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TITLE:

Growth of Human and Sheep Corneal Endothelial Cell Layers on Biomaterial Membranes

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KEYWORDS:

corneal endothelium, cornea, Descemet's membrane, explant, biomaterial membrane, culture chamber

SUMMARY:

This protocol describes the critical steps required to establish and grow corneal endothelial cell cultures from explants of human or sheep tissue. A method for subculturing corneal endothelial cells on membranous biomaterials is also presented.

ABSTRACT:

Corneal endothelial cell cultures have a tendency to undergo epithelial-to-mesenchymal transition (EMT) after loss of cell-to-cell contact. EMT is deleterious for the cells as it reduces their ability to form a mature and functional layer. Here, we present a method for establishing and subculturing human and sheep corneal endothelial cell cultures that minimizes the loss of cell-to-cell contact. Explants of corneal endothelium/Descemet's membrane are taken from donor corneas and placed into tissue culture under conditions that allow the cells to collectively

migrate onto the culture surface. Once a culture has been established, the explants are transferred to fresh plates to initiate new cultures. Dispase II is used to gently lift clumps of cells off tissue culture plates for subculturing. Corneal endothelial cell cultures that have been established using this protocol are suitable for transferring to biomaterial membranes to produce tissue-engineered cell layers for transplantation in animal trials. A custom-made device for supporting biomaterial membranes during tissue culture is described and an example of a tissue-engineered graft composed of a layer of corneal endothelial cells and a layer of corneal stromal cells on either side of a collagen type I membrane is presented.

INTRODUCTION:

The cornea is a transparent tissue that is situated at the front of the eye. It is composed of three major layers: an epithelial layer on the outer surface, a middle stroma layer, and an inner layer called the corneal endothelium. The corneal endothelium is a monolayer of cells that sits on a basement membrane called Descemet's membrane and it maintains the transparency of the cornea by regulating the amount of fluid that enters the stroma from the underlying aqueous humor. Too much fluid within the stroma causes corneal swelling, opacity and vision loss. The endothelium is therefore vital for maintaining vision.

The corneal endothelium can become dysfunctional for a number of reasons including aging, disease and injury, and the only current treatment is transplant surgery. During this surgery, the endothelium and Descemet's membrane is removed from the patient's cornea and replaced with a graft of endothelium and Descemet's membrane obtained from a donor cornea. Many endothelium grafts also contain a thin layer of stromal tissue to aid handling and attachment to the host cornea[1].

Worldwide, the demand for corneal donor tissue for transplant surgeries is greater than the amount that can be supplied by eye banks[2]. There has therefore been a drive to develop tissue-engineered corneal endothelium transplants that could be used to alleviate this shortfall[3]. The rationale for this is based on the fact that currently, endothelium from an individual cornea can only be transferred to a single patient, however, if the corneal endothelial cells were first expanded and grown on biomaterial scaffolds in tissue culture, they could be used to treat multiple patients.

Major challenges that need to be addressed before tissue-engineered corneal endothelium transplants become a feasible option for surgeons include: (1) establishing techniques for expanding corneal endothelial cells of high quality and for producing mature and functional corneal endothelial cell layers in vitro, and (2) establishing techniques for growing the cells on biomaterial scaffolds to produce tissue-engineered grafts that are equal to, or better than, the donor cornea-derived grafts that are currently used.

Corneal endothelial cells have a very low proliferative potential in vivo but can be stimulated to divide in vitro[4]. Nevertheless, they have a strong tendency to undergo in vitro epithelial-to-mesenchymal transition (EMT), which reduces their capacity to form a mature, functional endothelial layer. Known triggers for EMT in corneal endothelial cells include exposure to certain

growth factors and loss of cell-to-cell contact[5]. It is thus almost inevitable that corneal endothelial cell cultures that are enzymatically dissociated during subculture will undergo changes associated with EMT. Here, we present a cell culture method for human or sheep corneal endothelial cells that is designed to minimize disruption of cell-to-cell contacts during isolation, expansion and subculture stages, to reduce the potential for EMT. Furthermore, we demonstrate how tissue-engineered grafts that resemble donor cornea-derived endothelium/Descemet's membrane/stromal tissue grafts can be produced by growing cultured cell layers on both sides of a biomaterial membrane in a custom-made mounting device.

PROTOCOL:

Human corneas with donor consent for research were obtained from the Queensland Eye Bank and used with ethics approval from the Metro South Hospital and Health Service's Human Research Ethics Committee (HREC/07/QPAH/048). Sheep corneas were obtained from euthanized animals at the Herston Medical Research Facility of the University of Queensland under a tissue sharing agreement.

1. Preparation of dissection tools

1.1. Sterilize two pairs of number 4 watchmaker forceps by either soaking them in a solution of 70% ethanol for 5 min or by autoclaving them.

2. Preparation of culture medium and tissue culture plates

2.1. Prepare 100 mL of culture medium containing Opti-MEM 1 (1x) + GlutaMAX-1, 5% fetal bovine serum and 100 U/mL Pen/Strep. This culture medium is adequate for sheep corneal endothelial cell cultures, however, for human corneal endothelial cell cultures the medium should be supplemented with 50 µg/mL bovine pituitary extract, 0.08% chondroitin sulphate, 200 µg/mL calcium chloride and 0.3 mM L-ascorbic acid 2-phosphate. Culture medium can be stored in the dark at 4 °C for two weeks.

2.2. Coat the wells of a 6-well tissue culture plate with Attachment Factor using the manufacturer's instructions and then add 1 mL of culture medium to each coated well. Prepare one well for each cornea.

3. Explant dissection and cell culture procedure

3.1. Place the cornea, endothelium side up, into a sterile Petri dish on the stage of a dissecting microscope. Adjust the illumination so that the corneal surface is well-lit with sufficient contrast to highlight the endothelial layer. Adjust the zoom so that the edge and some central corneal endothelium is in view.

3.2. Use sterilized watchmaker forceps to gently lift and tear Descemet's membrane away from the underlying stroma (**Figure 1**). The membrane will detach from the stroma as a strip that immediately curls up. Place the strip into one well of a 6-well tissue culture plate that has been

coated with Attachment Factor and contains 1 mL of culture medium. The lid of the tissue culture plate should be kept on at all times, except when explants are being added to it, to reduce the risk of contamination.

3.2. Remove strips of Descemet's membrane from the extreme periphery of the endothelium first and then from central regions later. Place all strips from one cornea into a single well within the 6-well plate.

3.3. Incubate the explants in a humidified cell culture incubator set at 5% CO_2 and 37 °C. Leave the culture undisturbed for 2 days to allow the explants to settle and attach to the plate surface. Typically, up to one third of explants fail to attach to the plate.

3.4. Remove the medium and any unattached explants from the culture after 4 days and gently add 2 mL of fresh culture medium taking care not to disturb the attached explants. Culture medium should be changed twice per week.

4. Continuous production of corneal endothelial cells by serial explant culture

NOTE: Explants can be transferred to fresh tissue culture plates after 10 days to establish additional corneal endothelial cell cultures.

4.1. Place the explant culture onto the stage of a dissecting microscope and adjust the illumination and zoom so that the explants are visible.

4.2. Using sterilized watchmaker forceps, gently pluck each explant from its culture plate and transfer it to a fresh well of a 6-well tissue culture plate that has been coated with Attachment Factor and contains 1 mL of culture medium.

4.3. Allow the explants to settle onto the surface of their new plate for 4 days before replacing the culture medium with 2 mL of fresh culture medium. Change culture medium changed twice per week.

5. Growing corneal endothelial cells on glass coverslips for immunofluorescence analyses

NOTE: Cell cultures that are destined to be analyzed using immunofluorescence should be established on glass coverslips that can be mounted onto glass microscope slides following the staining procedure.

5.1. Sterilize a pack of round glass coverslips of 13 mm diameter in an autoclave or by soaking in 70% ethanol for 15 min followed by three rinses in phosphate-buffered saline (PBS).

