon rings also have potential for mounting some biomaterials and especially those being designed for application to the ocular surface.

Innovative In-House Designs

Modified Micro-Boyden Chambers

In 1962, the Australian immunologist/ecologist Stephen Boyden published a technique for measuring the directed migration of leukocytes toward chemotactic factors.⁶⁷ The apparatus as originally described by Boyden consisted of a porous polycarbonate membrane mounted between an upper and lower chamber constructed from Perspex. Over the years, numerous modifications have been made to this

technique including those leading to the routine manufacture of porous membranes bonded to the bottom of cell culture well inserts. Nevertheless, an alternative modification of Boyden's design, described by Australian pathologist Leon Bignold⁶⁸ (micro-Boyden chamber), has served as inspiration for one of our in-house designs for mounting experimental biomaterials (Figure 1).

One of the key elements in Bignold's micro-Boyden chamber design was to utilize components readily available in most laboratories and local hardware stores. As such, the upper chamber (a flat O-ring cut from a sheet of silicon rubber) and lower chamber (Perspex disk with a central well drilled into it) were clamped together by being assembled within the upper ~2 cm end of a 15-mL polypropylene centrifuge tube and its screw-on lid, respectively. An additional plastic O-ring was placed between the upper chamber and porous polycarbonate membrane to reduce torsion on the membrane during chamber assembly. In doing so, the goal was to create a watertight seal around the edges of the membrane, so that any diffusion of lower chamber contents (a chemotactic factor in this case) would be restricted to the pores within the polycarbonate membrane (vital to creation of a consistent concentration gradient). Incorporating these ideas, we designed an upper ring-shaped chamber component to accommodate a recessed silicone rubber O-ring and to directly thread into a lower ring-shaped base chamber component (Figure 1). Both chamber components are constructed from polytetrafluoroethylene (PTFE, Teflon) to support sterilization by use of autoclave or treatment with 70% ethanol. A central hole within the upper and lower chamber components, combined with the relatively low height profile, supports routine monitoring of cultures by phase-contrast microscopy when growing on either the upper or lower surface of assembled membranes. In the original design for this chamber, 4 channels were cut into the surface of each chamber component in an effort to facilitate the movement of growth medium. The channel within the upper chamber also provided a purchase point for tightening of the chamber using forceps. In our latest design, however, this concept has been developed further by essentially widening the channels to the point of leaving 4 small feet (castellations) and by beveling the inner chamber edges. The upper castellation points, as for grooves used in the initial design, provide a purchase point for tightening of the chamber using forceps.

Application to corneal epithelial cells. Our modified micro-Boyden chamber was originally designed for the purpose of preliminary testing of biomaterials for corneal epithelial cells.¹⁸ In particular, we have successfully used HAM and transparent membranes constructed from silk fibroin. The procedure for mounting HAM is as follows. Following removal of residual amniotic epithelial cells, the nitrocellulose backing paper is removed and the HAM flattened as best as possible in a dry sterile Petri dish (60 mm diameter or greater). A silicone O-ring is then placed underneath the

membrane. The upper chamber component is then pressed down onto the O-ring until the HAM becomes clamped in place. The surrounding HAM is subsequently trimmed to a distance of approximately 5 mm away from the edge of the O-ring. Finally, the upper chamber fitted with HAM is then gently screwed down into the lower chamber compartment until finger tight. A similar strategy has been used for fibroin membranes, although these are typically mounted when dry with the O-ring being placed on top. Once assembled, the dry fibroin membranes can be stored in sterile 50-mL centrifuge tubes and we have successfully shipped them to collaborators across the world. While the inner, smaller chamber is designed to be the upper chamber, the entire apparatus can be inverted to allow seeding of cells onto either surface. Using this approach, we have successfully produced cocultures of corneal epithelial cells and corneal stromal cells separated by fibroin-based scaffolds.⁶⁹ A final advantage of this design is that since all components are compatible with formalin, ethanol and xylene, the entire culture can be fixed and processed into paraffin in preparation for histology. As such, the chamber does not require disassembly until the cultures/membranes are ready for embedding.

Application to corneal endothelial cells. We routinely grow corneal endothelial cells on silk fibroin membranes that have been coated with collagen type IV. Collagen coating is achieved by pipetting a solution of collagen type IV onto one side of the dry silk fibroin membrane that has been placed within our modified micro-Boyden chamber. The whole assembly is then dried overnight at 40 °C under a vacuum of -70 kPa. The coated membrane within the modified micro-Boyden chamber can then be used for cell culture once it has been sterilized with 70% ethanol and washed with phosphate-buffered saline (PBS). Corneal endothelial cells form monolayers that can be easily examined using phase-contrast microscopy during growth on these suspended membranes. Once a complete monolayer has formed, the cell constructs can be removed from the modified micro-Boyden chamber to undergo functional testing in an Ussing chamber.

