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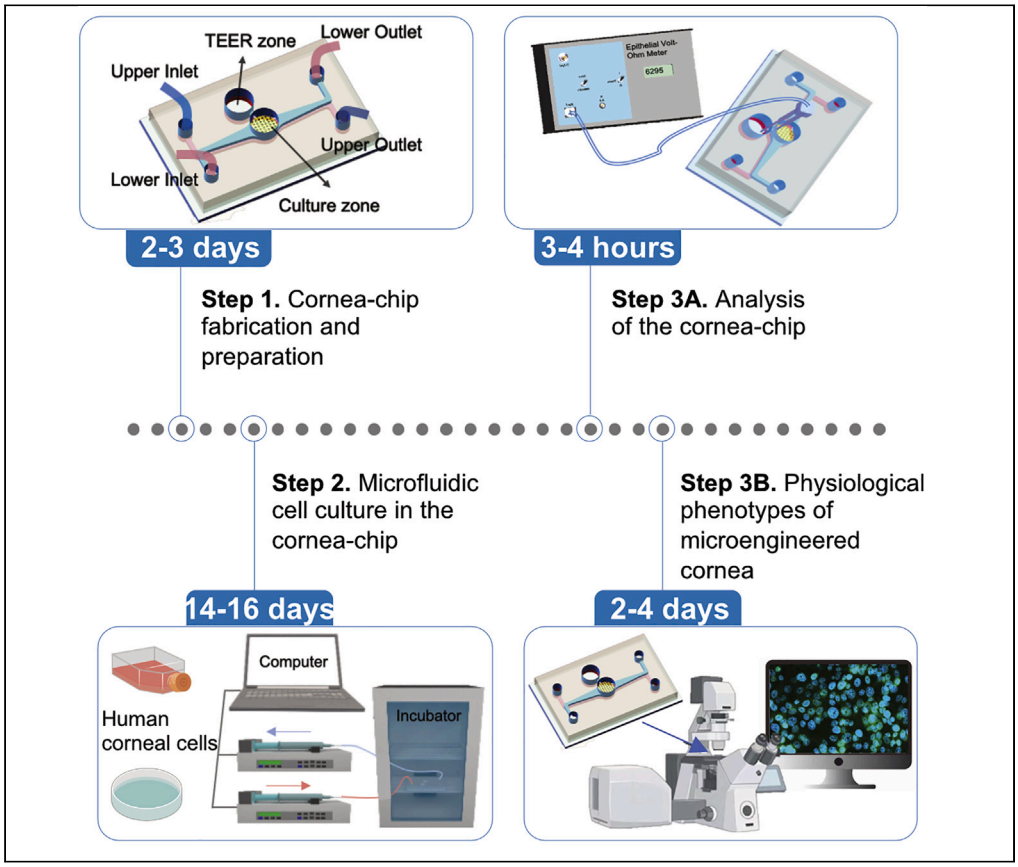
Protocol to develop a microfluidic human corneal barrier-on-a-chip to evaluate the corneal epithelial wound repair process



Organs-on-chips are microfluidic devices for cell culturing to simulate tissue- or organ-level physiology, providing new solutions other than traditional animal tests. Here, we describe a microfluidic platform consisting of human corneal cells and compartmentalizing channels to achieve fully integrated human cornea's barrier effects on the chip. We detail steps to verify the barrier effects and physiological phenotypes of microengineered human cornea. Then, we use the platform to evaluate the corneal epithelial wound repair process.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Combined human corneal cells and microfluidics to mimic ocular surface *in vitro*

Integrated human cornea's barrier effects on the microfluidic platform

Applying trans-epithelial electrical resistance techniques for co-culture analysis

Platform to study the interactions between epithelial and endothelial cells

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Protocol to develop a microfluidic human corneal barrier-on-a-chip to evaluate the corneal epithelial wound repair process

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SUMMARY

Organs-on-chips are microfluidic devices for cell culturing to simulate tissue- or organ-level physiology, providing new solutions other than traditional animal tests. Here, we describe a microfluidic platform consisting of human corneal cells and compartmentalizing channels to achieve fully integrated human cornea's barrier effects on the chip. We detail steps to verify the barrier effects and physiological phenotypes of microengineered human cornea. Then, we use the platform to evaluate the corneal epithelial wound repair process.

For complete details on the use and execution of this protocol, please refer to Yu et al. (2022).[1]

BEFORE YOU BEGIN

Background

In recent years, microphysiological systems have attracted much attention to establish standardized *in vitro* testing platforms for ophthalmology research. Organ-on-a-chip technology relies on our knowledge of human organs to engineer man-made constructs in which cells and their microenvironment are precisely controlled. The optical transparency of organ-on-a-chip device is another significant advantage over animal models because it enables direct real-time visualization and high-resolution quantitative analysis of diverse biological processes.[2] Besides, the design of compartmentalized channels for cocultivation in organ-on-chips allows independent fluidic access to different tissue types within a single device and assures parametric control of microenvironmental factors.[3] Microfluidic-based eye models that simulate eye events have been developed, such as corneal epithelium chips to evaluate eye drops, human blinking eye chips to study dry eye disease, and corneal barrier chips to emulate and evaluate an eye blinking shear force.[4–6] However, these models do not provide a complete human corneal structure, which is critical for recapitulating the micro-ocular physiological environment in corneal diseases and mass transport studies of ocular drugs. Cornea is a transparent avascular tissue that acts as a structural barrier and protects the eye against infections.[7] The barrier function is one of the most important functions of the cornea.[8] There are tight cellular barriers, corneal epithelium and endothelium, in the anterior and posterior parts of the eye that restrict the uptake of fluids and prevent penetration of foreign bodies.[9] Hence, we selected human corneal epithelial and endothelial cells to establish the cornea's barrier *in vitro*. Here, we design a microfluidic platform consisting of human corneal cells and a porous membrane to reproduce the physiological structure of the cornea (Figure 1). The protocol below details the use of

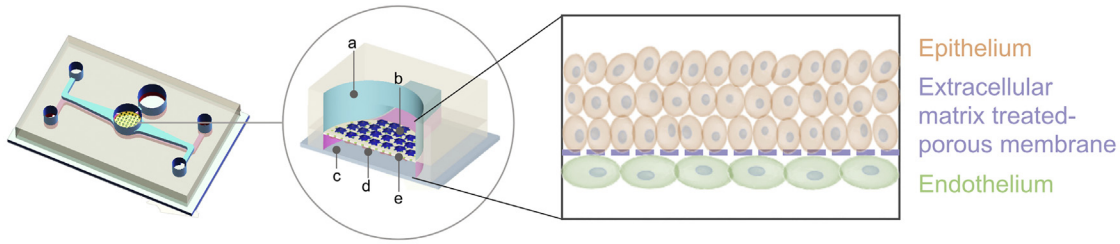

Figure 1. Schematic diagram of the human cornea-on-a-chip

The cornea-on-a-chip consists of an open top well with a top epithelial channel (a) comprised of human corneal epithelial (HCEpi) cells (b), as well as a bottom endothelial channel (c) comprised of human corneal endothelial (HCEnd) cells (d). The two channels are separated by an extracellular matrix treated-porous membrane (e).

human corneal epithelial cells and human corneal endothelial cells to create a corneal-barrier-on-a-chip and to perform the subsequent characterization.

Chip preparation and fabrication of SU-8 silicon master and its silanization

Timing: 1 day

This section describes the fabrication of SU-8 silicon master and its silanization. SU-8 is a commonly used epoxy-based negative photoresist, whereby the parts exposed to UV become cross-linked. At the same time, the unexposed film remains soluble and can be washed away during development. Silicon wafer is used as substrate to fabricate SU-8 master. Spin the SU-8 photoresist onto the wafer and pattern the photoresist to make the SU-8 silicon master. The SU-8 master is silanized to enhance its hydrophobicity, helping to demold polydimethylsiloxane (PDMS) parts, the latter are bonded to make the microfluidic devices.