5.2. Place individual coverslips into the wells of a 24-well tissue culture plate, coat them with Attachment Factor, and then add 0.5 mL of culture medium to each well.

5.3. Using sterilized watchmaker forceps remove explants from their tissue culture plates and transfer them to wells containing coverslips. Allow the explants to settle onto the coverslips for 4 days before changing the medium.

5.4. After the required incubation period, fix and stain the coverslip cultures within their culture wells. Mount the stained coverslips onto glass microscope slides for analysis under a fluorescence microscope.

6. Subculture of corneal endothelial cells using Dispase II

NOTE: Large fibroblastic cells can be selectively removed from explant cultures in 6-well plates before subculturing using this procedure. If all cells are to be subcultured, do not perform steps 6.2 to 6.4. The aim of this procedure is to transfer the cells to fresh plates while maintaining their cell-to-cell contacts as much as possible. The cells should be handled gently. Completely confluent wells should be passaged at a ratio of 1:2, while subconfluent wells should be passaged at a ratio of 1:1 or less.

6.1. Remove the medium from the culture and briefly rinse with DPBS.

6.2. Add 1 mL of Versene. Incubate at room temperature for 30 s.

6.3. Remove the Versene and add 1 mL of TrypLE. Incubate at 37 °C in the tissue culture incubator for 3 min.

6.4. Observe the culture using a phase contrast microscope. Gently tap the culture to dislodge cells from the plate. As soon as the large fibroblastic cells have detached from the plate remove them and the TrypLE using a 1 mL pipette. Wash residual TrypLE off the remaining cells by rinsing twice with 2 mL of DPBS.

6.5. Add 1 mL of 1 mg/mL Dispase II to the culture and incubate in the tissue culture incubator for 1 to 2 h or until all cells have detached from the plate. The cells should gradually float away from the plate in clumps.

6.6. Collect the cells using a 1 mL pipette and transfer to 20 mL of DPBS in a 50 mL centrifuge tube. Centrifuge for 5 min at 300 x *g* at room temperature.

6.7. Remove the supernatant and gently resuspend the cell pellet by trituration with a 1 mL pipette in 1 mL of culture medium. Transfer the cell suspension to either one or two wells of a 6-well plate, to passage at a ratio of either 1:1 or 1:2 respectively.

6.8. Top up the medium in each well to make 2 mL and place the cultures into the tissue culture incubator. Replace the medium with 2 mL of fresh medium twice per week.

7. Growth of corneal endothelial cell layers on biomaterial membranes

NOTE: The following procedure describes the steps involved in mounting a membranous biomaterial in a custom-made mounting device—called a micro-Boyden chamber—for cell culture. Please refer to our recent publication[6] for further information about the device and for purchasing details.

7.1. Assemble the upper chamber of the micro-Boyden chamber by placing a red O-ring into its center.

7.2. Use a trephine of 18 mm diameter to punch out a disc from a biomaterial sheet on a polytetrafluoroethylene (PTFE) cutting board. Place this disc over the red O-ring in the micro-Boyden chamber's upper chamber.

7.3. Screw the lower chamber onto the upper chamber, securing the biomaterial disc in between.

7.4. Soak the assembled micro-Boyden chamber in 70% ethanol for 1 h to sterilize it.

7.5. Completely immerse the assembled micro-Boyden chamber in sterile HBSS for 10 min to remove the ethanol. Repeat this wash step twice. Perform a final wash step for 10 min in unsupplemented culture medium.

7.6. Transfer the sterilized micro-Boyden chamber to culture medium in the well of a 6-well plate ensuring that the upper chamber is uppermost. Adjust the level of culture medium so that it contacts the lower surface of the biomaterial membrane but does not flow into the upper chamber.

7.7. Prepare a suspension of corneal endothelial cells using the procedure outlined in section 6. Sufficient cell density in the suspension should be achieved to allow a seeding density of at least 100,000 cells per cm^2 on the membrane in the micro-Boyden chamber. The culture surface area within the micro-Boyden chamber is 0.5 cm^2 and it can hold a volume of 100 µL. Therefore, a suspension containing 500,000 cells/mL should be prepared.

7.8. Pipette 100 µL of cell suspension (50,000 cells) onto the membrane in the micro-Boyden chamber. Incubate in the tissue culture incubator for 4 h before topping up the medium to completely submerge the chamber. Incubate the culture for the required period of time, replacing the medium twice per week.

NOTE: Once the corneal endothelial cells have attached to the upper surface of the biomaterial membrane the micro-Boyden chamber can be flipped over within the culture well so that the lower chamber is uppermost. More cells can then be added to the chamber to initiate cell cultures on the other surface of the membrane. For example, corneal stromal cells may be readily applied to the alternate surface thus mimicking the relative location of corneal cell types as seen within the posterior cornea.

REPRESENTATIVE RESULTS:

The method for isolating and expanding corneal endothelial cells from human or sheep corneas is summarized in **Figure 1** and **Figure 2**. Most explants that are derived from the corneas of 1 to 2-year-old sheep or human donors of less than 30 years of age will attach to Attachment Factor-coated tissue culture plates within a week, however, it is not unusual to find that up to one third of explants fail to attach within this time. These 'floating' explants can be removed from the cultures. Explants from human donors older than 30 years are less likely to attach to the plate and also less likely to produce cell cultures. Representative images of corneal endothelial cell cultures generated from sheep and human explants are shown in **Figure 3** and **Figure 4**. The cells that emerge from the explants generally remain in contact with each other as they migrate out onto the plate. This kind of migration is known as collective cell migration, and it is a feature of epithelial cells[7]. By 2 weeks of culture, patches of small, tightly-packed cells will have formed immediately next to many of the explants from both sheep and human corneas[8]. These patches of cells do not exhibit morphological characteristics of EMT and expand slowly over time. Larger cells with more irregular, fibroblastic shapes can be found outside of these patches. Once the cultures have been established, the explants can be removed using forceps and placed into fresh plates to establish new cultures.

Small, tightly packed cells within the corneal endothelial cell cultures are very resistant to digestion with TrypLE, while the larger fibroblastic cells are more sensitive to it. This difference in TrypLE resistance can be exploited to selectively remove large cells from the cultures before transferring the smaller cells to new plates. Representative images of human corneal endothelial cell cultures throughout the subculturing process using TrypLE and Dispase II are shown in **Figure 4**.

Immunofluorescence analyses can be conducted on corneal endothelial cell cultures to locate specific proteins in the cells. An example of this is presented in **Figure 5**. Explants from sheep and human corneas were placed onto Attachment Factor-coated glass coverslips in 24-well plates and cultured for 4 weeks. The explants were removed and then the cultures were analyzed using immunofluorescence for the presence of ZO-1, a tight junction protein, and N-cadherin, an adherents junction protein, according to our published protocol[9]. The same anti-ZO-1 and anti-N-cadherin antibodies were used for both sheep and human cells, and the results showed that both proteins were detected in the plasma membranes of cells from both species. ZO-1 is normally present as a distinct band at the cell border but becomes weak or absent in cells undergoing EMT. Therefore, the robust ZO-1 expression in these cultures indicated that the cells had not undergone EMT.

Our custom-made micro-Boyden chambers are designed to suspend a biomaterial membrane within the well of a 6-well tissue culture plate (**Figure 6**). The procedure for mounting a biomaterial membrane into a micro-Boyden chamber is shown in **Figure 7**. The design of the micro-Boyden chamber allows both sides of the membrane suspended within it to be used as cell culture surfaces simultaneously. To demonstrate this, sheep stromal cells derived from corneal stromal tissue were seeded at a density of 100,000 cells/cm^2 onto one side of a collagen type I

membrane and then 24 h later the chamber was flipped over and sheep corneal endothelial cells were seeded onto the other side of the membrane at a density of 400,000 cells/cm^2. The tissue-engineered cell layers were cultured for 4 weeks, then fixed with 10% neutral buffered formalin and stained using rhodamine phalloidin and Hoechst nuclear dye 33342. They were then examined and photographed using a confocal microscope (**Figure 8**). A cross-section view of the tissue-engineered cell construct revealed a single layer of corneal endothelial cells on one surface of the collagen membrane and a multi-layered culture of corneal stromal cells on the other surface.