Application to retinal pigment epithelial cells. We have successfully used our modified micro-Boyden chamber with membranes made from silk fibroin (3 µm thickness) to grow and study RPE cells.^{70,71} The modified chamber design facilitates proper cell culture conditions for this cell type, maintaining independent volumes of culture medium on either side of the membrane over an extended culture period (>4 mo has been tested). This culture setup allows development of appropriate polarization and maturation of the RPE culture. Cocultures of RPE cells and choroidal vascular endothelial cells can be established by sequential seeding of cells onto either side of the chamber.⁷² Moreover, this design enables ongoing functional testing to be performed during extended culture time. The apical and basal volumes of culture medium can be collected separately and examined for differences in growth factor concentration via

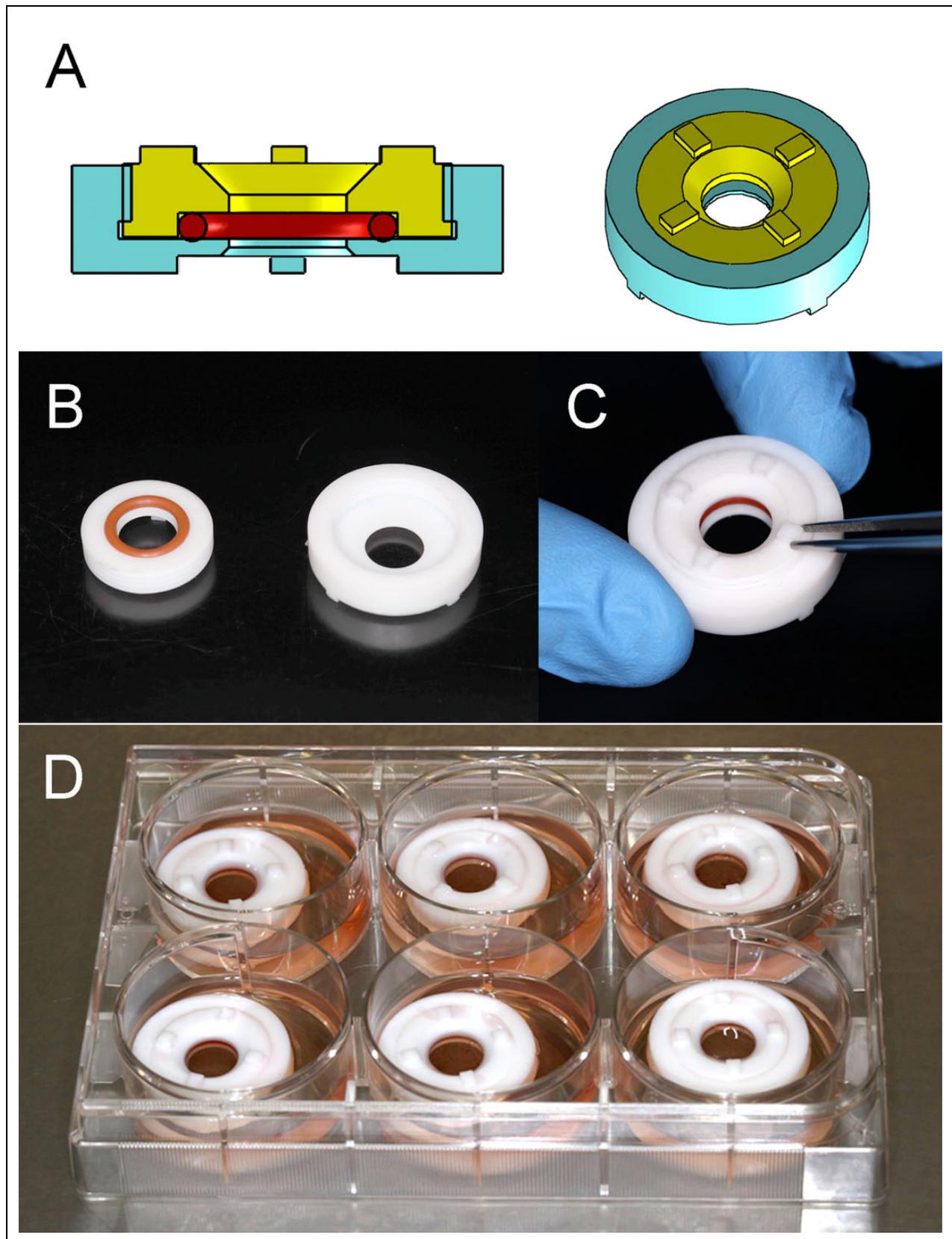


Figure 1. The modified micro-Boyden chamber. (A) Schematic displaying the micro-Boyden chamber design in cross-section and following assembly. The silicone O-ring is shown in red. (B) Appearance of upper and lower chamber prior to assembly. The silicone O-ring can be seen fitted within the upturned upper chamber. (C) Tightening of chamber components by threading together. The upper castellations provide a leverage point for tightening with forceps. (D) Example of chamber use in conjunction with experimental silk fibroin membranes within a standard commercial 6-well culture plate.

immunoassay. Our chamber also supports use of the EVOM2 Voltohmmeter (World Precision Instruments, Sarasota, FL, USA) using the chopstick electrode set (STX3) allowing routine measurement of a developing transepithelial resistance by the RPE cultures.⁷² In combination with noninvasive monitoring (i.e., phase-contrast microscopy), these functional assays inform readiness of the cultures for further experimentation. As for corneal cells, the modified micro-Boyden chambers also provide a convenient protective holder during fixation and storage of the cultures prior to subsequent examination by immunocytochemistry and electron microscopy.