1. Load a 4-inch silicon wafer vertically into a polytetrafluorethylene (PTFE) cleaning basket.
2. Get a 1,000 mL beaker which will fit the PTFE basket and the 4-inch wafer.

   Note: Put the basket in the empty beaker and add enough solution to cover the wafer. The solution will bubble, and you don't get it too close to the top. If the samples come within 10% of the top of the container, get a larger container.

3. Immerse the wafer-taking basket into the Piranha Clean (H_2SO_4:H_2O_2 = 3:1 ratio) for 30 min to remove all organics.

   Note: Process the Piranha Clean only in glass containers using PTFE or stainless-steel tools.

4. Transfer the sample carefully to a rinse beaker.
5. Let the wafer and tools soak in deionized (DI) water for 10 min.
6. Fill the second rinse beaker with DI water and rinse the wafer for another 5 min.
7. Dry the wafer by a nitrogen gun and dehydrate the wafer entirely by placing it on a hot plate maintained at 200°C for 10 min.
8. Remove the wafer from the hot plate and allow it to cool for 3 min.
9. Place the 4-inch silicon wafer on the chuck of a spin coater and apply vacuum. Ensure that vacuum is on and the wafer is centered.
10. Pour 1 mL of SU-8 2100 photoresist on the center of the wafer.
11. Wait for 2 min to allow the dispended SU-8 to spread over the wafer.
12. Start the spin coater and spin the wafer at 5 × *g* for 30 s.

13. Ramp up the spinning speed to 15 × *g* for 60 s to further hold the photoresist rotating and uniform due to the high viscosity of SU-8 2100. For the cornea-on-a-chip, increase the speed to 60 × *g* and hold for 40 s to achieve the desired thickness of SU-8 photoresist.
14. After spin-coating, slightly remove the photoresist accumulated at the wafer edges by dispensing SU-8 developer solution to the edges of the wafer.
15. Place the wafer on a hot plate at 65°C for 5 min.
16. Transfer the wafer to a hot plate at 95°C, and bake the wafer for 60 min.
17. Remove the wafer from the hot plate, and cool it at 22°C.
18. Load a photomask and the SU-8–coated wafer onto a mask aligner and bring them in a vacuum and hard contact.
19. Expose the wafer to UV light at 280 mJ/cm^2 in the EVG 610 Mask Aligner.
20. Place the exposed wafer on the hot plate at 65°C for 5 min, and then bake the wafer at 95°C for 5 min.
21. Place the wafer in SU-8 developer for 10 min.
22. Remove the wafer from the solution and spray it with an SU-8 developer to rinse off the undeveloped photoresist.
23. Spray and wash the wafer with isopropyl alcohol.
24. Use pressurized filtered nitrogen to dry the wafer.
25. Hard bake the wafer for 20 min at 150°C to increase the thermal, chemical and physical stability of developed resist structures for subsequent processes.
26. Place the wafer in a desiccator connected to a vacuum and place a glass coverslip adjacent to the wafer.
27. Put a small drop containing 35 μL of trimethylchlorosilane on the coverslip, and evacuate the chamber to induce the evaporation of silanizing agent.
28. Remove the wafer from the desiccator after 30 min silanization.

⚠ CRITICAL: To achieve the best results for fabrication of the SU-8 master, the user should obtain specific training in lithography and understand the basic procedures, or seek support from a specialist to perform the entire process. In addition, the photolithography process should be conducted in a clean room.

Culture cell lines

Timing: 30 min + 3–5 days

This section describes the preparation of two types of complete culture medium for human corneal cells and cell culture expansion.

Medium preparation

Timing: 30 min

29. Prepare appropriate cell culture media (detailed recipes are available in the materials and equipment section).
    a. Prepare DMEM/F12 Complete Medium contains DMEM/F12 medium with 6% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 10 ng/mL human epidermal growth factor (EGF).
    b. Prepare Prigrow I Complete Medium, Prigrow I medium supplemented with 10% FBS, 5 mg/L human insulin, 10 μg/mL human transferrin, 3 ng/mL sodium selenite, 10 nM hydrocortisone, 10 nM β-estradiol, 10 ng/mL human VEGF 165aa, 10 ng/mL human EGF, 10 ng/mL heparin sodium, 1% L-glutamine, and 1% penicillin-streptomycin.

Cell culture expansion

Timing: 3–5 days

30. Thaw human corneal epithelial cells in DMEM Complete Medium.
    a. Remove the cryovial containing the human corneal epithelial cells from liquid nitrogen storage and immediately place it into a 37°C water bath.
    b. Quickly thaw the cells by gently swirling the vial in the water bath within 1 min.
    c. Transfer the vial into a biosafety cabinet.
    d. Transfer the vial's content into a 15 mL centrifuge tube containing 10 mL of pre-warmed DMEM/F12 Complete Medium.
    e. Centrifuge the cell suspension at 200 × *g* for 5 min at 22°C.
    f. Discard the supernatant and resuspend the cell pellet in 5 mL DMEM/F12 Complete Medium.
    g. Transfer the contents into a T25 flask and incubate at a 37°C, 5% CO_2 incubator for 24 h.
    h. Aspirate out medium from the T25 flask and refresh with 5 mL pre-warmed DMEM/F12 Complete Medium the following day.
31. Thaw human corneal endothelial cells in Prigrow I Complete Medium.
    a. Quick thaw the cryovial containing the human corneal endothelial cells by placing it in a 37°C water bath for approximately 1 min.
    b. Transfer the vial's content into a 15 mL centrifuge tube containing 10 mL of pre-warmed Prigrow I Complete Medium.
    c. Centrifuge the tube at 200 × *g* for 5 min.
    d. Discard the supernatant and resuspend the cell pellet in 5 mL Prigrow I Complete Medium.
    e. Transfer the contents into a T25 flask and incubate at a 37°C, 5% CO_2 incubator.
    f. After 24 h, discard the medium from the T25 flask and refresh with 5 mL pre-warmed Prigrow I Complete Medium.
32. Maintain cultures and allow cells to grow until 90% confluency is reached.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-ZO1 tight junction protein rabbit polyclonal (1:500)	Abcam	ab221547
Anti-Cytokeratin 3/CK-3 mouse monoclonal (1:100)	Abcam	ab68260
FITC conjugated Goat Anti-Rabbit IgG (H+L) (1:200)	Servicebio	GB22303
Cy5 conjugated Goat Anti-Mouse IgG (H+L) (1:200)	Servicebio	GB27301
Chemicals, peptides, and recombinant proteins		
Polydimethylsiloxane (PDMS)	DOWCORNING	SYLGARDTM 184
SU-8	Microchem	2100
Photoresist developer	Microchem	Y020100
Trimethylchlorosilane	Sigma-Aldrich	89595
DMEM/F12	Gibco	C11330500BT
Prigrow I Medium	Applied Biological Materials	TM001
Certified fetal bovine serum (FBS)	BI	04-001-1A
Distilled water	WATSONS	N/A
Human EGF	Peprotech	AF-100-15-100
Penicillin-streptomycin	Gibco	15140-122
Recombinant human insulin	Applied Biological Materials	Z101065
Human transferrin	Sigma-Aldrich	T8158
Sodium selenite	Sigma-Aldrich	S5261
Hydrocortisone	Selleck Chemicals	S1696