FIGURE AND TABLE LEGENDS:

Figure 1. Technique for obtaining explants of endothelium/Descemet's membrane from fresh corneas. (**A**) The cornea is placed endothelium-side up in a Petri dish under a dissecting microscope. (**B**) Close up view of the area indicated by a red rectangle in (**A**). Watchmaker forceps are used to gently peel away Descemet's membrane from the underlying stroma.

Figure 2. Procedure for establishing and expanding cultures of corneal endothelial cells from endothelium/Descemet's membrane explants.

Figure 3. Representative phase contrast images of endothelial cell cultures during initial establishment from explants of sheep corneal endothelium/Descemet's membrane. (**A**) A sheep corneal endothelium/Descemet's membrane explant after 3 days in culture. Corneal endothelial cells have begun to migrate onto the plate. (**B**) A sheep explant culture after 1 week. The explant is surrounded by a confluent sheet of cells. (**C**) A sheep explant culture after 2 weeks. Small cells surround the explant while larger cells are located further away. (**D**) A sheep explant culture after 6 weeks. A region of small, tightly packed cells is seen next to a region of larger cells.

Figure 4. Isolation of small, tightly packed human corneal endothelial cells for subcultures. (**A**) A human corneal endothelium/Descemet's membrane explant culture after 7 weeks. Many small, tightly packed cells are present next to the explant. (**B**) Regions of small, tightly packed cells develop in human explant cultures in a similar manner to that observed in sheep explant cultures. (**C**) A human explant culture after removal of the explant and after 20 min exposure to TrypLE. Small, tightly packed cells have retained their attachment to the plate while the larger cells have floated away. (**D**) A human corneal endothelial cell culture after 1 h in Dispase II. Most cells have detached from the plate as free-floating clumps. (**E**) A human corneal endothelial cell subculture after 1 day. Cells have migrated outwards from cell clumps that were isolated from the original explant culture. (**F**) A human corneal endothelial cell subculture after 12 days. The cells have formed a confluent monolayer.

Figure 5. Localization of ZO-1 and N-cadherin proteins in the membranes of sheep and human corneal endothelial cells by dual-labelling immunofluorescence. Sheep and human corneal endothelium/Descemet's membrane explant cultures were established on Attachment Factor-coated glass coverslips and analyzed after 4 weeks. Both ZO-1 (green stain) and N-cadherin (red stain) were detected in the membranes of sheep (**A** and **B**) and human (**D** and **E**) corneal endothelial cells. Analysis of the merged images revealed that the two proteins were highly co-

localized within the cultures (**C** and **F**).

Figure 6. Diagram of a micro-Boyden chamber shown in cross section. Our custom-made micro-Boyden chamber consists of an upper chamber, a lower chamber and an O-ring. It can be used to suspend any type of membranous material within a tissue culture well.

Figure 7. The procedure for mounting a biomaterial membrane in a micro-Boyden chamber for tissue culture. (**A**) The equipment required for this procedure includes a polytetrafluoroethylene cutting board, a pair of forceps, a trephine of 18 mm in diameter, a custom-made micro-Boyden chamber and a biomaterial membrane. (**B**) Use the trephine to punch out a disc from the biomaterial membrane. (**C**) Place the O-ring into the upper chamber of the mounting device and then lay the biomaterial disc over it. (**D**) Screw the lower chamber onto the upper chamber of the mounting device. (**E**) The assembled micro-Boyden chamber is ready to be sterilized with 70% ethanol. (F) Immerse the sterilized micro-Boyden chamber in tissue culture medium in the well of a 6-well tissue culture plate.

Figure 8. Sheep corneal endothelial and stromal cells on opposing sides of a collagen type I membrane. The cells were stained with phalloidin rhodamine to visualize actin (red) and Hoechst to visualize nuclei (blue). The collagen type I membrane was not stained and is therefore not visible in these images that were collected using confocal microscopy. (**A**) A low magnification, cross section view of the tissue-engineered construct. A thin layer of actin representing a corneal endothelial cell culture is visible on the upper surface of the membrane, and a thicker layer of actin representing a stromal cell culture is present on the lower surface of the membrane. Blue nuclei are not shown in this image. (**B**) *En face* view of the corneal endothelial cell layer showing both actin and nuclei staining. (**C**) *En face* view of the corneal stromal cell layer showing both actin and nuclei staining.

DISCUSSION:

A significant technical challenge associated with establishing and expanding human corneal endothelial cells is preventing EMT from occurring in the cultures. EMT can be triggered in corneal endothelial cells by loss of cell-to-cell contact, yet most cell culture protocols for these cells involve enzymatic dissociation to single cells during isolation and subculture[10]. Here we present an alternative cell culture protocol for corneal endothelial cells that minimizes the risk of cells losing contact with each other during the isolation and subculture stages.

Our method for establishing corneal endothelial cell cultures involves placing explants of endothelium/Descemet's membrane from donor corneas into tissue culture plates under conditions that allow the cells to collectively migrate out from the membranes and onto the plates. For this to be successful, the explant must form a tight attachment to the tissue culture plate, and this is best achieved by not disturbing the plate for several days after the cultures have been set up. Another critical factor in the successful establishment of corneal endothelial cell cultures from humans is the age of the donor. Higher success rates tend to be achieved from donors younger than 30 years of age.

A disadvantage of using the explant culture method for establishing corneal endothelial cell cultures is the relatively long period that exists between setting up the cultures and obtaining large numbers of cells. So called 'peel-and-digest' methods involve stripping the endothelium from donor corneas and digesting it with enzymes to release the cells for culture[11]. These types of methods would produce cultures containing more cells initially than those established from explants.

Our explant culture method for corneal endothelial cells produces cultures containing very small, compact, mature cells of high quality. However, the cultures also contain larger, less ideal cells towards the periphery of the plate. The larger cells can be removed by digestion with TrypLE and discarded if desired, but this reduces the number of cells available for subculture. However, explants that have successfully initiated primary cell cultures are almost always able to initiate further cell cultures, and this ability can be exploited to obtain large numbers of high quality cells.

Our subculture method for corneal endothelial cells involves using Dispase II to gently lift cell clumps away from the tissue culture plate for transfer to fresh plates, and although this method is designed to minimize the possibility of EMT occurring in passaged cells, it should be noted that it does not reduce the risk to zero.

It has been the goal of many groups to develop tissue-engineered corneal endothelial cell layers for transplantation purposes. Many different materials have been trialed as carriers for the cells and a variety of different methods have been used to restrain the material from moving around in the culture plate during cell culture. Most methods involve anchoring the material to the surface of the tissue culture plate somehow, restricting cell growth to the upper surface of the membrane only. While these methods could be used to produce single layers of tissue-engineered corneal endothelium that would be equivalent to endothelium/Descemet's membrane grafts (DMEK grafts), they could not be used to produce tissue-engineered equivalents of the endothelium/Descemet's membrane/stroma grafts (DSEK or DSAEK grafts) that are most commonly used by surgeons currently. We have therefore developed a membrane mounting device called a micro-Boyden chamber that allows cells to be simultaneously grown on both surfaces of a suspended biomaterial membrane, and have used it to produce tissue-engineered grafts consisting of corneal endothelial cells and corneal stromal cells on opposite surfaces of collagen type I membranes. These dual-layered tissue-engineered grafts could potentially be used to replace donor cornea-derived grafts of endothelium and stromal tissue on either side of Descemet's membrane (DSEK or DSAEK grafts).

