Methods for Investigating Corneal Cell Interactions and Extracellular Vesicles In Vitro

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ABSTRACT:

Science and medicine have become increasingly 'human-centric' over the years. A growing shift away from the use of animals in basic research has led to the development of sophisticated in vitro models of various tissues utilizing human-derived cells to study physiology and disease. The human cornea has likewise been modeled in vitro using primary cells derived from corneas obtained from cadavers or post-transplantation. By utilizing a cell's intrinsic ability to maintain its tissue phenotype in a pre-designed microenvironment containing the required growth factors, physiological temperature and humidity, tissue-engineered corneas can be grown and maintained in culture for relatively long periods of time on the scale of weeks to months. Due to its transparency and avascularity, the cornea is an optimal tissue for studies of extracellular matrix and cell-cell interactions, toxicology and permeability of drugs, and underlying mechanisms of scarring and tissue regeneration. This paper describes methods for the cultivation of corneal keratocytes, fibroblasts, epithelial and endothelial cells for in vitro applications. We also provide detailed, step-by-step protocols for assembling and culturing 3D constructs of the corneal stroma, epithelial- and endothelial-stromal co-cultures and isolation of extracellular vesicles.

Basic Protocol 1: Isolating and culturing human corneal keratocytes and fibroblasts

Basic Protocol 2: Isolating and culturing human corneal epithelial cells

Basic Protocol 3: Isolating and culturing human corneal endothelial cells

Basic Protocol 4: 3D Corneal stromal construct assembly

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Basic Protocol 5: 3D Corneal epithelial-stromal co-culture assembly

Basic Protocol 6: 3D Corneal endothelial-stromal co-culture assembly

Basic Protocol 7: Isolating extracellular vesicles from corneal cell conditioned medium

Support Protocol 1: Cryopreserving human corneal fibroblasts, corneal epithelial cells, and corneal endothelial cells

KEYWORDS:

corneal epithelial cells, co-culture, extracellular vesicles, keratocytes, corneal endothelial cells

INTRODUCTION:

The adult human cornea is composed of three primary cell layers: a stratified corneal epithelium, stromal keratocytes, and a single-layered corneal endothelium. In addition, sensory nerves derived from the trigeminal ganglion innervate the stroma and epithelium. Resident immune cells have also been identified in the uninjured cornea. Over the years, approaches to isolate the different cell types found in the cornea have been developed with an emphasis on the epithelial, stromal, and endothelial layers. The protocols described in this paper detail methods to isolate and culture primary corneal keratocytes, fibroblasts, epithelial and endothelial cells from human tissue. These cells may be used for tissue engineering approaches to develop a functional human corneal tissue equivalent for in vitro pharmacological and disease mechanistic studies. We further describe applications utilizing isolated corneal fibroblasts to generate 3D stromal constructs that may be co-cultured with corneal epithelial or endothelial cells to study cell-cell interactions.

BASIC PROTOCOL 1

ISOLATING AND CULTURING HUMAN CORNEAL KERATOCYTES AND FIBROBLASTS

Primary human corneal keratocytes (hCKs) or fibroblasts (hCFs) may be isolated from human corneas provided by the National Disease Research Interchange (NDRI, Philadelphia, PA, USA), local eye banks, and ophthalmic surgery centers. The recovery of hCKs and hCFs from corneal tissue has been previously described in detail with varying phenotype depending on the concentration of fetal bovine serum (FBS) contained within the medium (Beales et al., 1999; Karamichos et al., 2013). Low serum conditions are required to maintain the keratocyte phenotype (e.g., high expression of keratocan and aldehyde dehydrogenase 3a), while exposure to high-serum conditions leads to differentiation to a fibroblast (e.g., low expression of keratocan markers with co-expression of α-smooth muscle actin) (Beales et al., 1999; Yam et al., 2017). In this protocol, the epithelial and endothelial layers are manually debrided from the surface of the stroma. The stromal tissue is then cut into small pieces and placed into a T25 flask and allowed to adhere to the bottom of the flask (**Figure 1**). Complete keratocyte (1% FBS) or fibroblast medium (10% FBS) is then added, and the

cultures are incubated for 2-4 weeks at 37˚C/5% CO_2 to allow for cell migration from the explant. Primary hCKs or hCFs may be frozen or subcultured up to passage 5-7. Alternative protocols using the addition of Dispase II or EDTA to chemically-remove the epithelial or endothelial cell layers have been reported (Trinkaus-Randall et al., 1985; Li et al., 2007). Collagenase-mediated degradation of the stromal collagen matrix has also been shown to aid in the recovery of corneal fibroblasts from intact tissue (Wu et al., 2014; Yam et al., 2017).

Materials:

Human corneal tissue in preservation medium (i.e., Optisol)

Dulbecco's phosphate-buffered saline (DPBS) (Gibco, cat. no. 14190-144)

Complete keratocyte medium -

1% FBS (Atlanta Biologicals, Flowery Branch, GA, cat. no. S11150)

Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA, cat. no. 30-2003)

1X Antibiotic-Antimycotic (Gibco, Grand Island, NY, cat. no. 15240-062)

Complete fibroblast medium -

10% FBS (Atlanta Biologicals, Flowery Branch, GA, cat. no. S11150)

EMEM (ATCC, Manassas, VA, cat. no. 30-2003)

1X Antibiotic-Antimycotic (Gibco, Grand Island, NY, cat. no. 15240-062)

0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (1X) (Gibco, cat. no. 25300-054)

Pipette

Forceps

100 mm petri dish

Scalpel

Razor blade

T25 and T75 cell culture flasks

Incubator with CO_2

15 mL polypropylene tubes

Centrifuge [Thermo IEC Centrifuge (Centra CL3R – rotor: IEC 243)]

Microscope (inverted light)

Hemocytometer

Optional: 1X Antibiotic-Antimycotic (Gibco, Grand Island, NY, cat. no. 15240-062)

Note: All steps must be performed under sterile conditions using a biosafety hood according to BSL2 universal precautionary guidelines. Obtaining human tissue should be performed in accordance with the Declaration of Helsinki and with approval from the appropriate Institutional Review Board. Sterilization of materials may be performed using 0.2 µm filtration (liquids), UV-irradiation (solids), or by autoclaving (solids or liquids). Cell culture medium should be stored at 4°C in the dark and warmed to room temperature prior to use in cell culture.

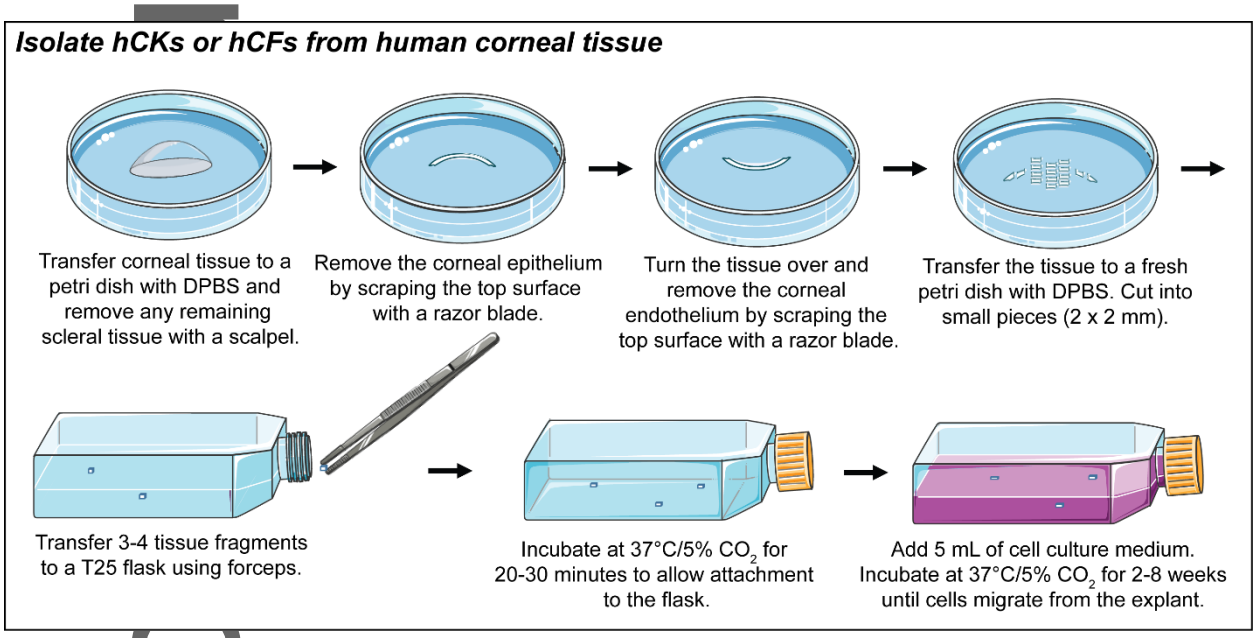

Figure 1. General experimental protocol for isolating hCKs or hCFs from human corneal tissue. Pictorials modified from Servier Medical Art based on a Creative Commons license available at https://smart.servier.com/.