The “Ludowici” Chamber

The inspiration for this second chamber design arose from our desire to translate our *in vitro* studies of corneal epithelial cells to clinical trials. More specifically, we required a chamber design that would accommodate larger sheets of membranes as required for application to the ocular surface of patients and large animal models (e.g., sheep and rabbits). The initial thought was simply to make a larger version of the modified micro-Boyden chambers. Nevertheless, a screw thread mechanism of clamping was considered to produce insufficient tension and promote rippling of the membrane surface. Discussion of alternative options with a local manufacturer (Ludowici Sealing Solutions, Brendale, Queensland, Australia) led to the design of double PTFE ring assembly that could be clamped together via use of an internal flange (within top ring) and matching gutter (in base ring; Figure 2). A series of castellations/feet were again used to encourage flow of culture medium when the entire chamber is laid flat in either orientation. Additionally, 4 small holes were placed through the base ring in order to assist with chamber disassembly. In theory, disassembly would be enabled through downward pressure against an aluminum block fitted with 4 small metal pins to match the position of holes located in the base ring. In practice, however, we have been able to routinely dislodge the inner/upper ring by twisting the entire apparatus until a small section becomes unlocked. We have successfully used this device for mounting either HAM or fibroin membranes. The critical steps involved with mounting of HAM are displayed in Figure 2. Once thawed and washed in sterile buffer, the entire sheet of HAM is placed on top of the lower ring such that approximately 5 mm resides outside the inner gutter. The top ring is then placed on top and downward pressure applied by hand until the upper flange snaps into place within the gutter. In order to maintain sterility during this procedure, the entire apparatus is placed within a sterile Petri dish as demonstrated in Figure 2C. Once mounted, and with the aid of a dissecting microscope, the backing paper is gently punctured with watchmaker forceps and peeled away in sections until the central field is entirely clear. The HAM can then be processed to remove remnants of amniotic epithelium by treatment of choice. In our case, we have used treatment for

5 to 10 min with 0.05% trypsin with 1 mM ethylenediaminetetraacetic acid. Vigorous pipetting of solution across the HAM surface is preferred to mechanical scraping. Given that the HAM is held taut and without backing paper, the extent of epithelial debridement can be effectively monitored by phase-contrast microscopy and repeated as often as necessary. Since fibroin membrane has less elasticity than HAM, we have found it necessary to initially overfill the membrane by indenting the center, which then subsequently becomes flat and taut as the 2 rings are snapped together. As for the smaller chambers, cells can be cocultured on either side of the mounted membrane simply by inverting the apparatus to the desired side when seeding. On the day of surgery, the entire chamber is transported in a sterile specimen container while immersed in culture medium. The chamber is then simply lowered into position across the ocular surface in the desired orientation. Application in rabbits is further facilitated by proptosing the eye prior to surgery (Figure 2E). Once again, the entire chamber can be processed into paraffin without disassembly.

The Dunphy Chamber

As with the other designs, the idea here was to create a flat surface for the *in vitro* culture of ocular cells (Figure 3). The initial motivation came from difficulties encountered with commercially available systems for membranes such as Scaffdex. Commercially available systems tended to tear fragile membranes prepared from electrospun fibers, often required some form of manipulation to render them suitable for bespoke applications, and offered little scope for coculture experiments. Furthermore, they are not reusable, making them a rather costly option. While the Dunphy chamber was originally designed for use with electrospun nanofiber scaffolds prepared from polyesters including poly(lactic-co-glycolic acid), it can also be used with other membranous scaffolds including HAM.

The initial design fits snugly into a 24-well plate leaving a small space between the well and chamber to accommodate media changes using a pipette. The entire culture area is approximately 0.5 cm², a suitable size for a corneal graft; however, the design could easily be scaled up to increase the culture area. Like the Ludowici chamber, the Dunphy chamber consists of 2 separate pieces clamped together: a biocompatible PTFE base and stainless steel retaining clip. To mount a membrane, circular discs of the biomaterial are first cut slightly larger than the diameter of the chamber, for example, using a 12-mm biopsy punch. The semiflexible metallic clip is open at one end, allowing it to be stretched with forceps and then placed around the membrane, which is clamped around the upper lip of the PTFE base. Once the clip is released, it snaps firmly into place to secure the membrane. Similar to a drum or embroidery ring, the membrane is gripped in tension as shown in Figure 3B to D. Additional groove features were added to the base to allow medium to flow under the chamber, and small “legs” were included on the “top side” of the PTFE to enable the chamber to stand