In summary, the methods presented in this article are designed for those who wish to obtain primary corneal endothelial cells of high quality for use in tissue engineering studies. Gentle culture methods are described that are designed to reduce the risk of the cells undergoing EMT and a method for growing the cells on suspended biomaterial membranes is presented. We hope that these methods may assist others towards their goals of producing tissue-engineered corneal endothelium transplants.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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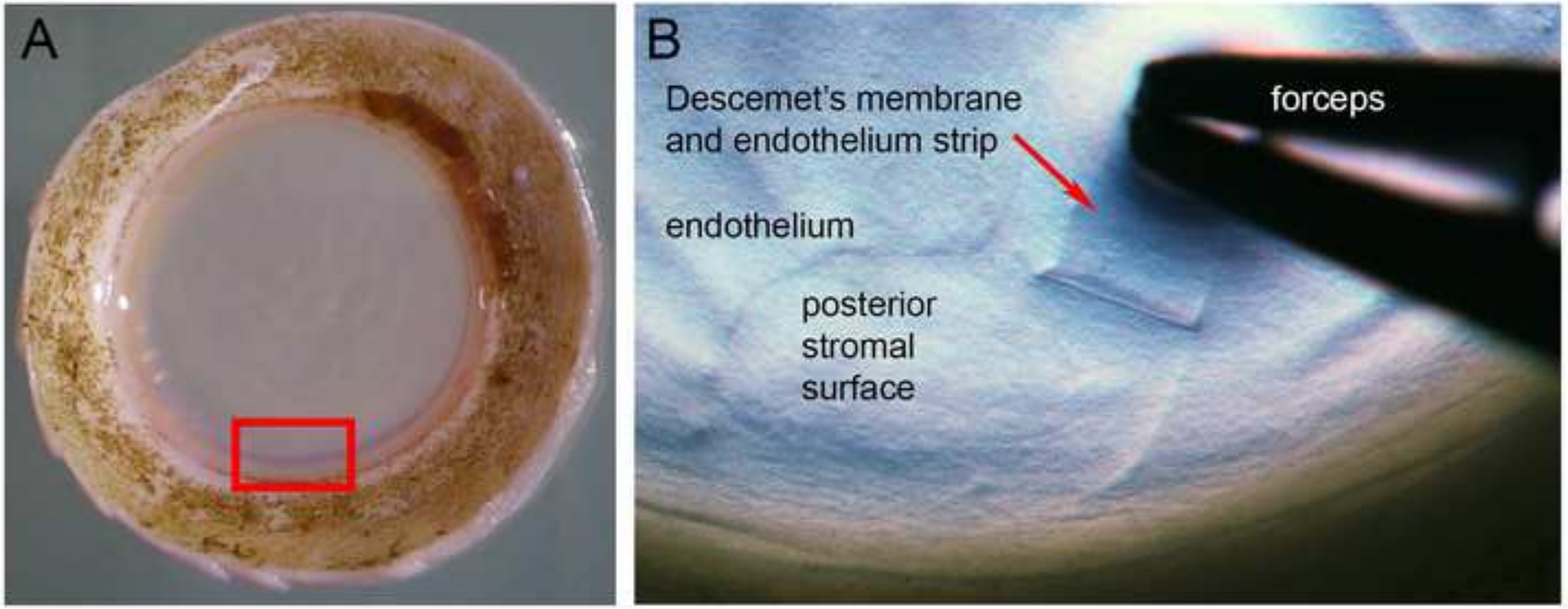
Figure 1



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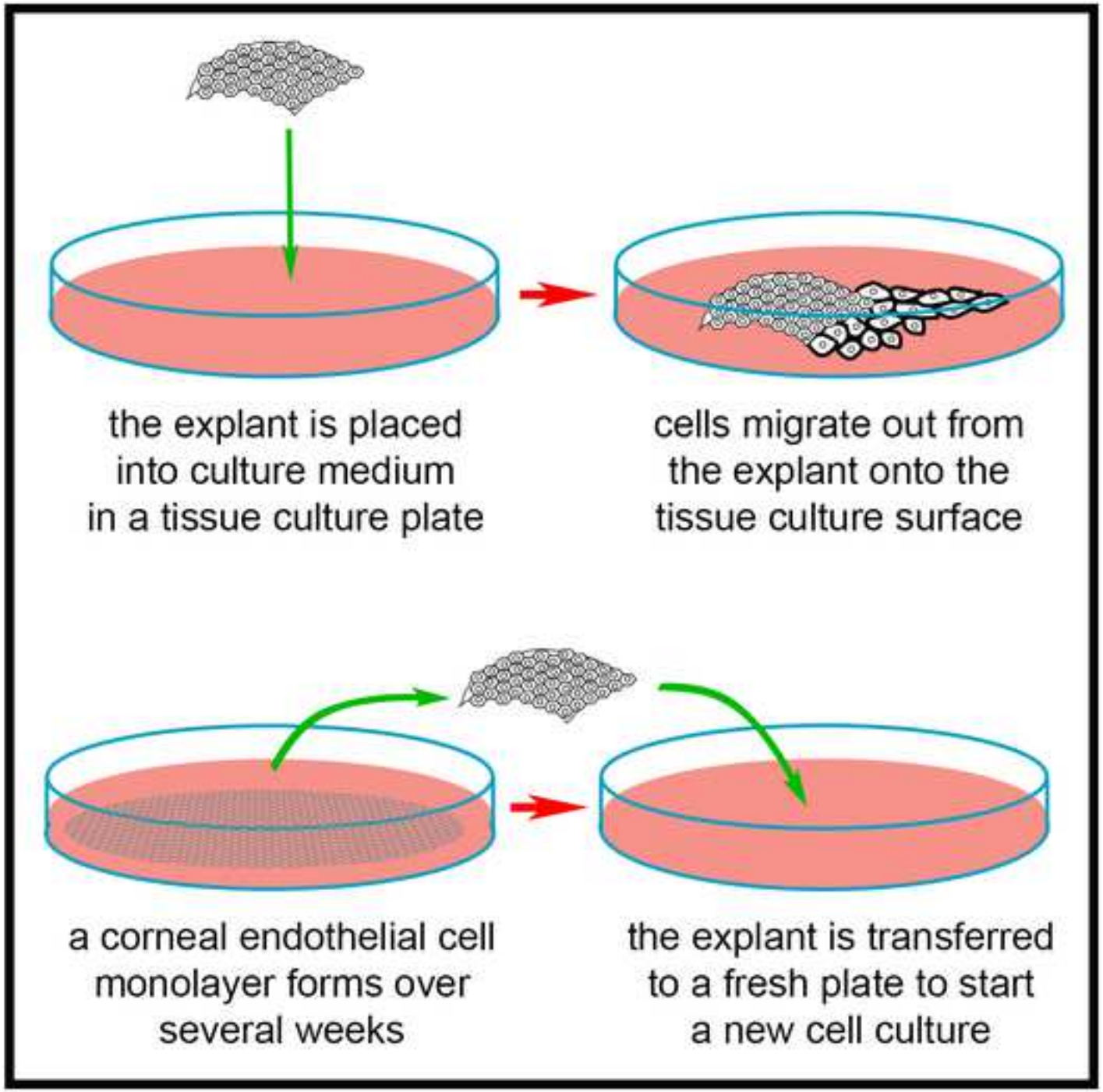