Isolate hCKs or hCFs from human corneal tissue

1. Retrieve the human corneal tissue and transfer to a petri dish with 5 mL DPBS.

Optional: Incubation of the tissue in an antibiotic-antimycotic (1X) solution for 3-5 minutes at room temperature may be performed to reduce the likelihood of contamination.

2. Using a scalpel, cut any remaining scleral tissue from the cornea and discard in the appropriate biohazard waste stream.

Note: The outer white portion of the eye is termed the sclera, while the cornea is clear.

3. Orient the cornea epithelial-side up and gently scrape the corneal epithelium using a razor blade (5X). Turn the tissue over, and repeat debridement of the endothelium by scraping (3-5X).

Note: Removal of the corneal epithelium and endothelium can be seen with the release of whitish material/cells into the DPBS solution. Repeat debridement as needed to fully remove any epithelial or endothelial layers.

4. Transfer the corneal tissue to a fresh petri dish containing DPBS. Cut the corneal tissue into small fragments (2 mm x 2 mm) using a scalpel. Transfer the 3-4 fragments using forceps to the bottom of a T25 flask.

Alternative: The tissue fragments may be transferred to wells of a 6-well plate to decrease the time required to obtain 90% confluence.

5. Allow the corneal tissue fragments to attach to the bottom of the flask by incubating at 37˚C/5% CO_2 for 20-30 minutes.

6. Gently add 5 mL of complete corneal keratocyte or fibroblast medium to each flask—take care not to detach the tissue from the flask. Transfer flasks to an incubator and maintain at 37˚C/5% CO_2 for 2-8 weeks.

Note: There is no need to change the medium of the flasks until cells have begun to migrate from the explant and proliferate. Take care to not disturb the tissue and cause detachment from the flask. Cells are unlikely to migrate from the explant if the tissue is floating in the medium.

7. Check for cell migration from the explant every other day by visualizing under the microscope. Once any cells are identified, change the medium by gently aspirating and adding fresh complete corneal keratocyte or fibroblast medium.

Note: It usually takes 2 weeks for hCF migration from the explant and 3-8 weeks for hCKs.

8. Upon 70% confluence, trypsinize the hCKs or hCFs by aspirating the medium, washing 1X with DPBS, and adding 2-3 mL of 0.05% trypsin-EDTA to the flask followed by incubation for 5-7 minutes at 37˚C/5% CO_2. Neutralize the trypsin by adding an equal volume of complete corneal keratocyte or fibroblast medium and transfer to a 15 mL polypropylene tube.

9. Centrifuge at 300x*g* (1250 rpm) for 5 minutes at 4˚C and aspirate the supernatant. The cells will be pelleted at the bottom of the tube and may then be subcultured (*Step 10*) by re-suspending cells in 1 mL of complete keratocyte or fibroblast medium or cryopreserved for long-term storage (*Supporting Protocol 1*).

Subculture hCKs or hCFs

10. Isolate hCKs or hCFs from culture (*Step 9*) or thaw frozen stock of hCKs or hCFs (*Support Protocol 1, Step 5*) and add to 10 mL of complete keratocyte or fibroblast medium in a 15 mL polypropylene tube.

Note: Due to the long culture period of the stromal constructs (4 weeks), utilization of younger passages of hCKs or hCFs (passage 1-4) are required to allow for sufficient proliferation and extracellular matrix (ECM) production over time.

11. Centrifuge at 300x*g* (1250 rpm) for 5 minutes at 4˚C, aspirate supernatant, and re-suspend cells in 1 mL of complete keratocyte or fibroblast medium.

12. Transfer cell solution to a T75 flask containing 12 mL of complete keratocyte or fibroblast medium and culture at 37˚C/5% CO_2 until 80% confluent with medium changes every other day.

13. To split or passage cells, aspirate the medium, wash 1X with DPBS, and add 3-5 mL of trypsin-EDTA to the T75 flask. Incubate for 5 minutes at 37˚C/5% CO_2 until all cells are detached, as visualized under the microscope. Increase incubation time by 2-3 minutes if cells are not completely detached.

14. Add an equal volume of complete keratocyte or fibroblast medium, transfer to a centrifuge tube, and centrifuge at 300x*g* (1250 rpm) for 5 minutes at 4˚C. Aspirate the supernatant and re-suspend cells in 1 mL of complete keratocyte or fibroblast medium.

15. Count cells using a hemocytometer and re-suspend in the appropriate volume of keratocyte or fibroblast medium based on the total number of cells/well needed.

Note: hCKs and hCFs are commonly seeded at the following concentrations for an experimental setup: 6-well plate – 10^6 cells/well; 12-well plate – $5x10^5$ cells/well; 24-well plate – $2.5x10^5$ cells/well.

Basic Protocol 2:

ISOLATING AND CULTURING HUMAN CORNEAL EPITHELIAL CELLS

Primary human corneal epithelial cells (hCECs) may be isolated from either corneal tissue or obtained from commercial sources. Limbal corneal epithelial cells serve as the ideal source of corneal epithelial cells due to their high proliferative capacity (Tovell et al., 2015). In addition, an SV40-immortalized human corneal epithelial cell line (hCE-TJ) has been developed and thoroughly described (Araki-Sasaki et al., 1995; Guo et al., 2017). This protocol details the medium requirements and functional steps involved in the isolation and expansion of hCECs obtained from corneal limbal tissue and the subculturing of hCE-TJs (**Figure 2**). Differentiated hCECs may likewise be isolated from the cornea proper or central cornea using this protocol. Isolation of limbal epithelial stem cells found in the crypts within the underlying basal region of the limbus requires collagenase and specialized culture conditions including a feeder layer (Tovell et al., 2015).

***Materials**:*

Human corneal limbal rim in preservation medium (i.e., Optisol)

DPBS (Gibco, Grand Island, NY, cat. no. 14190-144)

20 µg/mL gentamicin (Gibco, Grand Island, NY, cat. no. 15750060)

Dissociation medium -

Hank's Balanced Salt Solution (Sigma Aldrich, St. Louis, MO, cat. no. H9269)

5 µg/mL gentamicin (Gibco, Grand Island, NY, cat. no. 15750060)

25 caseinolytic units/mL dispase II (Gibco, Grand Island, NY, cat. no. 17105041)

Complete corneal epithelial cell medium (*See Reagents and Solutions*)

0.05% trypsin-EDTA (1X) (Gibco, Grand Island, NY, cat. no. 25300-054)

Optional: FNC Coating Mix® (Athena Environmental Sciences, Baltimore, MD, cat. no. 0407)

Forceps

100 mm Petri dish

Pipette

Scalpel

15 mL polypropylene tubes

Centrifuge [Thermo IEC Centrifuge (Centra CL3R – rotor: IEC 243)]

T25 and T75 flasks

Microscope

Hemocytometer

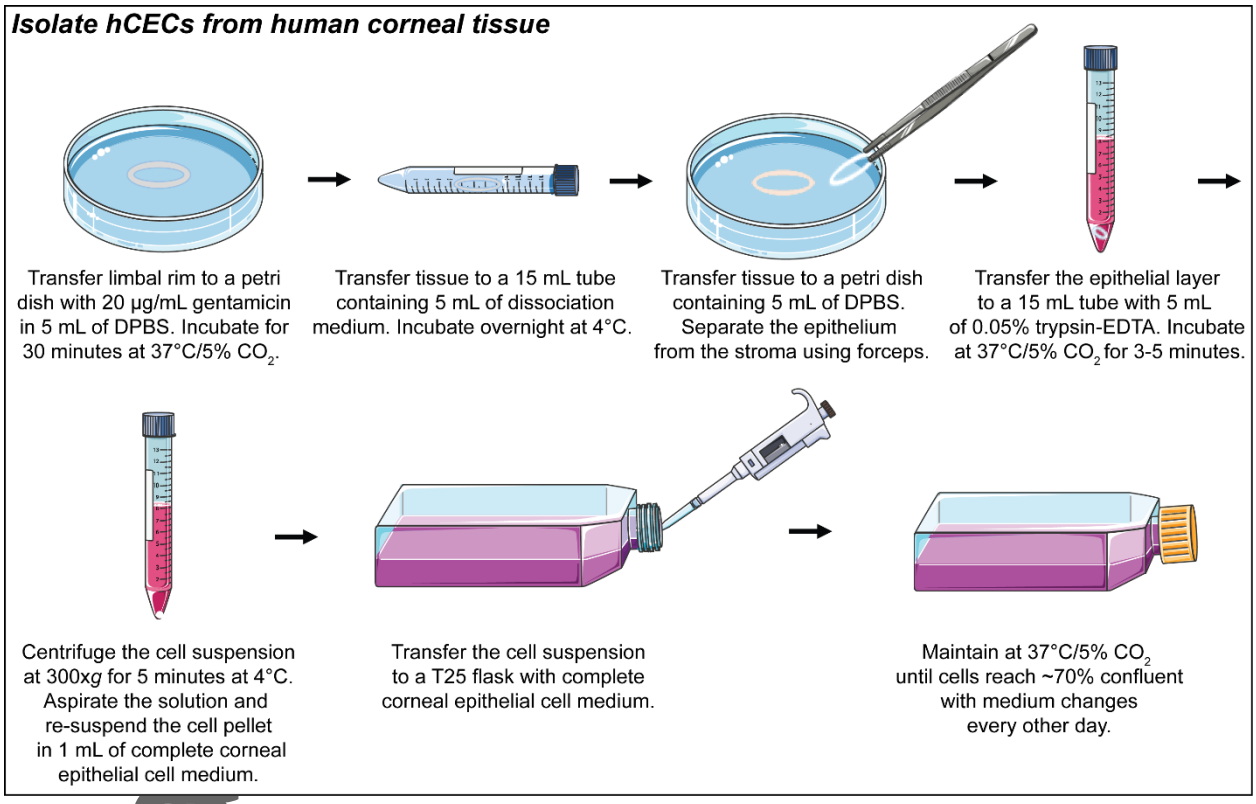

Figure 2. General experimental protocol for isolating hCECs from human corneal tissue. Pictorials modified from Servier Medical Art based on a Creative Commons license available at https://smart.servier.com/.