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
β-Estradiol	Sigma-Aldrich	E2758
Recombinant human VEGF (165aa)	Applied Biological Materials	Z100895
Heparin sodium	Selleck Chemicals	S1346
L-glutamine	Applied Biological Materials	G275
0.25% Trypsin-EDTA	Gibco	25200-072
Phosphate-buffered saline (PBS)	Gibco	C10010500BT
Hank's Balanced Salt Solution (HBSS)	Servicebio	G4204
Ethanol	Lingfeng Chemical	N/A
Acetic acid	Lingfeng Chemical	N/A
DMSO	Sigma-Aldrich	D4540
Collagen I, Rat Tail	Corning	354249
Paraformaldehyde	Sangon	E672002-0100l
Hematoxylin	Servicebio	G1005-1
Eosin	Servicebio	G1005-2
4′,6′-Diamidino-2-phenylindole hydrochloride (DAPI)	Invitrogen	D1306
Fluorescein isothiocyanate–dextran (average MW 3,000–5,000)	Aladdin	F121152
Antibody diluent	Servicebio	G2025
Experimental models: Cell lines		
Immortalized human corneal epithelial cells	RIKEN Biosource Center	N/A
Immortalized human corneal endothelial cells	Applied Biological Materials	T5077
Software and algorithms		
GraphPad Prism	GraphPad Software Inc.	Prism 8
Other		
0.5–10 μL clear tips	Kirgen	KG1031-L
1–200 μL clear tips	Kirgen	KG1232
100–1,000 μL clear tips	Kirgen	KG1333
5 mL serological Pipets	Corning	4487
10 mL serological Pipets	Corning	4488
0.22 μm syringe filter	Sartorius	16541-k
50 mL syringes	Hongda Medical	N/A
1.5 mL tubes	Axygen	MCT-150-C-S
15 mL tubes	Nest	601001
50 mL tubes	Nest	602001
100 mm plates	Corning	430167
T25 flasks	Corning	430649
T75 flasks	Corning	430641
6 well culture plates	Corning	3516
24 well culture plates	Corning	3526
Whatman porous membrane	Sigma-Aldrich	Z674087
Glass slide	CITOTESE	10127105A
Coverslip	CITOTESE	10212432C
Magic tape	3M Scotch	810
Histochemical pen	Gene Technology	GT1000
Countess cell counting chamber slides	Invitrogen	C10228
Electric heating thermostatic sink	Shanghai Jinghong	DK-320
4-inch silicon wafer	Suzhou RDMICRO	N/A
Spin coater	Laurell	WS-650MZ-23NPPB
Hot plate	CHEMAT	KW-4AH
Photolithography/mask aligner	EV Group	EVG 610
Oxygen plasma	WEIKE	PDC- MG
Automated cell counter	Thermo Scientific	Countess 3
CO_2 incubator	Thermo Scientific	HERAcell 150i
Biological safety cabinets	Thermo Scientific	1380

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Benchtop centrifuge	Hunan Xiangyi	L500-A
Syringe pump	CETONI	neMESYS290N
Millicell-electrical resistance system	Millipore	MERS00002
Inverted routine microscope	Carl Zeiss	Primovert
Inverted fluorescence microscope	Carl Zeiss	Axio Observer 7
Microtomes	Leica Biosystems	RM2016
Hybrid multi-mode reader	BioTek	Synergy H1

MATERIALS AND EQUIPMENT

DMEM/F12 Complete Medium		
Reagent	Final concentration	Amount
DMEM/F12	N/A	92.9 mL
FBS	6%	6 mL
Penicillin-streptomycin	1%	1 mL
Human EGF	10 ng/mL	100 μL
Total	**N/A**	**100 mL**

Note: Filter with a 0.22 μm syringe filter. DMEM/F12 Complete Medium can be stored at 4°C for 1 week.

Prigrow I Complete Medium		
Reagent	Final concentration	Amount
Prigrow I	N/A	86.6 mL
FBS	10%	10 mL
Penicillin-streptomycin	1%	1 mL
Human EGF	10 ng/mL	100 μL
Human Insulin	5 mg/L	500 μL
Human Transferrin	10 μg/mL	200 μL
Sodium Selenite	3 ng/mL	30 μL
Hydrocortisone	10 nM	100 μL
β-estradiol	10 nM	272.38 μL
Human VEGF 165aa	10 ng/mL	100 μL
Heparin Sodium	10 ng/mL	100 μL
L-glutamine	1%	1 mL
Total	**N/A**	**100 mL**

Note: Filter with a 0.22 μm syringe filter. Prigrow I Complete Medium can be stored at 4°C for 1 week.

Human EGF

Centrifuge vial prior to opening. Reconstitute 100 μg recombinant human EGF (hEGF) in 1 mL distilled water at a concentration of 100 μg/mL. Dilute 100 μg/mL hEGF in 5% trehalose to prepare a final concentration of 10 μg/mL for use.

Note: Do not vortex. Store working aliquots at −20°C or −80°C for extended storage. Avoid repeated freeze-thaw cycles.

Human insulin
Quickly spin the vial. Reconstitute 10 mg recombinant human insulin in 10 mL distilled water at a concentration of 1 mg/mL. Add 20 μL acetic acid to increase solubility.

Note: Prepare twenty 500 μL aliquots. Aliquots can be stored at −20°C for six months.

Human transferrin
Centrifuge vial prior to opening. Dissolve 10 mg of human transferrin in 2 mL distilled water to prepare a final concentration of 5 mg/mL for use.

Note: Prepare ten 200 μL aliquots. Aliquots can be stored at −20°C for three months.

Sodium selenite
Centrifuge vial prior to opening. Reconstitute 1 mg sodium selenite in 1 mL distilled water at a concentration of 1 mg/mL. Dilute 1 mg/mL sodium selenite in distilled water to prepare a final concentration of 100 ng/mL for use.

Note: Working aliquots can be stored at −20°C for six months.

Hydrocortisone
Dilute 10 mM hydrocortisone in distilled water to prepare a final concentration of 10 μM for use.

Note: Aliquots can be stored at −20°C for six months.

β-estradiol
Centrifuge vial prior to opening. Reconstitute 1 mg β-estradiol in 1 mL ethanol at a concentration of 1 mg/mL. Dilute 1 mg/mL β-estradiol in distilled water to prepare a final concentration of 1 μg/mL for use.

Note: Working aliquots can be stored at −20°C for six months.

Human VEGF 165aa
Centrifuge vial prior to opening. Reconstitute 10 μg recombinant human VEGF 165aa in 1 mL distilled water to prepare a final concentration of 10 μg/mL for use.

Note: Store working aliquots at −20°C for six months. Avoid repeated freeze-thaw cycles.

Heparin sodium
Centrifuge vial prior to opening. Dissolve 10 μg of heparin sodium in 1 mL distilled water to prepare a final concentration of 10 μg/mL for use.

Note: Store working aliquots at −20°C for six months. Avoid repeated freeze-thaw cycles.

Extracellular matrix (ECM)
Dilute collagen solution to 10 μg/cm^2 with a sterile diluting solution:

- Convert desired coating density (collagen/cm^2) to Collagen concentration (collagen/mL):

$$\frac{0.2826\ \text{cm}^2 \times 10\ \mu\text{g/cm}^2}{20\ \mu\text{L}} = 0.1413\ \mu\text{g}\Big/\mu\text{L}$$

- Calculate total collagen needed to make 2 mL of working solution:

$$2\ mL \times 0.1413\ mg/mL = 0.2826\ mg$$

- Calculate volume of stock solution needed:

$$\frac{0.2826\ mg}{3.17\ mg/mL} = 0.09\ mL$$

- Add 1.91 mL of diluting solution for a total of 2 mL.