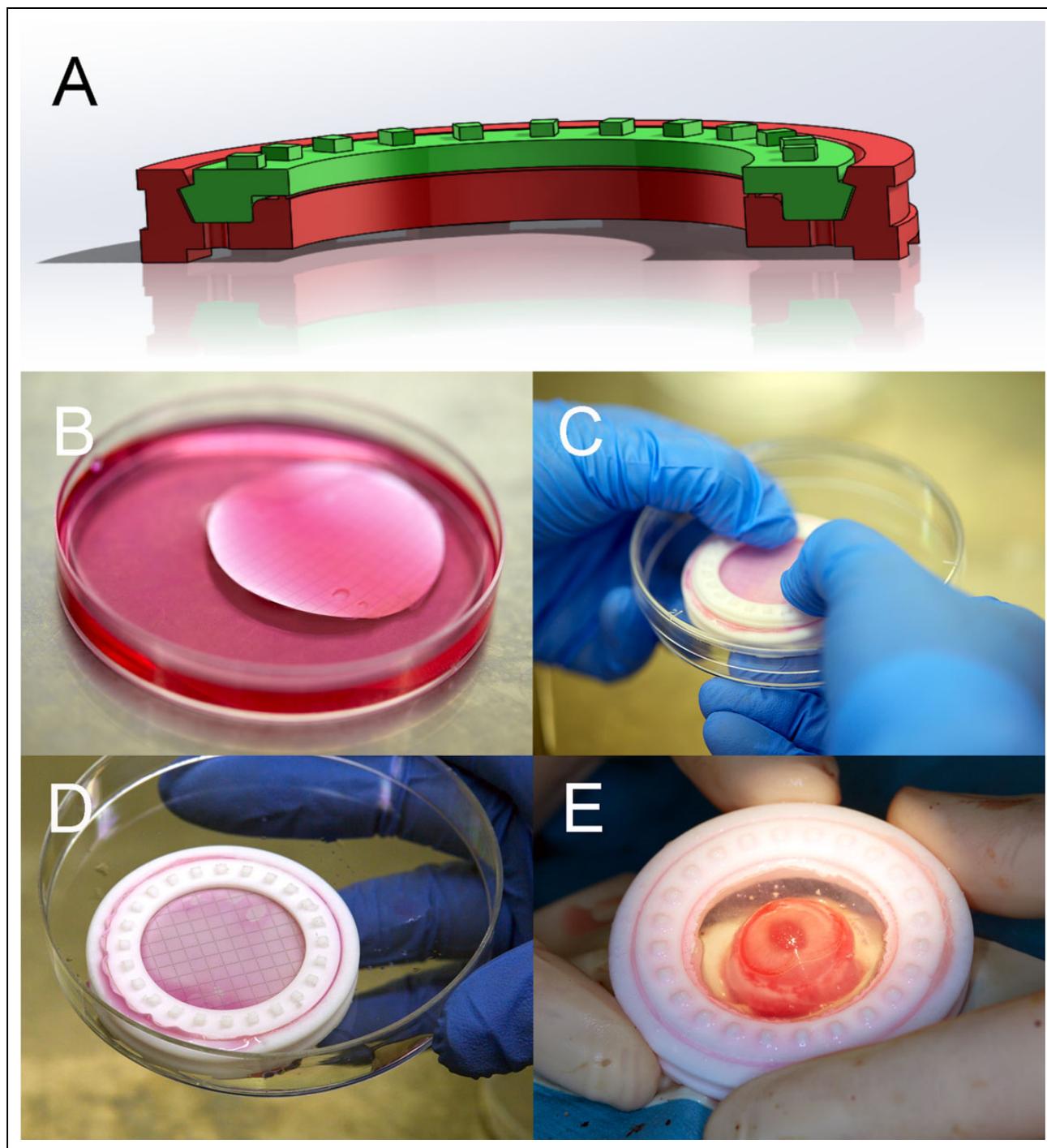


Figure 2. The Ludowici Chamber. (A) Schematic displaying Ludowici chamber design in cross-section. (B) Sheet of donor human amniotic membrane (HAM) after being thawed and washed in culture medium. (C) Assembly of HAM into Ludowici chamber by application of pressure through base and lid of sterile Petri dish. (D) Appearance of HAM following mounting into chamber. The backing paper is subsequently peeled away prior to further preparation. (E) Application of limbal epithelial cell culture grown on HAM onto the surface of proposed rabbit eye.

stably when inverted. This facilitates cell seeding on both sides of a single membrane or bilayered scaffold. Dimensions were chosen such that medium can flow under the chamber when upright or inverted to allow nutrients and oxygen to reach the cells, while maintaining the minimum

dimensional requirements such that culture medium could be used economically. In our experiments, we successfully used the chamber to coculture corneal epithelial and stromal cells on opposite sides of a bilayered membrane to mimic the native structure of the anterior cornea.