Isolate hCECs from human corneal tissue

1. Transfer the human corneal limbal rim to a small 100 mm petri dish containing 20 µg/mL gentamicin in 5 mL of DPBS for 30 minutes at 37˚C/5% CO_2.
2. Transfer the tissue to a 15 mL polypropylene tube containing 10 mL of dissociation medium. Incubate overnight at 4˚C horizontally.
3. Carefully remove the tissue pieces from the polypropylene tube using forceps and transfer to a petri dish containing DPBS.
4. Using forceps, remove the epithelial layer from the stromal tissue by gently lifting the epithelial layer from one side and peeling it away from the stroma. Transfer the epithelium to a fresh 15 mL polypropylene tube containing 5 mL of 0.05% trypsin-EDTA and incubate for 3-5 minutes at 37˚C/5% CO_2 to promote cell dissociation. Add an equal volume of complete corneal epithelial cell medium to neutralize the trypsin-EDTA and centrifuge at 300x*g* (1250 rpm) for 5 minutes at 4˚C.
5. Aspirate the supernatant and re-suspend the pelleted hCECs in complete corneal epithelial cell medium. Transfer cell suspension to a T25 flask.

Optional: The flask may be pre-treated with fibronectin to promote cell attachment by adding 3 mL of cFN to the flask, followed by swirling, and aspirating the cFN solution.

6. Culture hCECs until 70-80% confluent, then subculture (*Step 7*) or cryopreserve (*Support Protocol 1*).

Subculture hCECs or hCE-TJs

7. Isolate hCECs from culture (*Step 6*) or thaw cryopreserved hCECs/hCE-TJs (*Support Protocol 1*) and re-suspend in 10 mL of complete corneal epithelial cell medium in a 15 mL polypropylene tube.

NOTE: Since hCE-TJ is an established corneal epithelial cell line, these cells may be extensively passaged. Corneal epithelial marker expression (e.g., keratin-3 and -7) and cell morphology should be evaluated routinely to verify that the proper cell phenotype is maintained over time without undergoing an epithelial-to-mesenchymal transition. Further genotyping may also be performed to validate the cell line.

8. Centrifuge at 300x*g* (1250 rpm) for 5 minutes at 4˚C to pellet cells. Remove the supernatant and re-suspend the cell pellet in 10 mL of complete corneal epithelial cell medium. Transfer cell solution to a T75 flask and incubate at 37˚C/5% CO_2 to allow cell adhesion. Change the medium every other day until cells reach ~90-100% confluence.

9. To split a confluent culture of hCECs/hCE-TJs, aspirate the medium, wash the cells in DPBS 1X, and add 3 mL of 0.05% trypsin-EDTA to the flask. Incubate for 5-8 minutes at 37˚C/5% CO_2 to allow cell detachment.

10. Add an equal volume of complete corneal epithelial cell medium (3 mL) to the trypsinized cell solution to neutralize the trypsin. Transfer the cell suspension to a 15 mL tube and centrifuge at 300x*g* (1250 rpm) for 5 minutes at 4˚C.

Optional: A solution of 5% FBS in PBS may be used alternatively to neutralize trypsin.

11. Aspirate the supernatant and re-suspend the hCEC/hCE-TJ pellet in 5 mL of complete corneal epithelial cell medium. Transfer solution to a T75 flask.

Note: hCECs may be subcultured up to passage 5. Older passages will begin to proliferate slowly, thus making it difficult to obtain sufficient cell numbers to subculture.

Basic Protocol 3:

ISOLATING AND CULTURING HUMAN CORNEAL ENDOTHELIAL CELLS

Primary human corneal endothelial cells (hCEnCs) may be isolated from cadaveric corneal tissue by removing the endothelial layer and the collagenous-rich Descemet's membrane from the stroma followed by trypsinization. Isolated hCEnCs may then be passaged or seeded in co-cultures for further experiments.

***Materials**:*

Human cornea in preservation medium (i.e., Optisol)

DPBS (Gibco, Grand Island, NY, cat. no. 14190-144)

20 µg/mL gentamicin (Gibco, Grand Island, NY, cat. no. 15750060)

0.02% EDTA solution (Sigma Cat. #E8008)

Flame-polished pipette (VWR Cat. No. 14672-380)

Complete corneal endothelial cell medium (*See Reagents and Solutions*)

0.05% trypsin-EDTA (1X) (Gibco, Grand Island, NY, cat. no. 25300-054)

FNC Coating Mix® (Athena Environmental Sciences, Baltimore, MD, cat. no. 0407)

Forceps

35 mm and 65 mm petri dishes

Pipette

15 mL polypropylene tubes

Centrifuge [Thermo IEC Centrifuge (Centra CL3R – rotor: IEC 243)]

6-well plate

Microscope

Isolate hCEnCs from human corneal tissue

1. Wash the donor human cornea 3X in 6 mL of complete corneal endothelial cell medium in either a 35 mm or a 6-well plate prior to dissecting.

2. Under a dissecting microscope, remove the Descemet's membrane with the attached endothelium using forceps (the endothelium and the Descemet's should peel away from the stroma) and transfer to a 60 mm petri dish.

Note: Do not allow the cornea to dry out. Every few minutes, place the cornea back in the well containing medium to re-hydrate.

3. As the pieces of hCEnCs with Descemet's membrane are collected, immediately place them into a 15 mL tube containing 15 mL of complete corneal endothelial cell medium.

4. Centrifuge the cell suspension at 3000 rpm for 5 min at 4˚C. Remove the supernatant without disturbing the cell pellet.

5. Re-suspend the cell pellet in 8 mL 0.02% EDTA solution, and incubate at 37˚C for 1 hour to break the cell-cell junctions.

6. Triturate the cell suspension (~50 times) with a flame-polished pipette and centrifuge at 3000 rpm for 5 min at 4˚C. Remove the supernatant without disturbing the pelleted cells (may leave ~1 mL of EDTA with the pellet).

7. Re-suspend the pelleted cells in 4 mL of complete corneal endothelial cell medium, and vigorously triturate (~15 times) the cell suspension with another flame-polished pipette.

8. Transfer the cell suspension (2 mL/well) into a 6-well plate that has been coated with FNC.

9. In order to allow the cells enough time to attach to the plate, wait 3-5 days before changing the medium for the first time, then change the medium every other day (2 mL/well).

10. Culture until 70-80% confluent, then subculture in complete corneal endothelial cell medium or cryopreserve (*Support Protocol 1*).

Basic Protocol 4:

3D CORNEAL STROMAL CONSTRUCT ASSEMBLY

To develop a 3D corneal stromal construct, isolated hCFs are seeded onto a transwell membrane and stimulated with a stable Vitamin C-derivative to promote secretion and deposition of a collagen type I/V-rich matrix (**Figure 3**). This approach requires maintenance of the construct for at least 4 weeks to allow sufficient cell proliferation and ECM assembly to generate a corneal stromal tissue equivalent of 30-80 µm thickness. The stable Vitamin C-derivative promotes collagen expression and secretion leading to accelerated ECM deposition. Mature stromal constructs transferred to a non-Vitamin C containing medium will remain viable, albeit with an expected reduction in collagen expression. The advantages of the transwell system are that it permits increased air-perfusion at the surface of the construct to allow for stratification and maintains a surface stiffness more comparable to the human cornea, rather than the rigid stiffness of polystyrene, the dominant material present in cell culture plates, dishes, and flasks.