⚠ CRITICAL: Filter the collagen solution using 0.22 μm syringe filters and store at 4°C for one month.

Note: Prepare these ECM solutions immediately before use, and keep collagen solution at 4°C or on ice. Due to the viscosity of most Collagen, we recommend making a diluted stock solution first and then using this solution to make a working solution at the final desired concentration. If the stock is made at a high concentration, such as 3.17 mg/mL (our lab), it can be stored at −20°C for long-term storage or 4°C for short-term storage. This protocol refers to Corning Collagen Coating Transwell® Inserts from Corning.

Fluorescently labeled-dextran (FITC-dextran)

Dissolve 5 mg FITC-dextran (average MW 3,000–5,000) in 5 mL distilled water at a concentration of 1 mg/mL. Dilute 1 mg/mL FITC-dextran in culture medium to prepare a final concentration of 100 μg/mL for use.

Note: Aliquots can be stored at −20°C for one month.

STEP-BY-STEP METHOD DETAILS

The protocol below describes (1) fabrication of the cornea-on-a-chip, (2) human corneal epithelial (HCEpi) cells and human corneal endothelial (HCEnd) cells seeding to create a corneal-barrier-on-chip, (3) measurement of corneal permeability (trans-epithelial electrical resistance and apparent permeability index), (4) hematoxylin and eosin (H&E) staining of the stratified corneal epithelium, and (5) immunofluorescent staining of cytokeratin-3 and zona occludens-1 (ZO-1) tight junction protein expression (Figure 2).

Fabrication of cornea-chip

Fabrication of the SU-8 silicon master and its silanization was described in the before you begin section (Figure 3A). Thereafter, this section describes (1) preparation of PDMS, (2) creation of the upper and lower PDMS substrate, and (3) alignment and assembly of the cornea-chip.

Manual mixing and degassing of PDMS

Timing: 1 h

1. Prepare a degassed mixture of PDMS silicone elastomer base and curing agent for replica molding.
2. Place an empty disposable plastic cup on a scale with appropriate precision and pour silicone elastomer base and curing agent in a 10:1 (base: curing agent) weight ratio.
3. Mix silicone elastomer base and curing agent vigorously by hand using a disposable plastic fork for 5 min until PDMS appears white.
4. Place the cup containing the PDMS mixture in a vacuum desiccator for 30 min.
5. Remove the cup from the chamber to complete degassing.

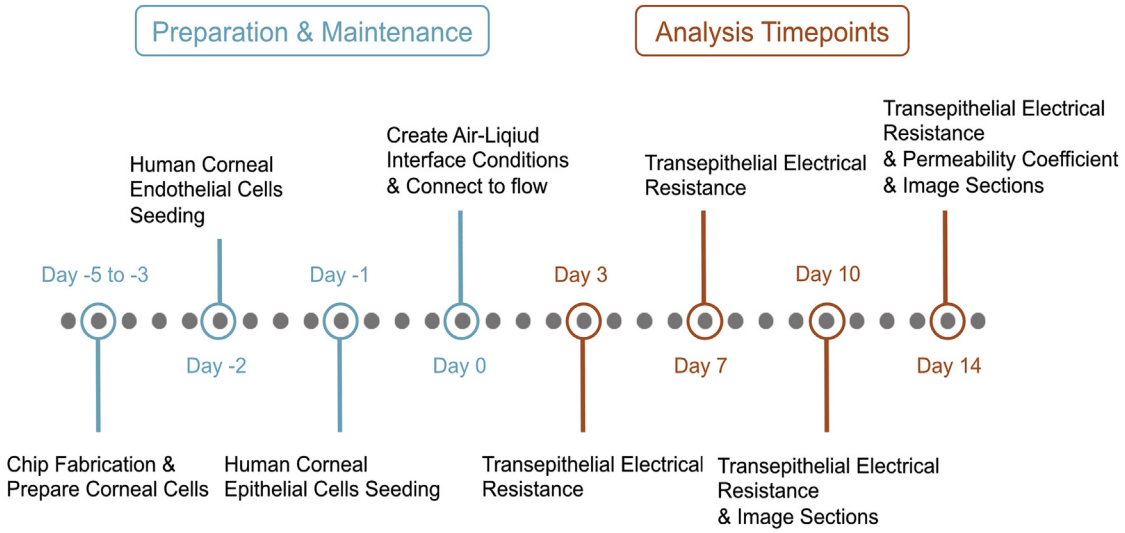

Figure 2. Experimental timeline

Detailed overview of key steps on critical days of chip preparation, cells seeding, analysis of barrier function and sections imaging.

Creation of the upper and lower PDMS substrate

Timing: 6 h

6. Attach a master silicon wafer with desired microfabricated channel features to the bottom surface of a Petri dish (100 mm) using double-sided tape.
7. Use compressed filtered air to gently blow off dust particles from the silicon master.
8. Pour 20 g of degassed 10:1 (wt/wt, prepolymer: curing agent) PDMS mixture onto the upper layer master. This produces a 2-mm thick PDMS slab with the features of the upper microchannels.
9. Pour 10 g of degassed 10:1 PDMS mixture onto the cleaned master of the lower layer. This produces a 1-mm thick PDMS slab with the lower microchannels.
10. Place the Petri dish with PDMS in a vacuum chamber to degas for 10 min and then on a level surface for 1 h to ensure uniform coverage of the master surface with PDMS.
11. Cover the Petri dish with a lid and put it in an oven at 80°C for 4 h to fully cure PDMS (Figure 3B).
12. Remove the dish from the oven and allow it to cool to 22°C.
13. Cut the fully cured PDMS along the edge of the silicon wafer using a scalpel and carefully peel it off.
14. Make holes through the PDMS slab using hole punchers. Use a 1-mm puncher to make holes through the PDMS slabs, providing access to the central upper and lower cell culture microchannels, respectively. Use a 6-mm puncher to create the "Culture zone" and the "TEER zone", respectively. Punch these holes from the micropatterned surface of the PDMS slab to ensure precise positioning of the holes relative to the microchannels.
15. Put magic tape on both sides of the PDMS block and then peel the tape off to clean the surfaces.
16. Wrap the final PDMS slab in magic tape to keep it clean until use.

Alignment and assembly of the cornea-on-a-chip

Timing: 1 h

Pause point: These devices can be stored at 22°C for up to one month before bonding to glass coverslip.