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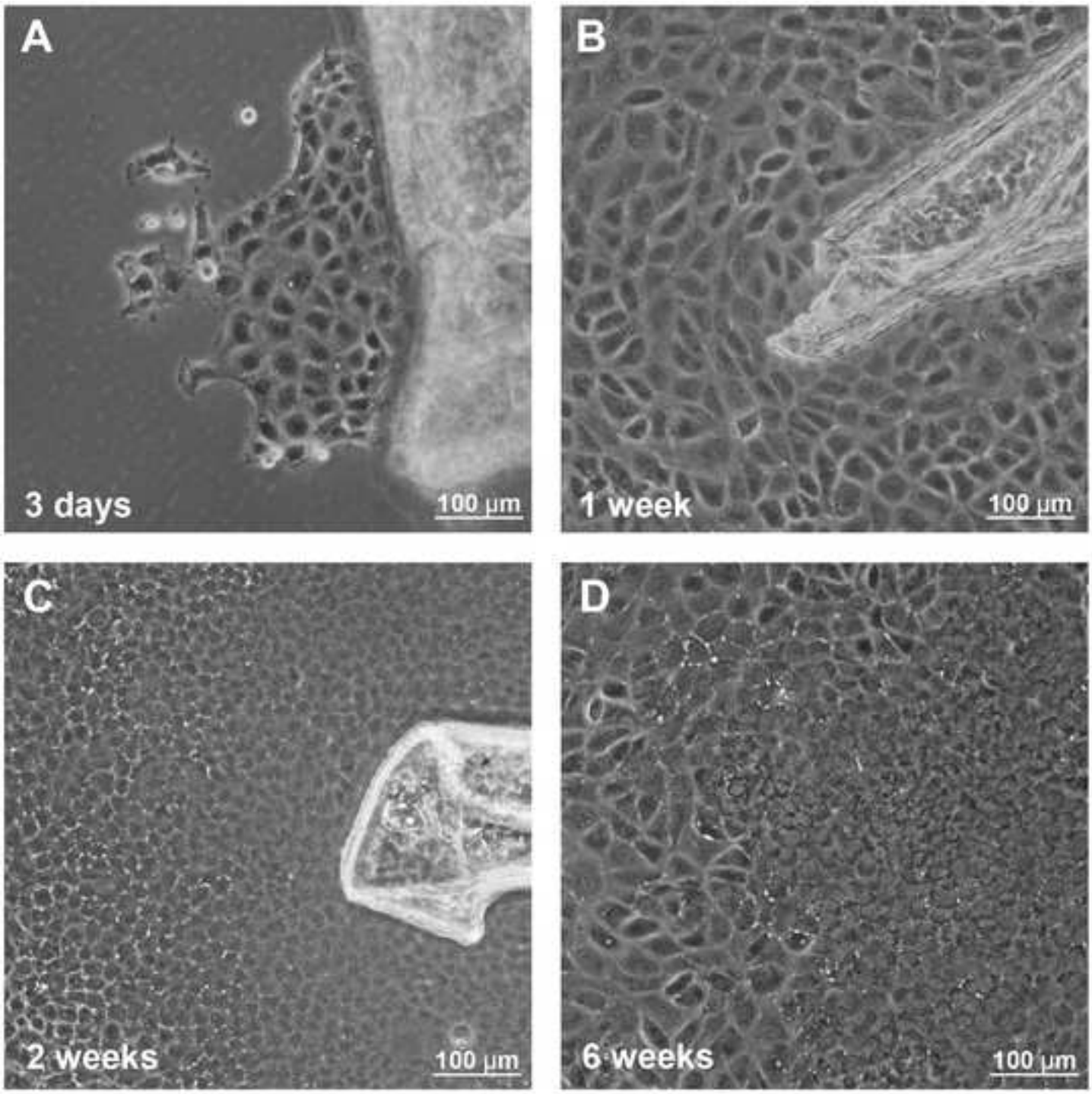

Figure 4

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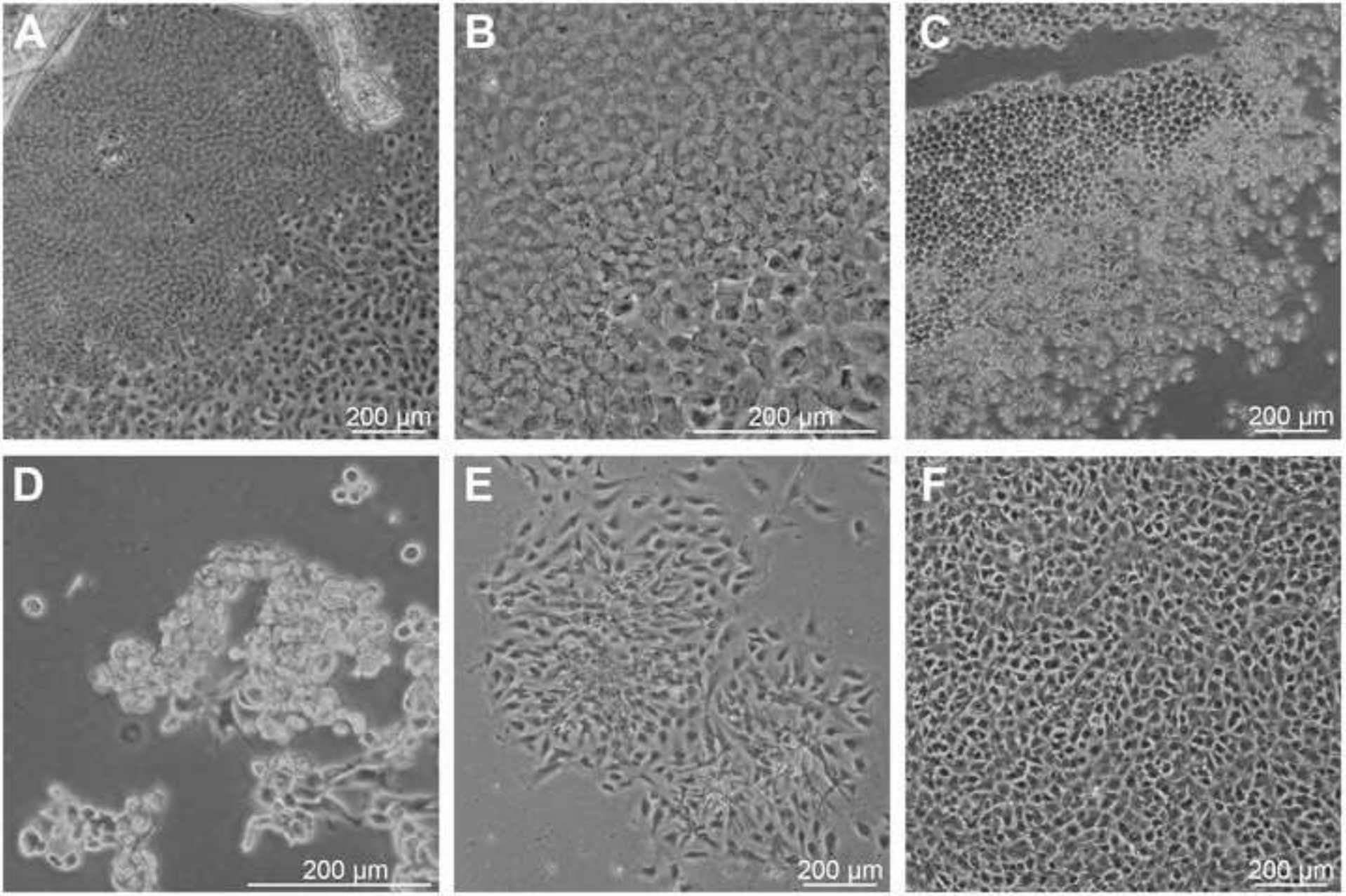