Materials:

hCFs suspended in complete corneal fibroblast medium (*Basic Protocol 1*)

Complete corneal fibroblast medium -

10% FBS (Atlanta Biologicals, Flowery Branch, GA, cat. no. S11150)

EMEM (ATCC, Manassas, VA, cat. no. 30-2003)

1X Antibiotic-Antimycotic (Gibco, Grand Island, NY, cat. no. 15240-062)

Complete corneal fibroblast medium with Vitamin C -

Complete corneal fibroblast medium

Vitamin C-derivative (0.5 mM 2-O-α-D-glucopyranosyl-L-ascorbic acid, Sigma Aldrich, St. Louis, MO, cat. no. SMB00390)

Pipette (5ml)

Hemocytometer

Microscope

Costar 0.4 µm polycarbonate transwell 6-well plate with 24 mm inserts (Corning, Kennebunk, ME, cat. no. 3412)

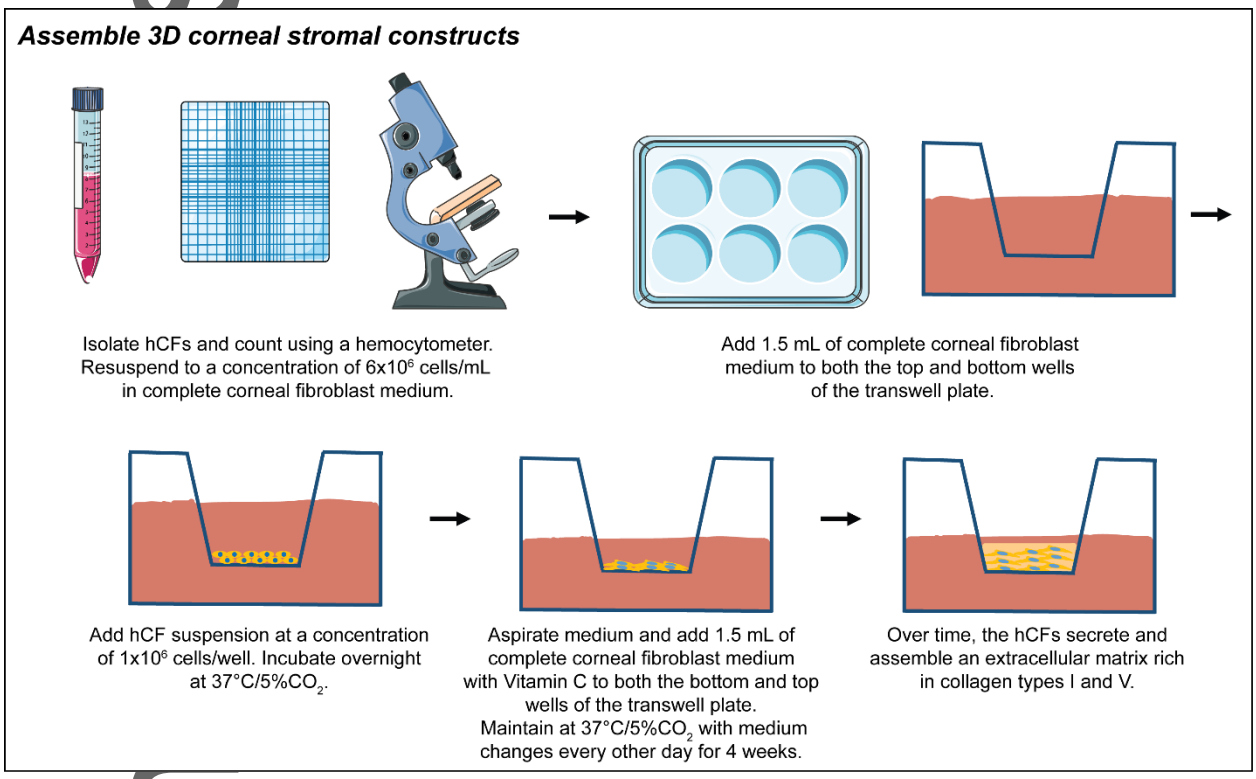

Figure 3. General experimental protocol for set-up of 3D corneal stromal constructs.

Seeding hCFs on transwell membrane

1. Isolate hCFs and resuspend in 1 mL of complete fibroblast medium. Count cells using a hemocytometer by adding 10 µL of the cell suspension to the glass slide and counting the number of cells per square, as visualized under a microscope. (Basic Protocol 1)
2. Resuspend hCFs to a final concentration of $6x10^6$ cells/mL in complete corneal fibroblast medium.

3. Add 1.5 mL of complete fibroblast medium to the top and bottom wells of a transwell plate. Add $1x10^6$ cells in suspension from step 2 to the medium in the top well. Gently, swirl the plate to ensure equal distribution of cells on the membrane.
4. Incubate overnight at 37˚C/5% CO_2 to allow cell attachment.

Stimulate with Vitamin-C to promote ECM deposition

5. At t=24 hours post-cell seeding, aspirate the medium from both the top and bottom wells. Then, add 1.5 mL of complete fibroblast medium with Vitamin C to both the top and bottom wells.
6. Change medium every other day for a total of 4 weeks to stimulate ECM deposition.

Note: An increase in construct thickness can be observed with the appearance of a translucent tissue layer on the transwell membrane as the hCFs begin to secrete and assemble a collagen-rich matrix.

Basic Protocol 5:

3D CORNEAL EPITHELIAL-STROMAL CONSTRUCT ASSEMBLY

A 3D co-culture approach has been developed to study corneal epithelial-stromal interactions (McKay et al., 2019). This model relies upon maturation of a corneal stromal construct as a substratum for a corneal epithelial layer (**Figure 4**). The stromal layer generated by hCFs is first cultured for 4 weeks in the presence of a stable Vitamin C-derivative to promote ECM deposition (Basic Protocol 4, Figure 3). Primary hCECs or an immortalized cell line (hCE-TJ) (Basic Protocol 2) are then seeded onto the stromal construct and cultured for an additional week at an air-liquid interface to allow maturation. Our lab has applied this model in the study of extracellular vesicle (EV) and provisional matrix secretion by different corneal cell populations (McKay et al., 2019).

Materials:

hCECs/hCE-TJs suspended in complete corneal epithelial cell medium (*Basic Protocol 2*)

Complete corneal epithelial cell medium (*See Reagents and Solutions*)

3D corneal stromal constructs (4 week hCF constructs seeded on polycarbonate transwell membranes) (*Basic Protocol 4*)

Complete fibroblast medium with Vitamin C (*See Reagents and Solutions*)

Pipette (5 ml)

Hemocytometer

Microscope

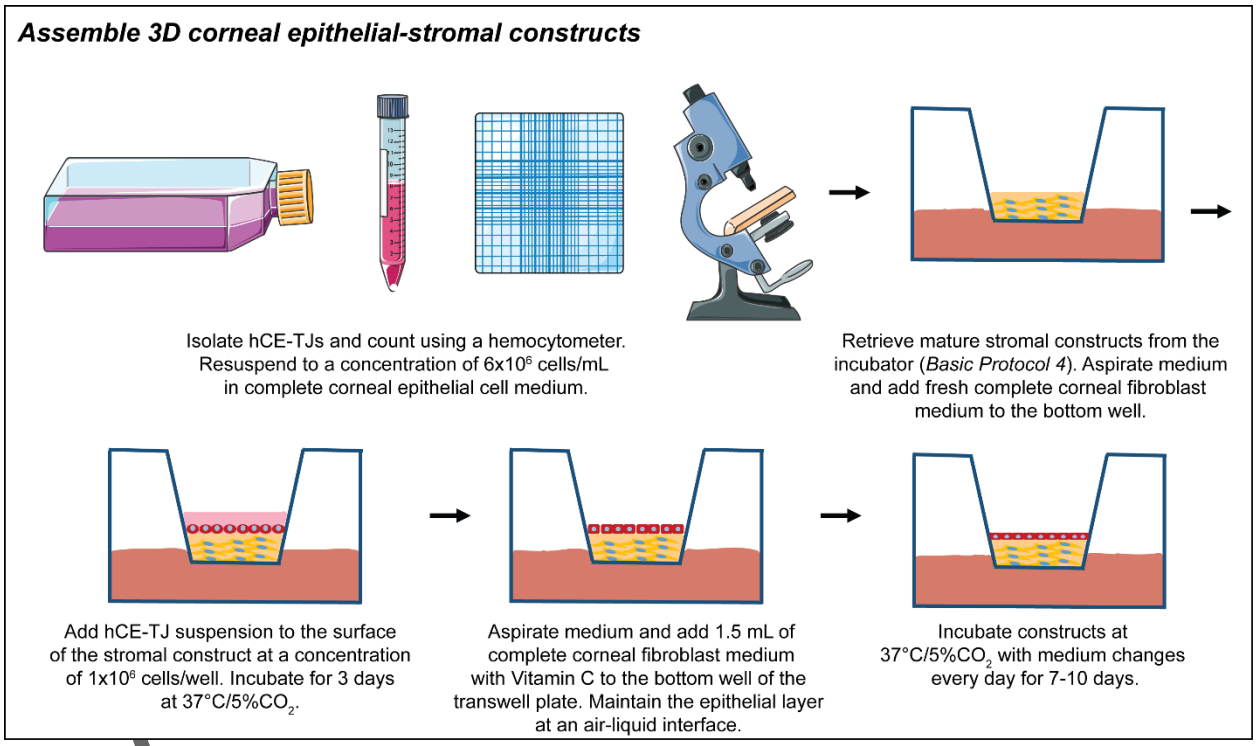

Figure 4. General experimental protocol for set-up of 3D corneal epithelial-stromal constructs.

Prepare hCECs or hCE-TJs for seeding

1. Isolate hCECs/hCE-TJs and suspend in complete corneal epithelial cell medium.
2. Using a hemocytometer, count the cells under the microscope and re-suspend the cells to a final concentration of 10^6 cells/mL in complete corneal epithelial cell medium.