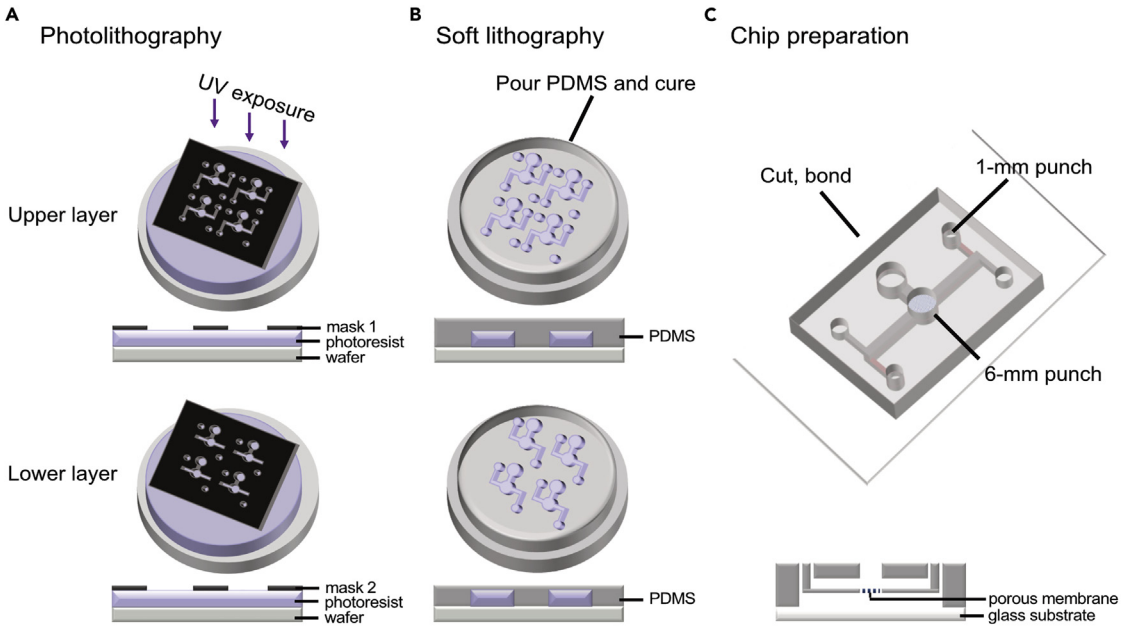

Figure 3. Overview of the process for microfabrication of the cornea-chip

(A) SU8 photoresist is spin-coated on the silicon wafers, patterned by UV exposure, and developed to generate SU8 masters.

(B) Polydimethylsiloxane (PDMS) is cured on the SU8 masters to make microfluidic structures.

(C) The PDMS slabs are peeled off and punched to make access holes and open wells. The lower PDMS slab and the glass substrate bond by oxygen plasma treatment. A porous polycarbonate membrane seals the open well in the lower PDMS slab. The upper PDMS slab is bonded to the lower layer to create the cornea-chip.

17. Peel the magic tape from the lower microchannel slab.
18. Treat the lower PDMS surface and a coverslip with plasma for 1 min.
19. Place the lower microchannel slab attached to a coverslip.
20. Treat the porous membrane surface with plasma for 1 min.
21. Place the porous membrane surface attached to the lower microchannel slab.
22. Remove the magic tape from the upper microchannel slab and expose the channel side to plasma for 1 min.
23. Overlay the upper microchannel slab on the membrane (Figure 3C).
24. When alignment is complete, press down the upper PDMS slab to eliminate trapped air pockets and to ensure firm contact between layers.
25. Insert the blunt needles into the four ports of the side microchambers.
26. Cut four pieces of silicone tubing to a length of 8 cm and connect them to the free ends of the needles.
27. Mix 5 min epoxy on a clean surface and apply it around each needle.
28. Wait for 30 min to allow the applied epoxy to set.

Preparation of the cornea-on-a-chip

Timing: 3.5 h

This section describes the preparation of the chip before cell seeding, including (1) chip sterilization, (2) channel hydrophilic treatment, and (3) extracellular matrix (ECM) pre-treated chip (preparation details are available in the materials and equipment section).

29. Transfer the chip into a biosafety cabinet and irradiate with UV light for 30 min for sterilization.

30. Add 100 μL of phosphate-buffered saline (PBS) to the chip's upper and lower inlets, add 50 μL of PBS to the middle chamber of the chip, and perform dynamic processing on a shaker for 5 min, with a rocking angle of at least 30 degrees.
31. Dilute collagen solution to 10 $\mu g/cm^2$ with a sterile diluting solution.
32. Add the 40 μL of diluted collagen solution to the upper and lower layers of the chip, respectively, and 20 μL of diluted collagen solution to the middle chamber.
33. Allow the chip to coat in a 37°C, 5% CO_2 incubator for 2 h.
34. Transfer the chip into a biosafety cabinet, aspirate any remaining collagen solution from the chip and air-dry for 30 min.
35. Rinse once with Complete Medium, and the chips are now ready for use or store at 4°C for later use.

Note: Pre-coated chips can be prepared and stored for one week at 37°C with 100 μL of PBS. Make sure to seal the chips with parafilm to avoid collagen from drying out.

⚠ CRITICAL: The collagen formation is critical for the growth of cells.

Cell seeding and 3D co-culture

Timing: 16 days

This section describes the seeding of HCEpi and HCEnd cells onto the chip to create a corneal-barrier-on-chip model. Considering the physiological characteristics of the human cornea and the feasibility of *in vitro* cell culture, the seeding density of corneal cells is 6×10^4 HCEpi cells and 1×10^4 HCEnd cells per chip.

Day 1

Timing: 40 min

36. Discard the supernatant and wash the cells with PBS three times.
37. Add 1 mL of 0.25% EDTA-Trypsin into the flask of the HCEnd cells (at least 90% confluency).
38. Transfer the flask into an incubator at 37°C, 5% CO_2 for 2 min.
39. Add 3 mL of Prigrow I Complete Medium to stop digestion.
40. Transfer the flask's content into a 15 mL centrifuge tube.
41. Centrifuge the tube at 200 × *g* for 5 min.
42. Count the cells and adjust the concentration of the cell suspension to 5×10^5 cells/mL.
43. Before cells seeding, add 20 μL of Prigrow I Complete Medium to the middle chamber, and then add 20 μL of HCEnd cells suspension in the lower inlet channel.
44. Quickly check the cells using microscope and then transfer the inverted chip into a horizontal position in the incubator at 37°C, 5% CO_2 for 24 h.

⚠ CRITICAL: Once cells are seeded into the chip, do not move the chip until the next medium change (24 h later), to allow the growth of cells.

⚠ CRITICAL: The endothelial cells do not grow out as efficiently.

Day 2

Timing: 40 min

45. Discard the supernatant and wash the cells with PBS three times.
46. Add 1 mL of 0.25% EDTA-Trypsin into the flask of the HCEpi cells (at least 90% confluency).

47. Transfer the flask into an incubator at 37°C, 5% CO_2 for 2 min.
48. Add 3 mL of DMEM/F12 Complete Medium to stop digestion.
49. Transfer the flask's content into a 15 mL centrifuge tube.
50. Centrifuge the tube at 200 × *g* for 5 min.
51. Count the cells and adjust the concentration of the cell suspension to 3 × 10^6 cells/mL.
52. Aspirate the medium from the lower outlet and refresh with 100 μL pre-warmed Prigrow I Complete Medium in the lower channel.
53. Add 20 μL of HCEpi cells suspension in the upper hole.
54. Transfer the chip to the incubator at 37°C, 5% CO_2 for 24 h.

⚠ CRITICAL: Once cells are seeded into the chip, do not move the chip until the next medium change (24 h later), to allow the growth of cells.

55. After the HCEpi cells proliferation on the membrane, the culture medium is gently aspirated from the upper hole, and the air is then filled in the epithelial compartment to create an air-liquid interface.
56. The lower channels are introduced into the DMEM/F12 Complete Medium and Prigrow I Complete Medium mix (1:1) at a continuous flow condition with a volumetric flow rate of 100 μL/h by using a programmable low-pressure syringe pump.
57. Maintain above conditions for 14 days and allow the HCEpi cells to grow until to form a stratified epithelium consisting of five to seven stacked cell layers.

Physiological phenotypes of the microengineered cornea

Timing: 3–4 h

This section describes (1) measurement of transepithelial electrical resistance (TEER), (2) permeability coefficient (Papp) of the corneal-barrier-on-chip model, (3) staining of paraffin sections of stratified corneal epithelium, and (4) immunofluorescence staining of the corneal cells.