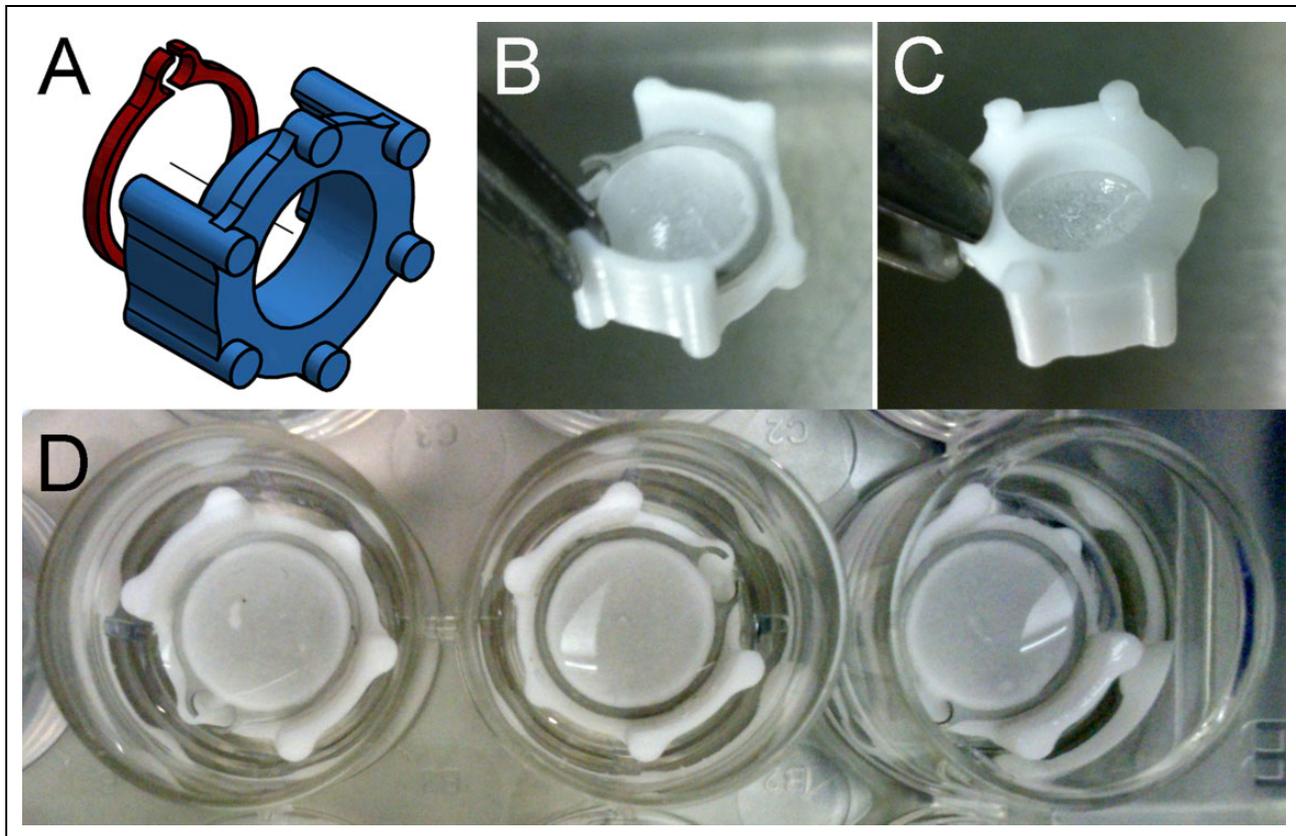


Figure 3. The Dunphy chamber. (A) Schematic displaying the Dunphy chamber components consisting of PTFE base (illustrated in blue) and a stainless steel retaining clip (illustrated in red). (B) Appearance of assembled chamber containing a synthetic electrospun polymer membrane (PLGA) when viewed from above. (C) Appearance of assembled chamber containing membrane when viewed from below. (D) Example of chamber use with 24-well culture plate. PLGA indicates poly(lactic-co-glycolic acid).

The idea behind the clamp-style mechanism is to prevent twisting, which can cause the membrane to break during the mounting process and may affect stresses across the membrane surface. Furthermore, the design has off-the-shelf potential; the entire system, including the membrane, can be sterilized and packaged for laboratory or clinical use, such that the user can simply choose a membrane size and type, place it into an appropriately sized culture plate with medium, and subsequently seed with the chosen cell type. This helps to avoid contamination issues often associated with manipulating biomaterials for use, both experimentally and clinically. In our hands, the membranes were mounted first and then sterilized by soaking the entire system in ethanol or by exposure to ultraviolet irradiation. Following a predetermined culture period, the membranes were easily dismantled by loosening the retaining ring with sterile forceps to release the membrane. The membrane can then be cut to suitable dimensions using a trephine or biopsy punch. Finally, the chamber can be sterilized for reuse, which was achieved by immersion in ethanol, ethylene oxide sterilization, or autoclaving, although the latter should be used with caution as the heating process can cause leaching from some polymeric materials to occur and may affect cell viability.

The Zakaria Chamber (Amnion Ring)

Once again the idea was to develop a fixation device for HAM that would immobilize this material during limbal epithelial stem cell culture, transportation, and surgical transplantation^{73,74} (Figure 4). The diameter of the ring is larger than both the modified micro-Boyden Chamber and the Dunphy chamber, requiring culture to be performed in a 60-mm Petri dish. The HAM is oriented epithelial side down, on the bottom of a culture dish. The inner ring is centered over the HAM and its edges pulled over the lip of the ring. The inner ring with membrane is grasped with forceps and flipped over. The outer ring is then positioned over the HAM and pressure applied to click the rings in place. The rings create a taut drum-like appearance of the membrane and the membrane surface are placed facedown in culture. A limbal biopsy is placed at the center of the HAM and cultured at the air-liquid interface. The design of the Zakaria chamber results in a separation of 1.6 to 2.1 mm between the membrane and the bottom of the culture dish to ensure a reservoir of medium for nutrient exchange. The basic mechanism here is similar to the other designs described previously, incorporating the idea of an embroidery drum to maintain immobility of the contained membrane. The advantage of this