Prepare 3D corneal stromal constructs

3. Retrieve the 3D corneal stromal constructs from the incubator. Aspirate the medium from the top and bottom wells of the transwell plate.
4. Add 1.5 mL of complete corneal fibroblast medium with Vitamin C to the bottom well.

Add hCECs or hCE-TJs to the surface of the stromal construct

5. Gently pipette hCECs/hCE-TJs to the top surface of the construct by adding 1 mL of the cell suspension in complete corneal epithelial cell medium to the surface.
6. Carefully, swirl the plate to equally distribute hCECs/hCE-TJs within the well. Add 0.5 mL of complete corneal epithelial cell medium to the top well. Incubate constructs at 37˚C/5% CO_2 overnight to allow cells to attachment.

Co-culture maintenance

7. For the first 3 days, change the medium daily by gently aspirating the medium from the top and bottom wells, and then adding 1.5 mL of complete corneal epithelial cell medium to the top well and 1.5 mL of complete corneal fibroblast medium with Vitamin C to the bottom well.

Note: hCE-TJs proliferate quickly and require frequent medium changes. To reduce pathological effects on the cell populations due to excessive glucose metabolism/lactate production, change the medium daily and replace with fresh epithelial and fibroblast medium in the respective chambers.

8. Generate an air-liquid interface by aspirating the medium from the top well. Maintain fresh complete corneal fibroblast medium in the bottom well by changing it every day. Culture for 7-10 days to allow for tissue maturation.

Note: Culturing hCECs at an air-liquid interface has been shown to promote corneal epithelial cell differentiation (Zieske et al., 1994), which may be characterized by the production of microvilli and keratin-3 by the surface epithelium.

Basic Protocol 6:

3D CORNEAL ENDOTHELIAL-STROMAL CONSTRUCT ASSEMBLY

Using a similar approach as the epithelial-stromal constructs, we have developed a corneal endothelial-stromal co-culture system that utilizes a mature hCF self-assembled construct cultured with an overlaying corneal endothelium (Hutcheon et al., 2019). This approach requires seeding of hCEnCs (*Basic Protocol 3*) on the surface of a 4-week hCF stromal construct (*Basic Protocol 4*) at a high cell number to allow formation of a cohesive endothelial layer over 5 to 14 days. A human corneal endothelial cell line (hCEnCL) (Griffith et al., 1999) has also been utilized in this model to successfully generate a confluent endothelial layer (Hutcheon et al., 2019).

Materials:

hCEnCs/hCEnCL suspended in complete corneal endothelial cell medium (*Basic Protocol 3*)

Complete corneal endothelial cell medium (*Refer to Reagents and Solutions*)

3D corneal stromal constructs (4 week hCF constructs seeded on polycarbonate transwell membranes) (*Basic Protocol 4*)

Complete fibroblast medium with Vitamin C (*Refer to Reagents and Solutions*)

Pipette (5 ml)

Hemocytometer

Microscope

Prepare hCEnCs or hCEnCL for seeding

1. Isolate hCEnCs/hCEnCL and suspend in complete corneal endothelial cell medium (*Basic Protocol 3*).
2. Using a hemocytometer, count the cells under the microscope and re-suspend cells to a final concentration of 10^6 cells/mL (for hCEnCs) or 5 X 10^5 cells/mL (for hCEnCL) in complete corneal endothelial cell medium.

Prepare 3D corneal stromal constructs

3. Retrieve the 3D corneal stromal constructs from the incubator (*Basic Protocol 4*). Aspirate the medium from the top and bottom wells of the transwell plate.
4. Add 1.5 mL of complete corneal fibroblast medium with Vitamin C to the bottom well.

Add hCEnCs or hCEnCL to the surface of the stromal construct

5. Gently pipette hCEnCs/hCEnCL to the top surface of the construct by adding 1 mL of the cell suspension in complete corneal endothelial cell medium to the surface.
6. Incubate constructs at 37˚C/5% CO_2 overnight to allow cell attachment.

Co-culture maintenance

7. The next day, gently aspirate the medium from the bottom and top wells and then add 1.5 mL of complete corneal endothelial cell medium to the top well and 1.5 mL of complete corneal fibroblast medium with Vitamin C to the bottom well. Repeat this every other day, for 5 to 14 days.

Note: hCEnCL proliferate more quickly than hCEnCs. Adding more hCEnCs to the construct will result in about the same endothelial cell density as hCEnCL on construct.

Basic Protocol 7:

ISOLATING EXTRACELLULAR VESICLES FROM CORNEAL CELL CONDITIONED MEDIUM

EVs are produced by corneal epithelium, stromal keratocytes and fibroblasts, and endothelium (McKay et al., 2019). EVs may be isolated from conditioned medium using gradient ultracentrifugation steps to first pellet out larger particles and aggregated proteins, and then, with the last ultracentrifugation step, a small colorless or slightly white pellet, which contains the EVs (e.g., microvesicles, exosomes, ectosomes, among others) is generated. This EV pellet may be stored in buffer at -80˚C for several weeks (**Figure 5**). Théry et al. published a detailed protocol for the isolation and characterization of EVs (Thery et al., 2006).

***Materials**:*

Conditioned medium

Phosphate buffered saline (PBS, Gibco, Grand Island, NY, cat. no. 10010023)

Pipette

50 mL sterile polypropylene tubes

Thermo IEC Centrifuge (Centra CL3R – rotor: IEC 243)

Polycarbonate ultracentrifuge tubes (Beckman Coulter, Brea, CA, 25x89 mm, capacity - 38 mL max, cat. no. 385631)

Beckmann Coulter Ultracentrifuge (rotor: 50.2 Ti)

1.5 mL microcentrifuge tubes

Freezer (-80°C)

[*Optional*: Total exosome isolation reagent (ThermoFisher, Waltham, MA, cat. no. 4478359)]

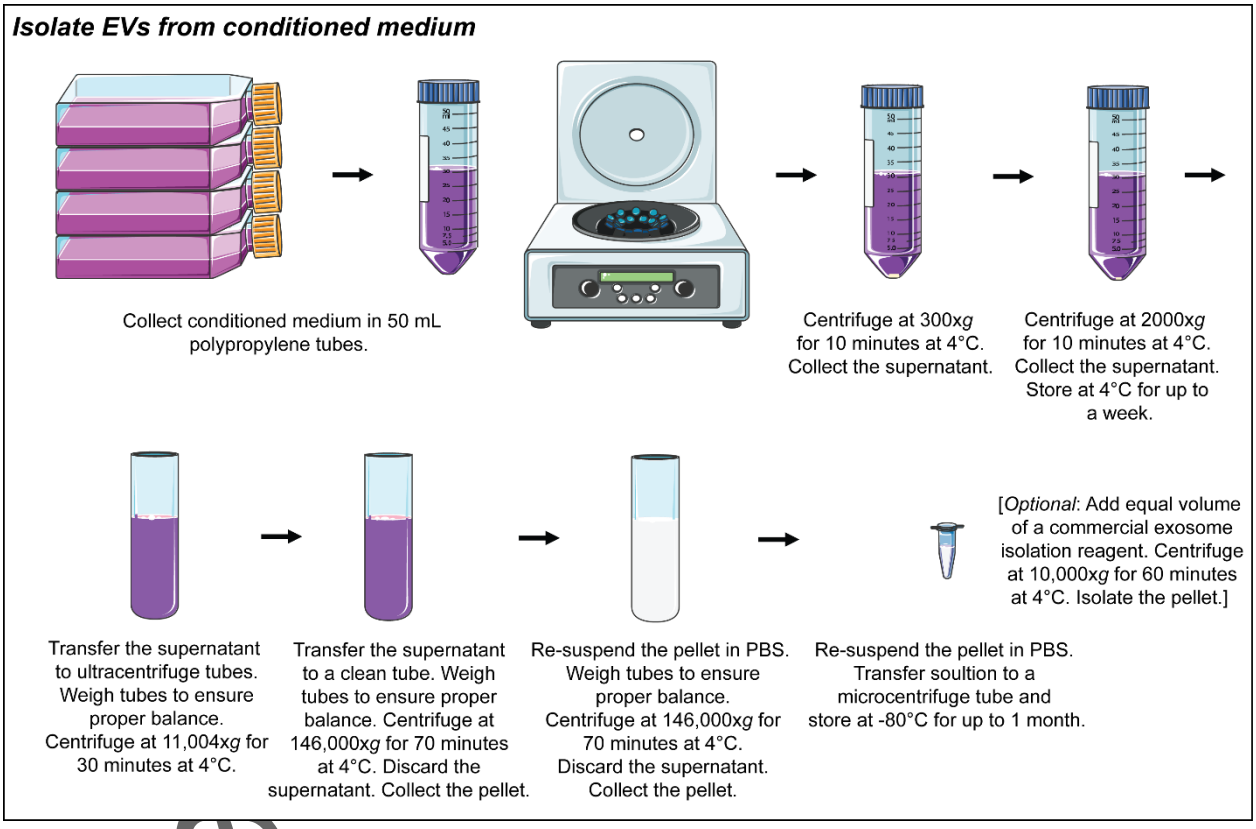

Figure 5. General experimental protocol for isolating EVs from conditioned cell culture medium. Pictorials modified from Servier Medical Art based on a Creative Commons license available at https://smart.servier.com/.