Measurement of transepithelial electrical resistance

Timing: 1–2 h

58. Sterilize the Millicell-electrical resistance system (Millicell-ERS) with 75% ethanol before placing it into the biosafety cabinet.
59. Connect the probe to the Millicell-ERS.
60. Sterilize the probe by submerged in 75% ethanol for 15 min.
61. Prepare the cornea-chip by removing the medium from the basal compartment.
62. Add 100 μL of PBS to the apical and basal compartments.
63. Prepare a blank sterilized microdevice without cells and inject PBS into the upper and lower culture channels.
64. Set the function switch to "Ohms".
65. Remove the probe from 75% ethanol and drip it off the electrode tips.
66. Wash the electrode tips in PBS.
67. Insert the long electrode tip into the "TEER zone".
68. Lower the probe until the long electrode tip touches the bottom of the well.
69. Keep the short electrode tip above the "Culture zone" surface.
70. Measure the resistance on the blank microdevice and record the value (Rblank).
71. Measure the resistance on the cornea-chip and record the value (Rtotal) (Table 1).
72. Remove the PBS from the apical and basal compartments.
73. Reconnect the microdevice to the low-pressure syringe pump and perfuse the lower channels with mixed culture medium at a volumetric flow rate of 100 μL/h.

Table 1. Example of raw transepithelial electrical resistance used to calculate TEER value

			Raw data		Calculation	
Time	Number	Condition	Rtotal (Ω)	Rblank (Ω)	TEER (Ω·cm^2)	Mean
Day 3	Control	Membrane		3924		
				3863		
				3884		
	Chip 1	HCEpi	4205		88.12	
			4283		109.96	
			4266		105.2	101.09
	Chip 2	HCEpi	4341		126.2	
			4407		144.68	
			4376		136	135.62
	Chip 3	HCEpi	4252		101.28	
			4273		107.16	
			4210		89.52	99.32
	Chip 4	HCEpi+HCEnd	4328		122.56	
			4254		101.84	
			4281		109.4	111.26
	Chip 5	HCEpi+HCEnd	4351		129	
			4383		137.96	
			4390		139.92	135.62
	Chip 6	HCEpi+HCEnd	4470		162.32	
			4428		150.56	
			4482		165.68	159.52
Day 7	Control	Membrane		3870		
				3852		
				3903		
	Chip 1	HCEpi	4563		192.64	
			4517		179.76	
			4553		189.84	187.41
	Chip 2	HCEpi	4734		240.52	
			4691		228.48	
			4724		237.72	235.57
	Chip 3	HCEpi	4634		212.52	
			4687		227.36	
			4655		218.4	219.43
	Chip 4	HCEpi+HCEnd	4706		232.68	
			4680		225.4	
			4673		223.44	227.17
	Chip 5	HCEpi+HCEnd	4845		271.6	
			4768		250.04	
			4859		275.52	265.72
	Chip 6	HCEpi+HCEnd	4975		308	
			4958		303.24	
			5004		316.12	309.12
Day 10	Control	Membrane		3924		
				3871		
				3903		
	Chip 1	HCEpi	6207		646.16	
			6179		6388.32	
			6158		632.44	638.97
	Chip 2	HCEpi	6405		701.6	
			6379		694.32	
			6429		708.32	701.41
	Chip 3	HCEpi	6273		664.64	

(*Continued on next page*)

Table 1. *Continued*

Time	Number	Condition	Raw data		Calculation	
			Rtotal (Ω)	Rblank (Ω)	TEER (Ω·cm^2)	Mean
			6281		666.88	
			6252		658.76	663.42
	Chip 4	HCEpi+HCEnd	6865		825.92	
			6786		830.4	
			6617		808.28	821.53
	Chip 5	HCEpi+HCEnd	6621		762.08	
			6614		760.12	
			6675		777.2	766.46
	Chip 6	HCEpi+HCEnd	6745		796.2	
			6804		813.32	
			6780		806.6	805.57
Day 14	Control	Membrane		3836		
				3906		
				3828		
	Chip 1	HCEpi	6155		643.64	
			6126		635.52	
			6103		629.08	636.08
	Chip 2	HCEpi	6079		622.36	
			6102		628.8	
			6085		624.04	625.06
	Chip 3	HCEpi	6315		688.44	
			6270		675.84	
			6268		675.28	679.85
	Chip 4	HCEpi+HCEnd	6617		773	
			6672		788.4	
			6636		778.32	779.9
	Chip 5	HCEpi+HCEnd	6835		834.04	
			6880		846.64	
			6812		827.6	836.09
	Chip 6	HCEpi+HCEnd	6752		810.8	
			6725		803.24	
			6801		824.52	812.85

74. Incubate at a 37°C, 5% CO_2 incubator.

Note: Keep the probe at a 90° angle. Allow time for the values to level off. Perform the TEER measurement procedure ideally within 5–10 min to ensure the optimal integrity of the cultures. If the device contains different media conditions, wash the probe with 75% ethanol and PBS between the readings. This protocol refers to the User Guide of Millicell® ERS-2 Electrical Resistance System.

75. The TEER of epithelium or cornea-chip calculates by: (Figure 4A and Table 1).

$$R_{tissue}\ (\Omega) = R_{total} - R_{blank}$$

$$TEER\ (\Omega \cdot cm^2) = R_{tissue}\ (\Omega) \times M_{area}\ (cm^2)$$

in this formula, R_{tissue} is tissue resistance, and M_{area} is the area of the "Culture zone".

Measurement of permeability coefficient

Timing: 2 h

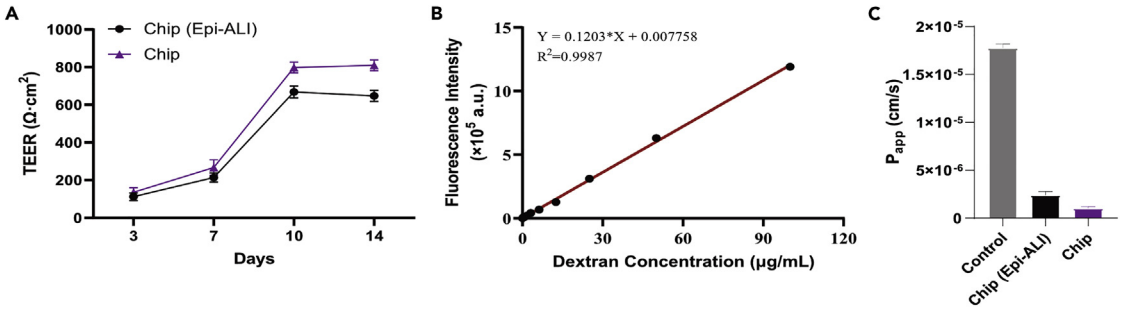

Figure 4. Analysis of the barrier function of microengineered cornea

(A) Measure trans-epithelial electrical resistance (TEER) values of the corneal epithelium at 3, 7, 10, and 14 days, respectively.

(B) The calibration curve on the relations between the concentration of FITC-Dextran (MV 3,000–5,000) and the fluorescent intensity. The equation expresses a linear correlation.

(C) Measure permeability coefficient (Papp) of the corneal epithelium by using 5 kDa FITC-Dextran. Control means the membrane without cells. "Chip (Epi-ALI)" represents HCEpi cells culture in the cornea-chip with an air-liquid interface (ALI) condition. "Chip" represents HCEpi cells and HCEnd cells co-cultivate in the cornea-chip with a suitable condition. (A and C) All data are mean ± SD from three independent experiments.

⚠ CRITICAL: When aspirating solution or collecting medium, ensure there are sufficient medium remaining over the pod vias to avoid introducing bubbles at this step.