Figure 5

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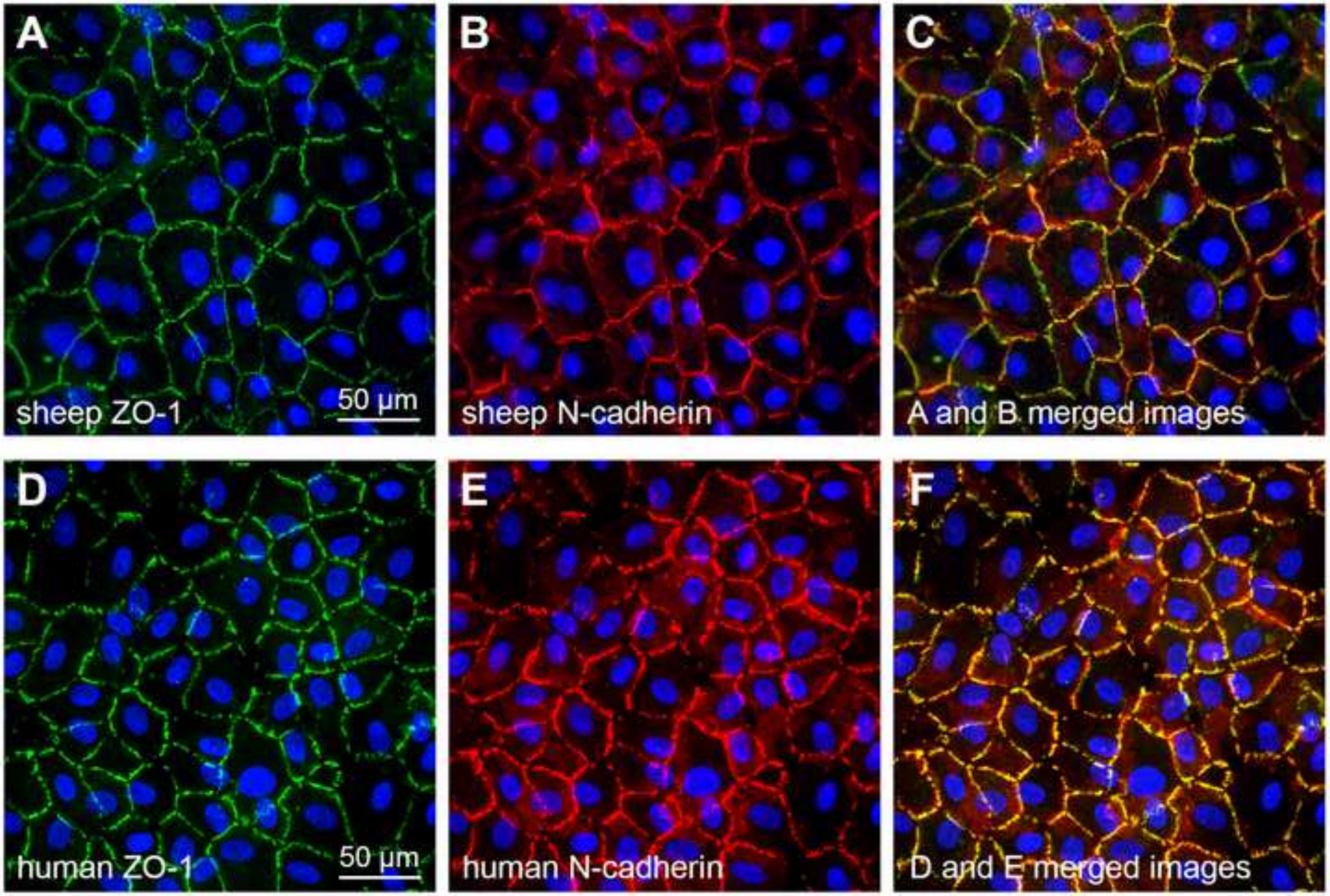