Isolate EVs from conditioned medium

1. Isolate conditioned medium in 50 mL sterile polypropylene tubes and centrifuge at 300x*g* for 10 minutes at 4˚C to pellet any floating cells (Thermo IEC Centrifuge).
2. Transfer the supernatant to a 50 mL tube and centrifuge at 2000x*g* (3150 rpm) for 10 minutes at 4˚C to remove any larger cell debris or fragments (Thermo IEC Centrifuge).
3. Transfer the supernatant to a 50 mL tube. At this point, either proceed to the next step or store the supernatant at 4˚C for up to 48 hours.
4. To isolate EVs from the medium, transfer the supernatant from step 3 to polycarbonate ultracentrifuge tubes. Weigh the medium-filled tubes to ensure equal weight between samples (≤ 0.001 g difference between samples). Centrifuge at 11,004x*g* (11,000 rpm) for 30 minutes at 4˚C to pellet larger particles (Beckmann Coulter Ultracentrifuge). Collect the supernatant and transfer into clean ultracentrifuge tubes.
5. Centrifuge the supernatant at 146,000x*g* (40,000 rpm) for 70 minutes at 4˚C to pellet the EVs (Beckmann Coulter Ultracentrifuge). Aspirate the supernatant and place to the side. Re-suspend the pellet in 10 mL of PBS.

6. Centrifuge the pellet suspension from step 5 at 146,000x*g* (40,000 rpm) for 70 minutes at 4˚C (Beckmann Coulter Ultracentrifuge). Remove the supernatant and place to the side. Re-suspend the pellet in 100 µL of PBS and transfer to a clean microcentrifuge tube and store at -80˚C until further use.

Optional: *A commercial exosome isolation reagent may be used to isolate EVs from conditioned medium. The larger particles are first removed using centrifugation (Steps 1-2) and the supernatant is mixed with the total exosome isolation reagent (1:1) in a microcentrifuge tube followed by incubation overnight at 4˚C. This solution is then centrifuged at 10,000xg for 60 minutes at 4˚C to pellet the EVs.*

Support Protocol 1

CRYOPRESERVING HUMAN CORNEAL FIBROBLASTS, CORNEAL EPITHELIAL CELLS, AND CORNEAL ENDOTHELIAL CELLS

For long-term storage, hCFs, hCEC/hCE-TJs, and hCEnCs/hCEnCLs may be cryopreserved using standard protocols. Due to the cytotoxicity of the cryoprotectant, the steps following suspension of the cells in freezing medium should be performed quickly to minimize exposure at room temperature. Further considerations regarding optimal handling and recovery of frozen cells have been described extensively (Ramos et al., 2014; Baust et al., 2017). A lower percentage of DMSO to cryopreserve hCECs or hCFs may also be used as the freezing medium (5% DMSO in 95% FBS).

Materials:

Freezing medium -

90% FBS (Atlanta Biologicals, Flowery Branch, GA, cat. no. S11150)

10% Dimethyl sulphoxide (DMSO) Hybri-Max® (Sigma Aldrich, St. Louis, MO, cat. no. D2650)

Pipette

1.5 mL cryotubes

Cryobowl

-20˚C freezer

-80˚C freezer

Liquid nitrogen storage dewar

Prepare cells for cryopreservation

1. Label cryotubes and set up cryobowl.

2. Following trypsinization and isolation of hCFs, hCEC/hCE-TJs, and hCEnCs/hCEnCLs, prepare fresh freezing medium.

3. Re-suspend cells in freezing medium (10^6 cells/mL) and quickly mix by trituration.

4. Add 1 mL of cell suspension to each labelled cryotube and transfer to the cryobowl.

5. Quickly transfer the cryobowl to the -20˚C freezer for 1 hour followed by incubation at -80˚C overnight.

6. Transfer cryotubes to the liquid nitrogen storage dewar for stable long-term storage.

REAGENTS AND SOLUTIONS:

Complete corneal endothelial cell medium -

OptiMEM-I (Gibco, Grand Island, NY, cat. no. 51985)

200 mg/L $CaCl_2$ (Sigma, cat. no. C2661)

800 mg/L Chondroitin Sulfate (Sigma, cat. no. C9819)

20 mg/L Ascorbic Acid (sigma, A4544)

50 mg/L Gentamicin (Gibco, cat no. 15710)

1X Antibiotic-Antimycotic (Gibco, Grand Island, NY, cat. no. 15240-062)

50 µg/L EGF (Millipore, 01-101)

8% FBS (Atlanta Biologicals, Flowery Branch, GA, cat. no. S11150)

Add $CaCl_2$, and chondroitin sulfate to 460 mL OptiMEM, and mix with a magnetic stir bar for 30 min at room temperature. Add ascorbic acid, FBS, gentamicin, antibiotic-antimycotic, and EGF to medium. Store at 4°C in the dark for up to 1 month.

Complete corneal epithelial cell medium -

Keratinocyte Serum-Free Basal Medium (Gibco, Grand Island, NY, cat. no. 10724-011)

0.05 mg/mL bovine pituitary extract (Gibco, Grand Island, NY, cat. no. 37000-015)

0.005 µg/mL epidermal growth factor (Gibco, Grand Island, NY, cat. no. 37000-015)

1X Antibiotic-Antimycotic (Gibco, Grand Island, NY, cat. no. 15240-062)

Add growth factors and antibiotic-antimycotic solutions to the basal medium. Store at 4°C in the dark for up to 1 month.

Complete fibroblast medium with Vitamin C -

10% FBS (Atlanta Biologicals, Flowery Branch, GA, cat. no. S11150)

EMEM (ATCC, Manassas, VA, cat. no. 30-2003)

1X Antibiotic-Antimycotic (Gibco, Grand Island, NY, cat. no. 15240-062)

Vitamin C-derivative (0.5 mM 2-O-α-D-glucopyranosyl-L-ascorbic acid, Sigma Aldrich, St. Louis, MO, cat. no. SMB00390)

Dissolve the Vitamin C-derivative in 10 mL of EMEM, mix thoroughly by vortex, and incubate 15 minutes at room temperature. Filter-sterilize the solution (0.2 µm filter) and add to the medium. Store medium at 4°C in the dark for up to 1 month.

COMMENTARY

BACKGROUND INFORMATION:

Primary corneal cell types, e.g., epithelial, stromal keratocytes and fibroblasts, and endothelial cells, may be isolated from human corneas (*Basic Protocol 1-3*) and cultured in 3D constructs (*Basic Protocol 4-6*) followed by EV isolation (*Basic Protocol 7*) for the study of cell-cell communication in vitro. Our laboratory and others have applied the self-assembled ECM approach to engineer a biomimetic of the human corneal stroma (Guo et al., 2007; Carrier et al., 2008; Ren et al., 2008; Karamichos et al., 2010; Couture et al., 2016). This method relies upon in situ collagen secretion and deposition by hCKs or hCFs stimulated with a Vitamin C-derivative. Our work over the years has focused heavily on hCFs due to their relatively easy isolation and expansion in culture compared to the more quiescent hCKs. The hCFs may be seeded on a permeable, micropatterned substratum to induce initial cell alignment, which appears to guide organization of the collagen matrix (Karamichos et al., 2014; Zareian et al., 2016). This model has been applied in the study of diseases that affect corneal structure, including fibrosis (Karamichos et al., 2010), keratoconus (Karamichos et al., 2012; McKay et al., 2017b), diabetes mellitus (Priyadarsini et al., 2016), and herpes simplex infections (Drevets et al., 2015), as well as to study the effects of microenvironmental changes during hypoxia (McKay et al., 2017a; Lee et al., 2018) and UV-mediated crosslinking (Sharif et al., 2017). Further applications with co-cultures of epithelial cells has shown evidence of a presumptive basement membrane, though with disperse laminin expression, and provisional matrix deposition along the epithelial-stromal interface (McKay et al., 2019). In the 3D corneal endothelial-stromal co-cultures, ZO-1, a tight junctional protein, and laminin are present at the endothelial-stromal interface in this model, which is suggestive of the formation of a basement membrane (Hutcheon et al., 2019). Both epithelial- and endothelial-stromal constructs are useful tissue engineered-corneal models that allow for the study of cell-cell interactions, including EV secretion and

uptake between different cell populations (McKay et al., 2019), in a minimalist, yet 3D microenvironment containing a physiological ECM.

CRITICAL PARAMETERS:

The quality of the corneal explant contributes to the success of cell isolation for the individual cell layers and is dependent on the time from post-mortem (for cadaveric tissue) or post-transplantation to culture, as well as endothelial cell count, proper tissue preservation after collection and during transportation, and systemic disease presence that may contribute to corneal defects (e.g. diabetes mellitus). Generally, isolation of cells within 24 hours post-mortem and storage of the tissue in preservation medium, such as Optisol, under sterile conditions contributes to a high success for quality cell isolation. Other considerations include the low migratory and proliferative ability of quiescent keratocytes compared to corneal fibroblasts. We have found that isolation of keratocytes requires a prolonged time period (up to 8 weeks) for sufficient cell numbers to migrate from the explant. Also, storing the medium and growth factors properly and using only freshly prepared medium is required for maintaining healthy and stable primary cells in culture.