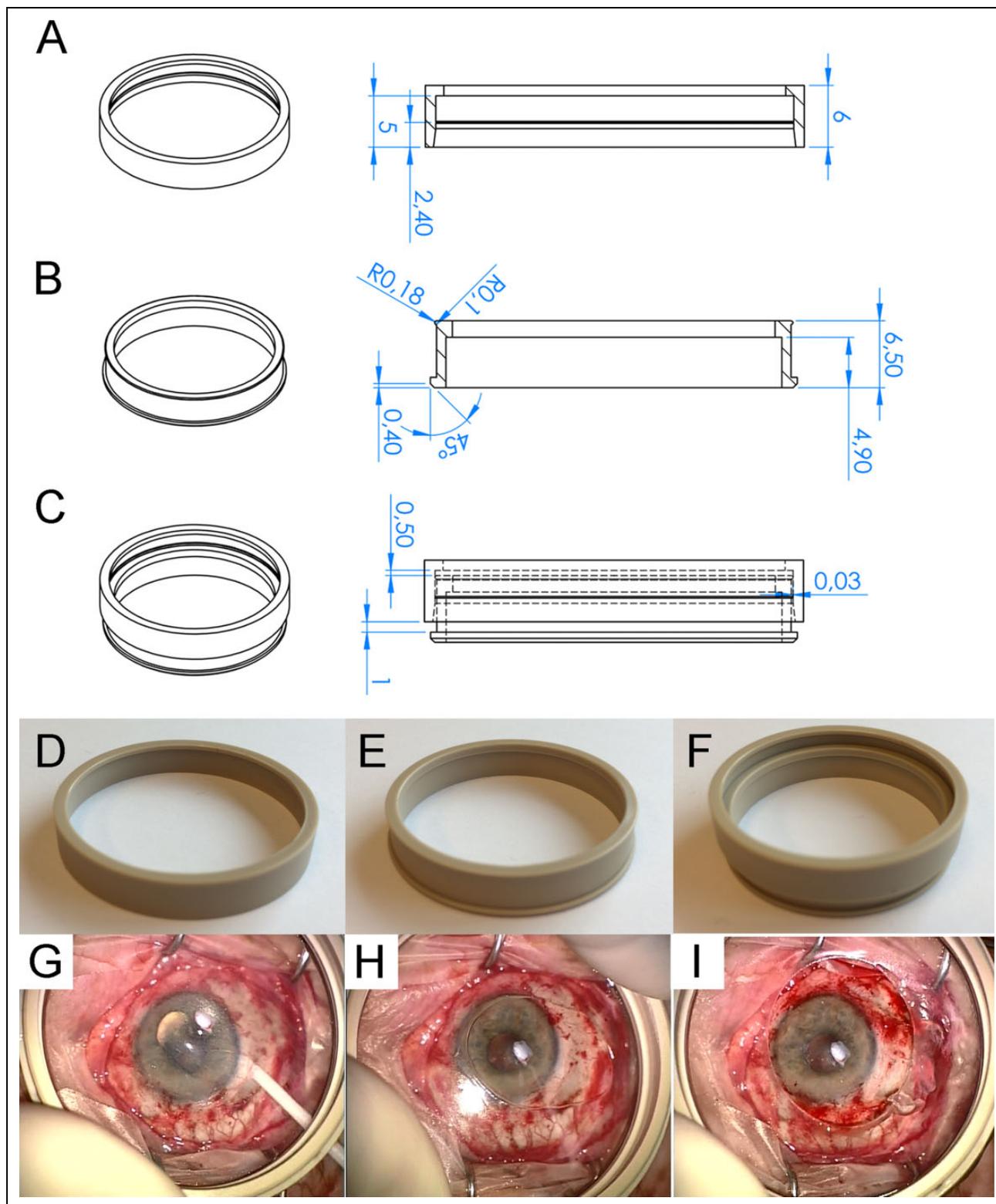


Figure 4. The Zakaria chamber (amion ring). (A to C) Schematic displaying the upper and lower ring components when separate and combined. Distance measurements are displayed in millimeters. (D to F) Photographs of ring components when displayed separately and combined. Prior to assembly, a sheet of amniotic membrane (not shown) is draped across the lower ring component. (G to I) Photographs illustrating the “no-touch” transplantation technique for limbal epithelium cultured on human amniotic membrane (HAM). (G) A drop of fibrin glue is placed onto the corneal surface and the HAM positioned over it. (H) Gentle downward pressure is applied sealing the surfaces together. (I) A trephine is used to free the graft from the chamber and the excess tissue is retained for quality control.