76. Stop the syringe pump, and aspirate the medium from the outlet.
77. Incubate cells with hanks balanced salt solution (HBSS) to equilibrate for 20 min in the incubator.
78. Replace the solution with 100 μL of 100 μg/mL fluorescently labeled dextran (FITC-dextran).
79. Aspirate out the solution from the lower compartment and refresh with 100 μL HBSS.
80. 20 μL of samples collect from the basal side at time 0 and 60 min.
81. Prepare a calibration curve of serial dilution range of 0.39–100 μg/mL FITC-dextran. HBSS serves as the zero standard (0 μg/mL).
82. The fluorescence intensity measurement of FITC-dextran is performed by a hybrid multi-mode microplate reader (excitation/emission wavelengths: 488/525 nm).
83. Plot a graph with the fluorescence intensity measurements as the dependent variable (Y-axis) and the known concentration of FITC-dextran as the independent variable (X-axis), resulting in an equation formatted as follows: $Y = 0.1203X + 0.0078$ ($R^2 = 0.9987$), where solving for X determines the FITC-dextran concentration of the sample. (Figure 4B).
84. Prepare a blank device as a control, whose data is subtracted from all readings before data analysis.
85. The permeability coefficient (Papp) of epithelium or cornea-chip calculates by: (Figure 4C).

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0}$$

where, dQ/dt is the slope of the cumulative fraction absorbed versus time (in seconds), A is the area of the culture zone (cm^2), and C_0 is the initial concentration in the apical chamber (μg/mL).

Hematoxylin-eosin staining of the paraffin sections of corneal epithelium

Timing: 2 days

86. The stratified corneal epithelium on the membrane is carefully removed from the PDMS device for further processing.
87. Add 1 mL 4% paraformaldehyde (PFA) to the membrane to fix for 30 min.
88. Rinse with PBS three times gently and incubate in PBS for 12 h at 4°C.

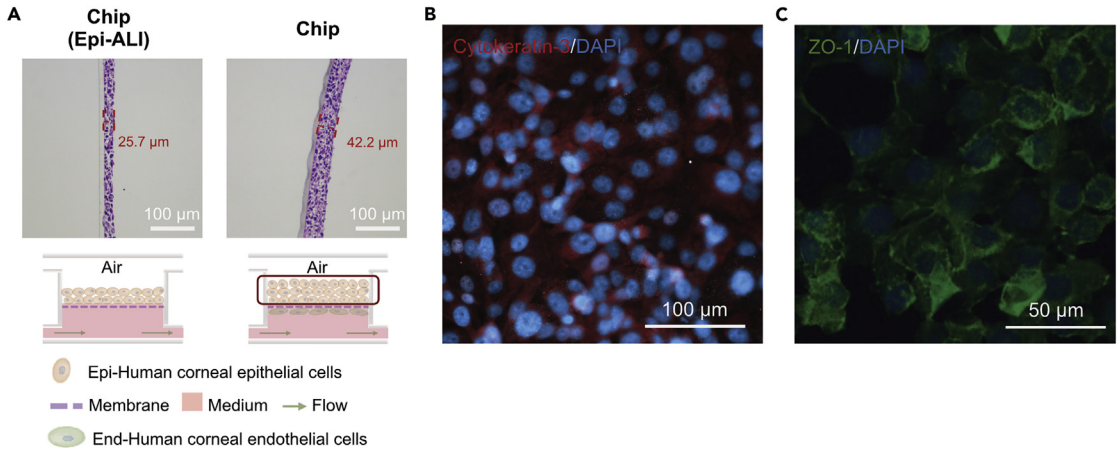

Figure 5. Characterization of human corneal epithelium culture on-chip

(A) H&E images on the stratified corneal epithelium in different conditions after 14 days. "Chip (Epi-ALI)" represents HCEpi cells cultivate in the cornea-chip with an air-liquid interface (ALI) condition. "Chip" represents HCEpi cells and HCEnd cells co-cultivate in the cornea-chip with a suitable condition. Red dotted lines and numbers indicate the thickness of the stratified corneal epithelium.

(B) Immunofluorescence images of cytokeratin-3 expression in the corneal epithelial cells.

(C) Immunohistochemical analysis of tight-junction protein expression shows the lattice-like structure of the epithelial typical tight junction between cells.

89. For tissue dehydration, use 70% ethanol—80% ethanol—95% ethanol—100% ethanol each for 30 min, then in 100% ethanol for another 45 min twice, to gradually remove water from the tissue.
90. The dehydrated film is incubated with xylene for 30 min to be transparent.
91. Transfer to a mixture of molten paraffin wax for incubation at 56°C for 30 min and then in 100% molten wax for another 60 min twice.
92. After embedding, the membrane is sectioned into slices of 3 μm in thickness.
93. The paraffin-embedded sections are deparaffinized first in a clearing agent and rehydrated from high-to-low-concentration ethanol.
94. Rinse with distilled water for 1 h.
95. Place the slides with the section in a metal staining rack.
96. Immerse sections in the hematoxylin for 1 min.
97. Remove the rack to a beaker with tap water.
98. Exchange tap water until the water is clear.
99. Immerse sections in the eosin for 40 s.
100. Remove rack to a beaker with tap water.
101. Exchange tap water until the water is clear.
102. The slides are rinsed with 95% ethanol twice for 1 min each, 100% ethanol for 1 min, and the clearing agent for 1 min.
103. Mount coverslip onto the section on the glass slide.
104. The slides are photographed by a Zeiss Axio Observer microscope (Figure 5A).

⚠ CRITICAL: Keep same intensity settings between all conditions and for different slides.

Immunofluorescence staining of specific corneal epithelium protein and intercellular junctions

Timing: 2 days

105. After 14 days, the membrane is carefully removed from the PDMS device.

106. Incubate the membrane in 4% PFA for 30 min at 22°C.
107. Rinse with ice-cold PBS three times gently.
108. Place the membrane on glass slides.
109. Draw a circle around the membrane with a histochemical pen to prevent the solution flow away.
110. Add 0.25% Triton X-100 for 10 min at 22°C.
111. Wash cells in PBS three times for 5 min.
112. Block by 1% BSA for 1 h.
113. Dilute the primary cytokeratin 3 antibody in antibody diluent at 1/100 dilution for use.
114. Dilute the secondary Cy5-conjugated goat anti-mouse IgG antibody in antibody diluent at 1/200 dilution for use.
115. Dilute the primary ZO1 antibody in antibody diluent at 1/500 dilution for use.
116. Dilute the secondary FITC conjugated goat anti-rabbit IgG antibody in antibody diluent at 1/200 dilution for use.
117. Incubate cells in the diluted primary cytokeratin 3 antibody in a humidified chamber 12 h at 4°C.
118. Decant the solution and wash the cells three times in PBS, 5 min each.
119. Incubate cells with the diluted secondary Cy5-conjugated goat anti-mouse IgG antibody for 1 h at 22°C in the dark.
120. Decant the secondary antibody solution and wash the cells three times with PBS for 5 min each in the dark.
121. Incubate cells in the diluted primary ZO1 antibody in a humidified chamber for 12 h at 4°C.
122. Decant the solution and wash the cells three times in PBS, 5 min each.
123. Incubate cells with the diluted secondary FITC conjugated goat anti-rabbit IgG antibody for 1 h at 22°C in the dark.
124. Decant the secondary antibody solution and wash the cells three times with PBS for 5 min each in the dark.
125. Incubate cells with 2 mg/mL 4′, 6′-diamidino-2-phenylindole hydrochloride (DAPI) for 10 min at 22°C in the dark to stain the nucleus.
126. Add coverslip with fluorescent mounting medium for 5 min in the dark.
127. Photo the cells with a Zeiss Axio Observer fluorescent microscope (Figures 5B, 5C, and 6).

⚠ CRITICAL: Keep laser intensity settings equal between all conditions and for different timepoints.