Figure 6

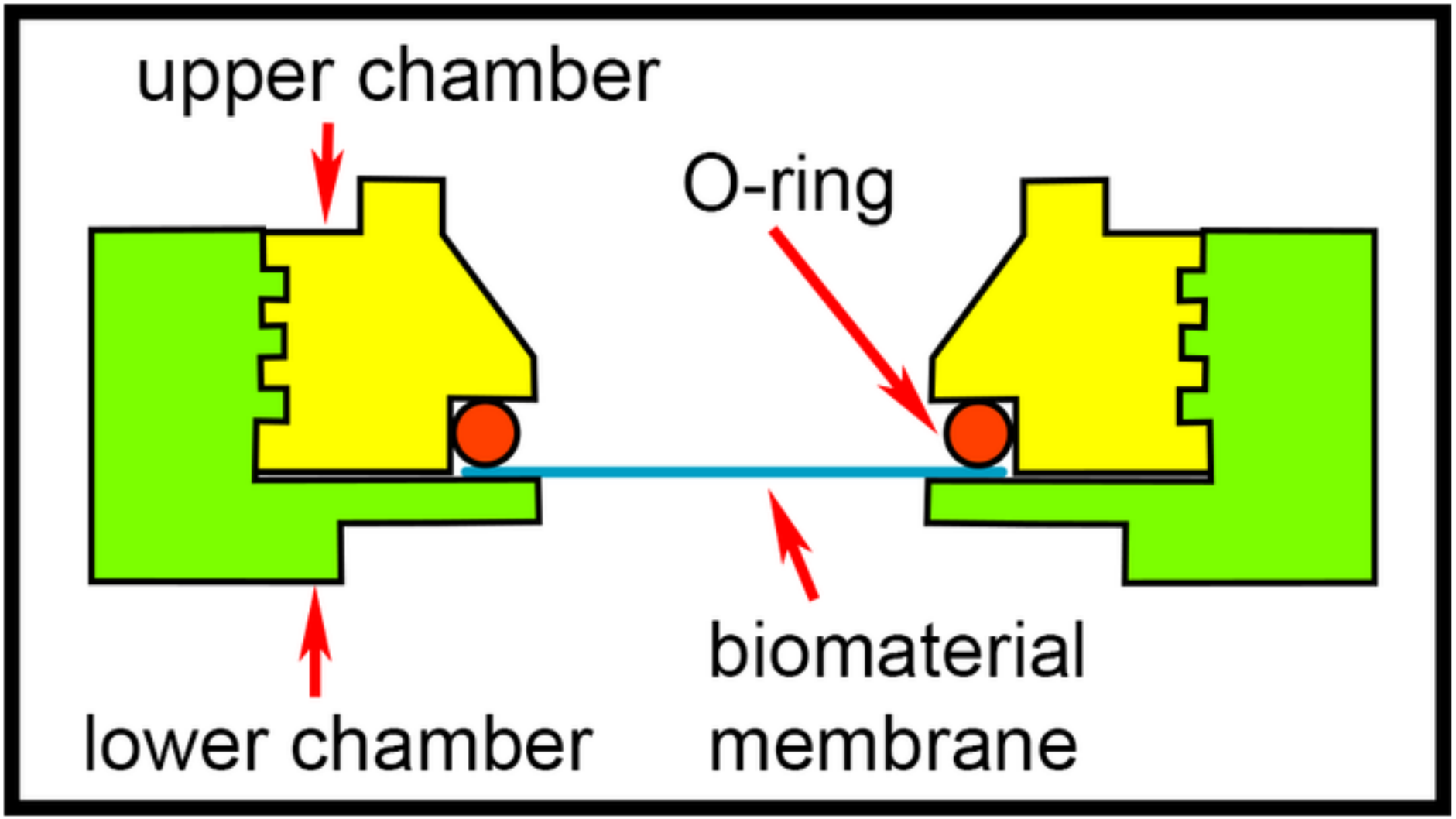


Figure 7

Click here to access/download;Figure;Fig 7.psd

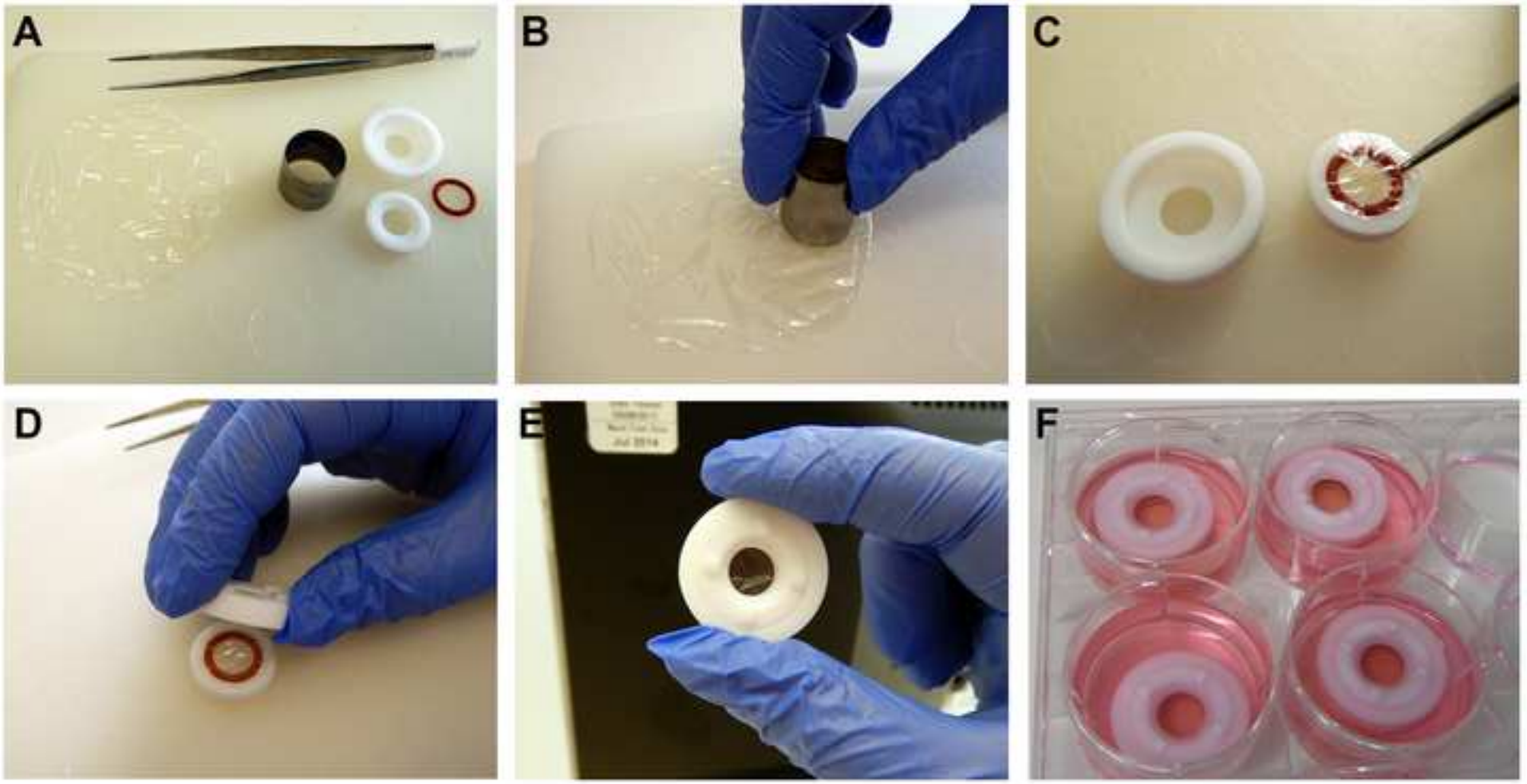
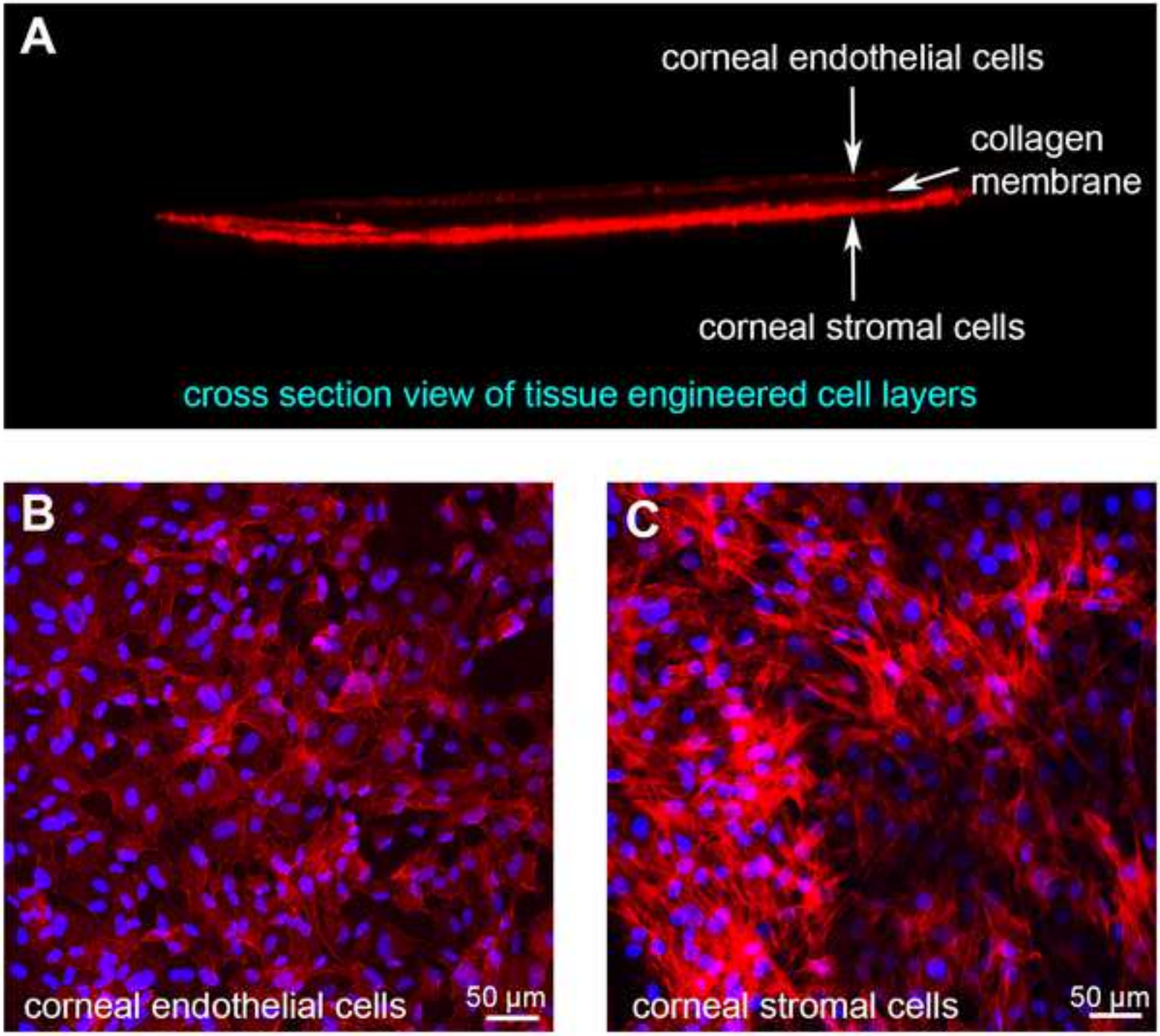