Table 2. Materials and Components Used for In-house Designs.

| Design | Manufacturer/Supplier | Component/Cat. No. | Price ^a |
|-------------------------------|--|--|----------------------|
| Modified micro-Boyden chamber | CNC Components Pty. Ltd. (Geebung, Queensland, Australia) | Upper ring: QUT-0002-0006 Base ring: QUT-0002-0007 Both made from PTFE | A\$11.53 A\$11.37 |
| | Ludowici Sealing Solutions (Brendale, Queensland, Australia) | Silicone rubber O-ring: RSB012 All supplied nonsterile | A\$0.23 |
| Ludowici chamber | Ludowici Sealing Solutions | Upper ring: L140841 Base ring: L140840 Both made from PTFE. Supplied nonsterile | A\$34.4 A\$38.52 |
| Dunphy chamber | Medical Engineering Unit (Queens Medical Centre, Nottingham, United Kingdom) | Per set (nonsterile) PTFE base Stainless steel retaining clip Both supplied nonsterile | £16.02 |
| Zakaria chamber | O&O Medical Device Consultants (Frascati, Rome, Italy) | Per pair/sterile and packed: Lower: Ø36,8*Ø33,2*LG6 Upper: Ø35,6*Ø31,3*LG6,5 Made from medical grade PEEK | €34.40 |

Abbreviations: PTFE, polytetrafluoroethylene (Teflon); PEEK, polyether ether ketone (Ketrion).

^aAll prices are subject to change.

Table 3. Quick Reference Chamber Guide According to Required Application.

| Chamber Design/Supported Applications | Modified Micro-Boyden Chamber | Ludowici Chamber | Dunphy Chamber | Zakaria Chamber |
|--|-------------------------------|------------------------|----------------|-----------------|
| Direct imaging of cells during culture | ✓ | ✓ | ✓ | ✓ |
| Creation and maintenance of apical and basal culture compartments | ✓ | Not ideal ^a | ✓ | Not ideal |
| Measurement of polarized growth factor secretion | ✓ | Not ideal | Not tested | Not ideal |
| TER measurements | ✓ | Not ideal | Not tested | Not ideal |
| Cell types tested | | | | |
| Corneal/limbal epithelium | ✓ | ✓ | ✓ | ✓ |
| Corneal stroma | ✓ | ✓ | ✓ | |
| Corneal endothelium | ✓ | | | |
| RPE cells ^b | ✓ | | | |
| Materials mounted | | | | |
| Amniotic membrane | ✓ | ✓ | ✓ | ✓ |
| Fibroin membrane | ✓ | ✓ | ✓ | |
| Collagen membrane | ✓ | | | |
| Electrospun fibrous mats | ✓ | | | |
| Medical device for applying materials/cells to ocular surface during surgery | No | ✓ | No | ✓ |

Abbreviations: ✓, Yes; TER, transepithelial resistance; RPE, retinal pigment epithelium.

^a“Not ideal” due to the relatively shallow and larger width of chamber compartments.

^bNumerous sources of RPE cells have been successfully used including ARPE-19 cells, RPE cells derived from cadaveric tissue, and RPE cells derived from either embryonic stem (ES) cells or induced pluripotent stem (iPS) cells.

system is that once the rings have “clicked” together, the HAM is contained and cannot slip out or become detached. This ensures an anchored membrane during culture and transportation to the operating room. A 33.2-mm diameter size for the ring was chosen to not only ensure adequate surface area for outgrowth of the limbal epithelial cells but also aid in the transplantation procedure itself. Following corneal pannus dissection, the surgeon can apply fibrin glue to the surface of the cornea, position the graft over it, and trephine out the required graft size without manipulating the graft in any way. The “no-touch” surgical technique helps maintain viability of transplanted cells. The

remainder of the graft is embedded in paraffin and stored as a retained sample, a requirement for good manufacturing practice (GMP).

Conclusions

Membranes are a popular choice of scaffold for ophthalmic cell therapies and also support studies of ocular cell function in vitro. Ideally, the choice of membrane mounting technique should support a flat surface with adequate tension to facilitate optimal cell attachment and growth. In some cases (e.g., for RPE cells), it is advantageous to create