EXPECTED OUTCOMES

The cornea is a transparent avascular tissue that acts as a structural barrier and protects the eye against infections.[7] Tight cellular barriers, corneal epithelium and endothelium in the anterior and posterior parts of the eye restrict the uptake of fluids and prevent penetration of foreign bodies.[9] In the cornea, type I collagen, the major component of the corneal stroma, occupies the extracellular space and provides structural support to the keratocytes.[10] Because ECM has been an essential contributor to regulate cell functions *in vivo*, an ECM-coated membrane was used in our chip to promote cell attachment and differentiation.[1,11] Therefore, HCEpi cells and HCEnd cells are seeded on the upper and lower side of the membrane, respectively, and cultured in nutrients that are injected into different microchannels (Figure 1). The open "Culture zone" provides the necessary ALI condition for differentiating corneal epithelial cells into multilayer cells and forming a natural barrier function, thereby simulating the physiological environment of the ocular surface more precisely.

The barrier function is one of the most important functions of the cornea.[8] Trans-epithelial electrical resistance (TEER), an acknowledged method for evaluating corneal barrier function *in vitro*, can be measured *in situ* through the "TEER zone" to evaluate barrier integrity for 3, 7, 10, and 14 days (Figure 4A and Table 1). When the value exceeds 650 $\Omega \cdot cm^2$, the corneal chip establishes successfully.

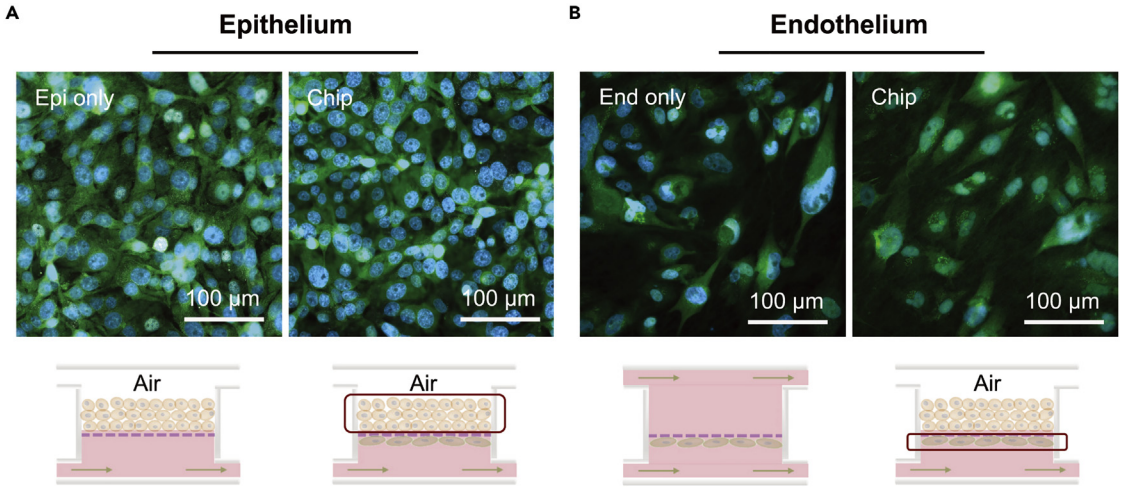

Figure 6. Construction of physiological phenotypes of the human cornea

Immunofluorescent characterization of human corneal cells culture in different conditions after 14 days.

(A and B) "Epi only" or "End only" represents a single type of cells culture in the chip. "Chip" means the coculture of the HCEpi cells and HCEnd cells in the chip. Green: ZO-1; Blue: DAPI.

Then, physiological phenotypes of the microengineered cornea are further characterized by paraffin sections and immunofluorescence staining of specific proteins.

QUANTIFICATION AND STATISTICAL ANALYSIS

The TEER value of HCEpi cells and HCEnd cells in the top epithelial and bottom endothelial channels are quantified using a Millicell-ERS electrical resistance system. The R_{blank} value is the resistance of the blank microdevice without cells. The R_{total} value is the resistance of the cornea-chip. The R_{tissue} value is the R_{total} value minus the R_{blank} value, representing tissue resistance. The TEER ($\Omega \cdot cm^2$) of epithelium or cornea-chip calculated by R_{tissue} (Ω) multiply M_{area} (cm^2) (the area of the "Culture zone"). Experiments should be performed with three chips per condition and replicated three times.

LIMITATIONS

One limitation is that the cornea-chip relies on a programmable syringe pump to recapitulate the microenvironment *in vivo*, which depends on an external device during the coculture process and limits the system flexibility. In the future, we hope to use the culture module containing reservoirs to sustain the life of cells within our chip, automating the precise conditions needed to culture more chips.

Furthermore, due to the limited "TEER zone", the TEER values of the cells in the middle of the circle can be precisely measured but cannot detect the edge cells. For special applications, you may consider customizing the mini-size electrode tips.

Finally, we hope the tools will become available to allow for live, dynamic imaging of the chips while still subject to fluid flow and cyclic stretching.

TROUBLESHOOTING

Problem 1

Failure to perfectly align the walls of the microchannels in the upper and lower PDMS slabs (steps 19–23).

Potential solution

Lift off the upper PDMS slab slowly and repeat alignment until the complete alignment of the microchannel walls is achieved. Take great care when peeling off the slab, as this may cause the PDMS membrane to tear.

Problem 2

Introduce air bubbles into the channel when injection of the cell suspension solution (step 43).

Potential solution

Ensure that the needle attached to the syringe tightly fits into the inlet and outlet tubing. Before injection of the cell suspension solution, gently rinse the channel with the medium while aspirating the droplet that forms near the outlet. Then, injection of the cell suspension solution should be performed very slowly and carefully to prevent the introduction of air bubbles. If the bubbles persist, gently flush the channels with a fresh culture medium to remove the bubbles, and re-seed cells.

Problem 3

Human corneal epithelial cells are not stratified into corneal epithelium (steps 51–57).

Potential solution

To overcome the problem that the corneal epithelial cells are not stratified into corneal epithelium, first to confirm that the seeded cells adhere well and have good viability. It is recommended to extend the time of the submerged culture of the corneal epithelial cells in the chip to 48 h to ensure a tight and even distribution of the first layer of corneal epithelial cells. Then, provide enough air and nutrient-rich medium to promote the differentiation of corneal epithelial cells into multi-layered cells.

Problem 4

Bubbles persist within channel prior to pump connection (step 56).

Potential solution

Using a P200 pipette, push a small volume of PBS into the channel, forcing air bubbles out from the other side. If the air bubbles persist, depress the plunger of the P200 and insert the pipette tip into the channel containing the air bubbles. Gently pull up on the plunger to pull the PBS back into the channel until it is just behind the bubble. Then gently push the PBS back into the channel to squeeze the air bubbles out of the channel.

Problem 5

Difficult to distinguish the white polycarbonate porous membrane from the color of the reagent when making paraffin sections or frozen sections (steps 91–92).

Potential solution

Try to embed the membrane in the center position. When cut off the membrane, the adhesion between the reagent and the membrane is not good, and there will be partial separation. Then, according to the slice thickness, predict the center position of the membrane, and focus on retaining 5–10 slices before and after the center position.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Hui Yang (hui.yang@siat.ac.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All needed data to evaluate the conclusions are present in the paper. This paper does not report the original code. Any additional information required in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

H.Y. and Y.Z. conceived the study. H.Y. supervised the project. Z.Y. and H.Y. designed the experiments. Z.Y., R.H., and X.C. performed the experiments. Z.Y., R.H., X.C., L.M., and H.Y. wrote the manuscript. H.Y. edited the manuscripts.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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