For 3D stromal construct assembly, it is necessary to seed a sufficient number of hCFs in order to promote ECM deposition over 4 weeks, as well as utilize fresh solution of the Vitamin C derivative. In addition, changing the medium regularly (every other day) is essential to maintain the constructs and reduce myofibroblast differentiation and contraction, which may be driven by lowered pH and elevated lactate levels present in expended medium. This strict adherence to maintaining fresh medium strongly applies to the epithelial- and endothelial-stromal co-culture systems, which include epithelial or endothelial cells that are generally cultured in serum-free conditions. The transwell system allows the epithelial or endothelial cells on top of the stromal construct to be separated from the 10% serum found in the complete fibroblast medium. This medium feeds the construct from below; however, exposure of the epithelial layer to the serum medium may occur via diffusion through the stromal construct, but detrimental effects (epithelial-to-mesenchymal transition) may be minimized due to the relatively short co-culture period (7 days) that is still long enough to allow for epithelial maturation.

TROUBLESHOOTING:

Common issues encountered during Basic Protocols 1-2, 4-5, and 7 are described in **Table 1**.

Protocol	Issue	Reason	Solution
Basic Protocol 1	No or little hCK or hCF isolation from corneal tissue	Lack of tissue adhesion to flask or plate	Increase the incubation time before adding medium to allow the tissue to adhere to the flask or plate

		Time allowed for cell migration was too short	Maintain explants in medium for at least 8 weeks for hCK and 4 weeks for hCF migration from the tissue. Refresh medium after 2 weeks.
	Contamination of hCK or hCF culture with hCEC or hCEndo	Incomplete debridement of the anterior and posterior cell layers from the stromal tissue	Increase scraping of the cell layers to remove the epithelium and endothelium. Add additional washes, as needed, in separate petri dishes with PBS to remove any remaining adhered epithelial or endothelial cells
Basic Protocol 2	No or little hCEC isolation from corneal tissue	Lack of adhesion of cells to the flask	To improve cell adhesion, coat flask or plate with fibronectin (FNC) or collagen prior to cell seeding.
		Low proliferation of hCECs	Prepare fresh complete corneal epithelial medium with inclusion of epithelial growth factors
Basic Protocol 4	hCF cell layer appears to bundle into a whitish material at the edge of the well	During the first two week, hCFs may contract, this is occurs if the cells are seeded too densely or are stressed	Seed hCFs at a density of 10^6 cells/well for a 24mm diameter transwell. Change medium 3X per week using freshly prepared complete corneal fibroblast medium with the Vitamin C derivative
Basic Protocol 5	A thinner stromal matrix is present in	The hCE-TJ cell line proliferates quickly	Change the medium frequently (daily) to

	the epithelial-stromal co-cultures than expected	and may induce apoptosis of the underlying hCF layer	reduce nutritional deficiencies
Basic Protocol 7	High abundance of mitochondrial and nuclear proteins detected in isolated EVs	High-speed centrifugation of non-adherent or dead cell fragments will lead to cell lysis and the release of membrane-bound organelles	Perform low-speed centrifugation to pellet any non-adherent or dead cells prior to high-speed centrifugation -Isolate EVs from conditioned medium prior to freezing

Table 1. General troubleshooting approaches for common issues encountered during corneal cell isolation, 3D model assembly, and EV isolation. [Abbreviations: human corneal keratocyte (hCK), human corneal fibroblast (hCF), human corneal epithelial cell line (hCE-TJ), extracellular vesicle (EV)]

UNDERSTANDING RESULTS:

The unwounded corneal stroma contains keratocytes, which are quiescent and express crystallin proteins thought to be important in promoting cellular transparency. When the cornea is wounded, keratocytes become activated and differentiate into fibroblasts characterized by lower crystallin protein expression and co-expression of α-SMA. The fibroblast phenotype can be further differentiated into myofibroblasts following stimulation with transforming growth factor-β1 or platelet-derived growth factor (Fini, 1999). Morphological differences can be observed between hCKs, which exhibit a dendritic-like appearance with short cell extensions, and hCFs, which are larger in size and more contractile (Petroll et al., 2015). Biochemical differences are also present between these cell types and can be distinguished based on cell markers (**Table 2**). In order to maintain the keratocyte phenotype, hCKs are isolated in low serum conditions to reduce differentiation to a fibroblast phenotype. Therefore, isolation of hCKs requires significantly more time to allow for cell migration from the explant (up to 8 weeks) compared to hCFs that may be isolated within 2 weeks in 10% FBS-enriched medium.

Cell Type	Morphology*	Protein Marker Expression
Primary human corneal keratocyte (hCK)	Dendritic-like cell with short cell-extensions	Keratocan, aldehyde dehydrogenase 3a, lumican
Primary human corneal fibroblast (hCF)	Elongated cells with significant cell spreading and large cytoplasm in non-confluent cultures and spindle-like in confluent cultures	Low amounts of keratocan and α-SMA co-expression, high amounts of collagen types I and V
Primary human corneal	Small, cuboidal-shaped cells	Keratin-3 and -12

epithelial cell (hCEC) and human corneal epithelial cell line (hCE-TJ)	with close cell-cell contacts in confluent cultures and patches of cell clusters in non-confluent cultures	
Primary human corneal endothelial cell (hCEnC)	Small, polygonal-shaped cells with close cell-cell contacts in confluent cultures	ZO-1, Na^+/K^+ ATPase, Prdx-6 and CD166

Table 2. Cellular morphology and protein marker expression of the major corneal cell types. *The cell morphology may vary depending on the stiffness of the matrix or substratum and the presence or absence of growth factors.

We have shown that hCE-TJ-derived EVs isolated using the ultracentrifugation approach (*Basic Protocol 7)* exhibit a relatively consistent size and expression of key exosomal proteins, e.g. CD9, as well as provisional matrix proteins, fibronectin and thrombospondin-1 (**Table 3**). Further characterization of hCE-TJ-derived EVs showed that stimulation of hCFs promoted myofibroblast differentiation based on α-SMA expression (McKay et al., 2020).

Cell Source	Average Size	Total EV Protein	Dominant Protein Cargo	Functional Effects
Human corneal epithelial cell line (hCE-TJ)	58 ± 32 nm	12-27 µg	Fibronectin, thrombospondin-1, translational proteins, exosomal markers, cytoplasmic proteins	↑hCF contractility, ↑α-SMA

Table 3. Characterization of human corneal epithelial cell line (hCE-TJ)-derived extracellular vesicles (EVs) isolated from conditioned medium by ultracentrifugation (*Basic Protocol 7*). Data reported in (McKay et al., 2020).

TIME CONSIDERATIONS:

The most time-consuming methods described in this manual are the 3D corneal co-cultures (epithelial-stromal and endothelial-stromal models) and EV isolation process. In addition, the time spent preparing and changing cell culture medium should be considered and are not included in the active and total time estimates described in **Table 4**.

Protocol	Active Time Spent	Total Time Spent*
Basic Protocol 1: Isolating and culturing human corneal keratocytes and fibroblasts	30 min per cornea for cell isolation	1 hr
Basic Protocol 2: Isolating and culturing human corneal epithelial cells	1 hr per cornea for cell isolation	1 hr + overnight incubation
Basic Protocol 3: Isolating	1 hr per cornea for cell	1 hr

and culturing human corneal endothelial cells	isolation	
Basic Protocol 4: 3D Corneal stromal construct assembly	1 hr for (1) 6-well plate of constructs	1 hr
Basic Protocol 5: 3D Corneal epithelial-stromal construct assembly	2 hr for (1) 6-well plate of constructs	2 hr
Basic Protocol 6: 3D Corneal endothelial-stromal construct assembly	2 hr for (1) 6-well plate of constructs	2 hr
Basic Protocol 7: Isolating extracellular vesicles from corneal cell conditioned medium	2 hr	5 hr + overnight incubation

Table 4. Description of time considerations for Basic Protocols 1-7. *Total time spent describes the time required for cell isolation and seeding and does not include medium changes 3X per week for 4-8 weeks. The active and total times may vary heavily depending on the user and the total number of samples.

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LITERATURE CITED:

Araki-Sasaki, K., Ohashi, Y., Sasabe, T., Hayashi, K., Watanabe, H., Tano, Y., & Handa, H. (1995). An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci, 36*(3), 614-621.

Baust, J. M., Campbell, L. H., & Harbell, J. W. (2017). Best practices for cryopreserving, thawing, recovering, and assessing cells. *In Vitro Cell Dev Biol Anim, 53*(10), 855-871. doi: 10.1007/s11626-017-0201-y

Beales, M. P., Funderburgh, J. L., Jester, J. V., & Hassell, J. R. (1999). Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture. *Invest Ophthalmol Vis Sci, 40*(8), 1658-1663.