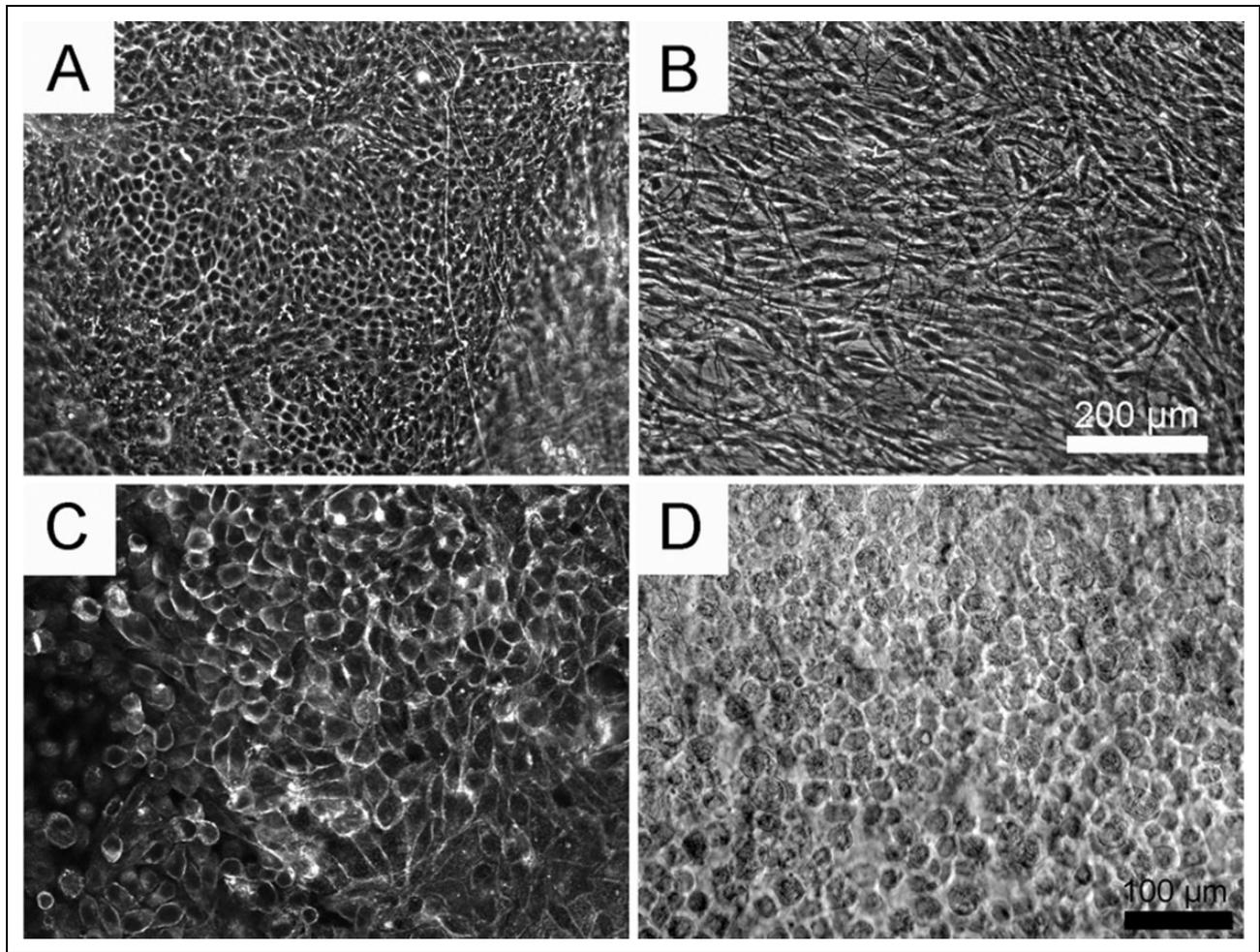


Figure 5. Examples of culture visibility and growth achieved using each chamber design. (A) The human retinal pigment epithelium (RPE) cell line ARPE-19 after 2 mo growth on a collagen-coated fibroin membrane, while mounted within the modified micro-Boyden chamber (phase-contrast optics). (B) Rabbit limbal mesenchymal stromal cells after 4 days growth on a sheet of denuded HAM, while mounted within the Ludowici chamber (phase-contrast optics). (C) SV40-immortalised human corneal epithelial cells stained with phalloidin after 2 wk growth on a nanofibrous PLGA scaffold, while mounted within the Dunphy chamber (confocal fluorescence microscopy is used owing to the poor transparency of the scaffold). (D) Human limbal epithelial cells after 14 days outgrowth from a tissue biopsy attached to denuded HAM, while mounted within the Zakaria chamber (phase-contrast optics). The approximate scale for part figures (A) and (B) is displayed in (B) (200 μm bar). The approximate scale for part figures (C) and (D) is displayed in (D) (100 μm bar).

separate upper and lower membrane compartments for studies of polarized cell function (e.g., barrier function or growth factor secretion). Although a small number of mounting devices are available commercially (e.g. Snapwell and Cell-Crown), the limitations associated with these products (including size range, sealing properties, and incompatibility with standard tissue processing techniques) have prompted the development of several in-house designs (Table 2). Table 3 demonstrates the varying suitability of our chamber designs according to required application. The ability to directly monitor cultures by phase-contrast microscopy while mounted within these chambers is highly desirable (Figure 5). The modified micro-Boyden chamber and Dunphy chamber are best suited to *in vitro* studies of cell function on bespoke membranes of choice. In contrast, the

Ludowici chamber and Zakaria chamber (amnon ring) support handling of larger membranes during cell culture and subsequent application to the ocular surface. As an added advantage, all of these designs are made from materials (e.g., PTFE or PEEK) that are readily amenable to sterilization (a regulatory requirement for clinical manufacturing) and are compatible with standard tissue processing methods into paraffin blocks (thus facilitating quality assurance tests on final product). In achieving these requirements, we recommend our chamber designs to others working within the field of ophthalmology as well as those working with other epithelial cells of clinical significance. For example, we consider that our chamber designs may well assist the development of tissue for repairing portions of the respiratory tract (e.g., trachea), the gastrointestinal tract (e.g.,

esophagus), the genitourinary tract (e.g., bladder epithelium), and tympanic membranes.

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