Figure 8



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Attachment factor	Gibco	S006100	A 1X sterile solution containing gelatin that is used to coat
Bovine pituitary extract	Gibco	13028014	A single vial contains 25 mg. Freeze in aliquots.
Calcium chloride	Merck	C5670	Dissolve in HBSS to make a 1 mM stock solution. Filter steri
Centrifuge tube, 50 ml	Labtek	650.550.050	
Chondroitin sulphate	LKT Laboratories	C2960	This is bovine chondroitin sulphate. Dissolve in HBSS to ma
Dispase II	Gibco	17105-041	Dissolve in DPBS to make a 2 mg/mL stock solution. Filter s
Ethanol	Labtek	EA043	100% undenatured ethanol should be diluted to 70% in dei
Foetal bovine serum	GE Healthcare Australia Pty Ltd	SH30084.03	This is a HyClone brand of foetal bovine serum.
Coverglass No. 1, Ø 13 mm	Proscitech	G401-13	Place sterilised cover slips into 24-well plates for tissue cult
HBSS	Gibco	14025-092	Hank's balanced salt solution, 1X, containing calcium chlori
L-ascorbic acid 2-phosphate	Merck	A8960	Dissolve in HBSS to make a 150 mM stock solution. Filter st
Micro-Boyden chamber	CNC Components Pty. Ltd.	Upper ring: QUT-0002-0006, Base ring: QUT-0002-0007	Both components are made from polytetrafluoroethelyne (
O-ring for micro-Boyden chamber	Ludowici Sealing Solutions	RSB012	Composed of silicon rubber.
Opti-MEM 1 (1X) + GlutaMAX-1	Gibco	51985-034	A reduced serum medium containing glutamine.
DPBS	Gibco	14190-144	Dulbecco's phosphate buffered saline, 1X, without calcium
Pen Strep	Gibco	15140-122	A 100X antibiotic solution containing 10,000 Units/mL peni
Petri dish	Sarstedt	82.14473.001	Sterile Petri dish, 92 X 16 mm, for tissue dissections.
Tissue culture plate, 24 well	Corning Incorporated	Costar 3524	A plate containing 24 wells, each with a surface area of 2 cr
Tissue culture plate, 6 well	Corning Incorporated	Costar 3516	A plate containing 6 wells, each with a surface area of 9 cm
TrypLE Select	Gibco	12563-011	A 1X enzyme solution for dissociating cells.
Versene	Gibco	15040-066	A 1X EDTA solution for dissociating cells.
Watchmaker forceps	Labtek	BWMF4	Number 4 watchmaker forceps work well for removing strip

tissue culture surfaces. Store at 4 °C.

ilise.

ke a 0.08 g/mL stock solution. Filter sterilise and freeze in aliquots.
terilise and freeze in aliquots.
ionised water for sterilising instruments and surfaces.

:ure.
de and magnesium chloride.
erilise.

(PTFE).

chloride and magnesium chloride.
cillin and 10,000 µg/mL streptomycin.

m^2.

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ps of endothelium/Descemet's membrane from corneas.

The Editor
JoVE

RE: JoVE60762: Growth of human and sheep corneal endothelial cell layers on biomaterial membranes.

Dear Editorial Team,

We thank the reviewers for their constructive feedback and welcome the opportunity to submit a revised version of our manuscript for further consideration by JoVE. Please find listed below our responses to the reviewers' comments and the changes that we have made to the manuscript.

Yours sincerely,

Dr Jennifer Young.

Rebuttal

Editorial comments

1. The manuscript has been proofread for spelling and grammar issues.
2. The manuscript does not contain figures that have already been used in previous publications.
3. Steps 3.2 and 6.7 have each been divided into two steps to shorten them.
4. Step 5.4 has been rewritten in the imperative tense.
5. A short description has been added to the Figure Legend for Figure 6.
6. Journal titles in the references section have been written in full.

Reviewer #1

Comment: Why do you choose collagen type I membrane？
Response: We chose to use membranes composed of collagen type I in this study because corneal endothelial cells grow well on them. Collagen type I can be used as a control substrate when testing other biomaterial substrates for their ability to support corneal endothelial cell growth.

Comment: Have you ever tried other biomaterials?
Response: Yes, we have grown corneal endothelial cells on other biomaterials. We have only shown data for one biomaterial in this study as this is sufficient for demonstration purposes.

Comment: From the experimental data, it seems no EMT phenomenon. Do you try to stain EMT markers？
Response: One of the major characteristics of EMT in corneal endothelial cells is a fibroblastic morphology, which can be can be seen using phase contrast optics. Based on our visual inspections, our explant cultures contain cells that display no morphological signs of EMT (those

surrounding the explants) and cells that have some EMT-like characteristics (those at the edges of the explant cultures). ZO-1 is detected as a distinct band at the borders of corneal endothelial cells *in vivo*, and this pattern of expression is evident in cultured cells that do not have morphological signs of EMT. However, ZO-1 expression in cells with EMT characteristics is different: it is weak or absent at borders, and can be present in the cytoplasm. The altered presence and distribution of ZO-1 expression can therefore be used as an EMT marker in corneal endothelial cells. We have provided some images of ZO-1 immunostaining in cultured corneal endothelial cells in our manuscript to demonstrate a typical result that would be obtained in cells without morphological signs of EMT. We have modified lines 278 - 279 and 298 - 300 to emphasise the fact that we found little evidence of EMT in our representative cultures.

Comment: Higher success rates tend to be achieved from donors younger than 30 years of age. Any data to provide this conclusion?
Response: No data, just years of experience of trying to grow corneal endothelial cells from corneal tissue from donors older than 30 years of age.

Comment: Some grammatical errors and typos.
Response: We have proofread the manuscript and corrected the grammatical errors and typos that we found.

Reviewer #2

Comment: 1.In INTRODUCTION:
I didn't see the results "In this procedure, the endothelium and Descemet's membrane is removed from the patient's cornea and replaced with a graft of endothelium and Descemet's membrane obtained from a donor cornea." , which is irrelevant to this article.
Response: We have rewritten this sentence so that it does not imply that the manuscript contains a procedure for transplant surgery.

Comment: (1)In PROTOCOL, "The membrane will detach from the stroma as a strip that immediately curls up." and "human donors of less than 30 years of age". In RESULTS, "one third of explants fail to attach to the plate" and " a corneal endothelial cell monolayer forms over several weeks" . All these mean that the isolation efficacy of corneal endothelial cells is low, especially when compared to other protocols, such as "peel-and-digest" methods (Peh GS, et al. Cultivation of human corneal endothelial cells isolated from paired donor corneas. PLoS One. 2011;6(12):e28310.)
Response: It would be interesting to compare our explant culture method with a peel-and-digest method using paired corneas to determine which method results in the most cells of high quality over the short and long term from a single donor. However, we agree that the peel-and-digest method would result in more cells in the cultures initially and have added a paragraph and reference to the Discussion to indicate this (lines 390 - 395).

Comment: (2)In Figure 8. Sheep corneal endothelial and stromal cells on opposing sides of a collagen type I membrane: There were not identification of corneal stromal cells. And we can't clearly see "a single layer of corneal endothelial cells on one surface of the collagen type I membrane while multiple layers of stromal cells had grown on the opposing surface". There

were not both cell phenotype or nuclear DAPI staining in Fig. 8 A and not multiple layers of stromal cell demonstration in Figure 8.
Response: We agree that the Figure legend for Figure 8 contained descriptions of results that were not visible in the Figure. We have completely rewritten this legend to better describe the images that are in this Figure.

Comment: The arm of article is "designed to minimise disruption of cell-to-cell contacts during isolation, expansion and subculture stages, to reduce the potential for EMT." However, authors didn't detect the EMT markers like αSMA, fibronectin, and so on. Therefore, it is better to add EMT related examination.
Response: Corneal endothelial cells that are undergoing EMT lose their regular polygonal shape and become more fibroblastic. EMT in corneal endothelial cells can therefore by assessed by morphological examinations. Our images of corneal endothelial cell cultures show that many of the cells, particularly around explants, have a very uniform polygonal shape. ZO-1 expression can also be used to indicate EMT in corneal endothelial cells. It is normally present as a distinct band at the cell border but becomes weak or absent in cells undergoing EMT. Our representative images of corneal endothelial cell cultures show robust ZO-1 expression at cell borders. We feel that the data that we have already provided to demonstrate a lack of EMT characteristics in our cell cultures is sufficient to make our point. We agree, however, that this point was not strongly conveyed in our Results section. We have therefore modified lines 278 - 279 and 298 - 300 to address this issue.

Comment: 1.In the INTRODUCTION: The sentence "The corneal endothelium is a monolayer of epithelial cells" is not so right. Authors should distinguish the definition between "endothelium" and "epithelium".
Response: The corneal endothelium is not an endothelial tissue but rather a fluid-transporting epithelium. Its name ‘corneal endothelium’ causes a lot of confusion. We have therefore removed the reference to the corneal endothelium as an epithelial layer from the Introduction.

Comment: 2. Grammatical tenses should be unified.
Response: We have proofread the manuscript and corrected the grammatical errors that we found.