Carrier, P., Deschambeault, A., Talbot, M., Giasson, C. J., Auger, F. A., Guerin, S. L., & Germain, L. (2008). Characterization of wound reepithelialization using a new human tissue-engineered corneal wound healing model. *Invest Ophthalmol Vis Sci, 49*(4), 1376-1385. doi: 10.1167/iovs.07-0904

Couture, C., Zaniolo, K., Carrier, P., Lake, J., Patenaude, J., Germain, L., & Guerin, S. L. (2016). The tissue-engineered human cornea as a model to study expression of matrix metalloproteinases during corneal wound healing. *Biomaterials, 78*, 86-101. doi: 10.1016/j.biomaterials.2015.11.006

Drevets, P., Chucair-Elliott, A., Shrestha, P., Jinkins, J., Karamichos, D., & Carr, D. J. (2015). The use of human cornea organotypic cultures to study herpes simplex virus type 1 (HSV-1)-induced inflammation. *Graefes Arch Clin Exp Ophthalmol, 253*(10), 1721-1728. doi: 10.1007/s00417-015-3073-4

Fini, M. E. (1999). Keratocyte and fibroblast phenotypes in the repairing cornea. *Prog Retin Eye Res, 18*(4), 529-551. doi: 10.1016/s1350-9462(98)00033-0

Griffith, M., Osborne, R., Munger, R., Xiong, X., Doillon, C. J., Laycock, N. L., Hakim, M., Song, Y., & Watsky, M. A. (1999). Functional human corneal equivalents constructed from cell lines. *Science, 286*(5447), 2169-2172. doi: 10.1126/science.286.5447.2169

Guo, X., Hutcheon, A. E., Melotti, S. A., Zieske, J. D., Trinkaus-Randall, V., & Ruberti, J. W. (2007). Morphologic characterization of organized extracellular matrix deposition by ascorbic acid-stimulated human corneal fibroblasts. *Invest Ophthalmol Vis Sci, 48*(9), 4050-4060. doi: 10.1167/iovs.06-1216

Guo, X., Hutcheon, A. E. K., Tran, J. A., & Zieske, J. D. (2017). TGF-beta-target genes are differentially regulated in corneal epithelial cells and fibroblasts. *New Front Ophthalmol, 3*(1). doi: 10.15761/nfo.1000151

Hutcheon, A. E. K., Zieske, J. D., & Guo, X. (2019). 3D in vitro model for human corneal endothelial cell maturation. *Exp Eye Res, 184*, 183-191. doi: 10.1016/j.exer.2019.04.003

Karamichos, D., Funderburgh, M. L., Hutcheon, A. E., Zieske, J. D., Du, Y., Wu, J., & Funderburgh, J. L. (2014). A role for topographic cues in the organization of collagenous matrix by corneal fibroblasts and stem cells. *PLoS One, 9*(1), e86260. doi: 10.1371/journal.pone.0086260

Karamichos, D., Guo, X. Q., Hutcheon, A. E., & Zieske, J. D. (2010). Human corneal fibrosis: an in vitro model. *Invest Ophthalmol Vis Sci, 51*(3), 1382-1388. doi: 10.1167/iovs.09-3860

Karamichos, D., Rich, C. B., Zareian, R., Hutcheon, A. E., Ruberti, J. W., Trinkaus-Randall, V., & Zieske, J. D. (2013). TGF-beta3 stimulates stromal matrix assembly by human

corneal keratocyte-like cells. *Invest Ophthalmol Vis Sci, 54*(10), 6612-6619. doi: 10.1167/iovs.13-12861

Karamichos, D., Zareian, R., Guo, X., Hutcheon, A. E., Ruberti, J. W., & Zieske, J. D. (2012). Novel in Vitro Model for Keratoconus Disease. *J Funct Biomater, 3*(4), 760-775. doi: 10.3390/jfb3040760

Lee, A., Karamichos, D., Onochie, O. E., Hutcheon, A. E. K., Rich, C. B., Zieske, J. D., & Trinkaus-Randall, V. (2018). Hypoxia modulates the development of a corneal stromal matrix model. *Exp Eye Res, 170*, 127-137. doi: 10.1016/j.exer.2018.02.021

Li, W., Sabater, A. L., Chen, Y. T., Hayashida, Y., Chen, S. Y., He, H., & Tseng, S. C. (2007). A novel method of isolation, preservation, and expansion of human corneal endothelial cells. *Invest Ophthalmol Vis Sci, 48*(2), 614-620. doi: 10.1167/iovs.06-1126

McKay, T. B., Hjortdal, J., Priyadarsini, S., & Karamichos, D. (2017a). Acute hypoxia influences collagen and matrix metalloproteinase expression by human keratoconus cells in vitro. *PLoS One, 12*(4), e0176017. doi: 10.1371/journal.pone.0176017

McKay, T. B., Hjortdal, J., Sejersen, H., & Karamichos, D. (2017b). Differential Effects of Hormones on Cellular Metabolism in Keratoconus In Vitro. *Sci Rep, 7*, 42896. doi: 10.1038/srep42896

McKay, T. B., Hutcheon, A. E. K., Zieske, J. D., & Ciolino, J. B. (2020). Extracellular Vesicles Secreted by Corneal Epithelial Cells Promote Myofibroblast Differentiation. *Cells, 9*(5). doi: 10.3390/cells9051080

McKay, T. B., Karamichos, D., Hutcheon, A. E. K., Guo, X., & Zieske, J. D. (2019). Corneal Epithelial-Stromal Fibroblast Constructs to Study Cell-Cell Communication in Vitro. *Bioengineering (Basel), 6*(4). doi: 10.3390/bioengineering6040110

Petroll, W. M., & Lakshman, N. (2015). Fibroblastic Transformation of Corneal Keratocytes by Rac Inhibition is Modulated by Extracellular Matrix Structure and Stiffness. *J Funct Biomater, 6*(2), 222-240. doi: 10.3390/jfb6020222

Priyadarsini, S., Sarker-Nag, A., Rowsey, T. G., Ma, J. X., & Karamichos, D. (2016). Establishment of a 3D In Vitro Model to Accelerate the Development of Human Therapies against Corneal Diabetes. *PLoS One, 11*(12), e0168845. doi: 10.1371/journal.pone.0168845

Ramos, T. V., Mathew, A. J., Thompson, M. L., & Ehrhardt, R. O. (2014). Standardized cryopreservation of human primary cells. *Curr Protoc Cell Biol, 64*, A.3i.1-8. doi: 10.1002/0471143030.cba03is64

Ren, R., Hutcheon, A. E., Guo, X. Q., Saeidi, N., Melotti, S. A., Ruberti, J. W., Zieske, J. D., & Trinkaus-Randall, V. (2008). Human primary corneal fibroblasts synthesize and deposit proteoglycans in long-term 3-D cultures. *Dev Dyn, 237*(10), 2705-2715. doi: 10.1002/dvdy.21606

Sharif, R., Hjortdal, J., Sejersen, H., Frank, G., & Karamichos, D. (2017). Human in vitro Model Reveals the Effects of Collagen Cross-linking on Keratoconus Pathogenesis. *Sci Rep, 7*(1), 12517. doi: 10.1038/s41598-017-12598-8

Thery, C., Amigorena, S., Raposo, G., & Clayton, A. (2006). Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol, Chapter 3*, Unit 3.22. doi: 10.1002/0471143030.cb0322s30

Tovell, V. E., Massie, I., Kureshi, A. K., & Daniels, J. T. (2015). Functional limbal epithelial cells can be successfully isolated from organ culture rims following long-term storage. *Invest Ophthalmol Vis Sci, 56*(6), 3531-3540. doi: 10.1167/iovs.14-15429

Trinkaus-Randall, V., & Gipson, I. K. (1985). A technique for obtaining basal corneal epithelial cells. *Invest Ophthalmol Vis Sci, 26*(2), 233-237.

Wu, J., Du, Y., Mann, M. M., Funderburgh, J. L., & Wagner, W. R. (2014). Corneal stromal stem cells versus corneal fibroblasts in generating structurally appropriate corneal stromal tissue. *Exp Eye Res, 120*, 71-81. doi: 10.1016/j.exer.2014.01.005

Yam, G. H., Williams, G. P., Setiawan, M., Yusoff, N. Z., Lee, X. W., Htoon, H. M., Zhou, L., Fuest, M., & Mehta, J. S. (2017). Nerve regeneration by human corneal stromal keratocytes and stromal fibroblasts. *Sci Rep, 7*, 45396. doi: 10.1038/srep45396

Zareian, R., Susilo, M. E., Paten, J. A., McLean, J. P., Hollmann, J., Karamichos, D., Messer, C. S., Tambe, D. T., Saeidi, N., Zieske, J. D., & Ruberti, J. W. (2016). Human Corneal Fibroblast Pattern Evolution and Matrix Synthesis on Mechanically Biased Substrates. *Tissue Eng Part A, 22*(19-20), 1204-1217. doi: 10.1089/ten.TEA.2016.0164

Zieske, J. D., Mason, V. S., Wasson, M. E., Meunier, S. F., Nolte, C. J. M., Fukai, N., Olsen, B. R., & Parenteau, N. L. (1994). Basement Membrane Assembly and Differentiation of Cultured Corneal Cells: Importance of Culture Environment and Endothelial Cell Interaction. *Experimental Cell Research, 214*(2), 621-633. doi: https://doi.org/10.1006/excr.1994.1300