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Assembly and Application of a Three-Dimensional Human Corneal Tissue Model

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The cornea provides a functional barrier separating the outside environment from the intraocular environment, thereby protecting posterior segments of the eye from infection and damage. Pathological changes that compromise the structure or integrity of the cornea may occur as a result of injury or disease and can lead to debilitating effects on visual acuity. Over 10 million people worldwide are visually impaired or blind due to corneal opacity. Thus, physiologically relevant in vitro approaches to predict corneal toxicity of chemicals or effective treatments for disease prior to ocular exposure, as well as to study the corneal effects of systemic, chronic conditions, such as diabetes, are needed to reduce use of animal testing and accelerate therapeutic development. We have previously bioengineered an innervated corneal tissue model using silk protein scaffolds to recapitulate the structural and mechanical elements of the anterior cornea and to model the functional aspects of corneal sensation with the inclusion of epithelial, stromal, and neural components. The purpose of this unit is to provide a step-by-step guide for preparation, assembly, and application of this three-dimensional corneal tissue system to enable the study of corneal tissue biology. © 2019 by John Wiley & Sons, Inc.

Keywords: 3D in vitro model • cornea • sensory nerves • silk biomaterials • tissue engineering • toxicology

How to cite this article:
McKay, T. B., Ford, A., Wang, S., Cairns, D. M., Parker, R. N., Deardorff, P. M., Ghezzi, C. E., & Kaplan, D. L. (2019). Assembly and application of a three-dimensional human corneal tissue model. *Current Protocols in Toxicology*, *81,* e84. doi: 10.1002/cptx.84

INTRODUCTION

Due to the highly innervated nature of the cornea and the presence of multiple cell types, the development and application of relevant in vitro tissue models have been limited in scope and complexity. To address this need in the field, we have bioengineered an advanced, innervated human corneal tissue model containing a stratified corneal structure with significant strength and rigidity, transparency, and sustainable culture conditions (Wang et al., 2017, 2018). This tissue model takes advantage of the highly versatile nature of silk protein as a biocompatible scaffold to construct a multi-layered, pre-assembled tissue structure. The cellular components of the model consist of human corneal epithelial cells (hCECs) seeded onto a silk film mimicking Bowman's layer, an underlying stromal layer composed of human corneal stromal stem cells (hCSSCs) seeded on three silk films, and differentiated sensory nerves innervating the central cornea from the periphery

CURRENT PROTOCOLS
A Wiley Brand
Current Protocols in Toxicology e84, Volume 81
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cptx.84
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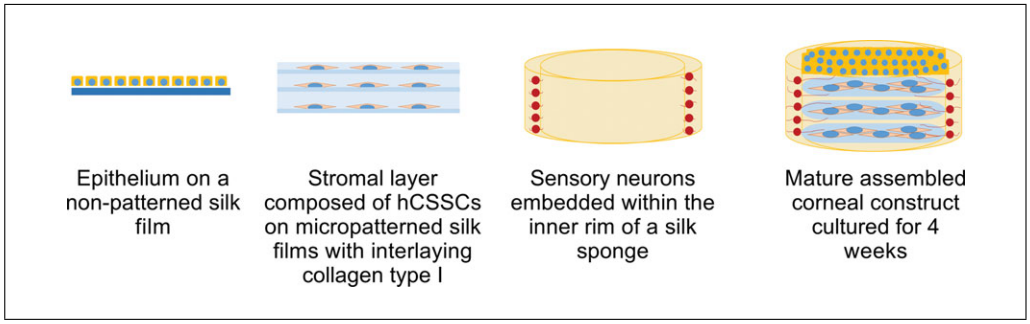

Figure 1 Schematic depicting assembly of the different layers that compose the corneal tissue model. Each scaffold component, i.e., the non-patterned silk film, micropatterned silk films, and silk sponge, is prepared from isolated silk fibroin and functionalized via a collagen, RGD-peptide, or PDL coating, respectively, prior to cell seeding. Maturation of the construct, characterized by stromal ECM deposition and innervation of the sensory nerves into the stromal layer, occurs over an ideal 4-week timeframe.

(Fig. 1). Addition of a collagen-type-I coating between the layers and on the surface serves to mediate cohesiveness of the construct. In our previous work, we have applied different sensory sources, including dorsal root ganglions (DRGs) (Wang et al., 2017; Wang, Ghezzi, White, & Kaplan, 2015) and human induced neural stem cells (hiNSCs) (Deardorff et al., 2018; Wang et al., 2018), to promote corneal sensory innervation in co-cultures of hCECs and hCSSCs. Utilization of hiNSCs has many advantages, including well-defined differentiation protocols that generate a gradual transition to differentiated nerves with elongated neuronal fibers (Cairns et al., 2016; Deardorff et al., 2018). Herein, we describe the application of hiNSCs in this system and detail culture conditions for isolation, subculture, and differentiation protocols.

The scaffold implemented in this corneal tissue model is protein based, using fibroin (silk), a naturally occurring biomaterial produced by the silkworm, *Bombyx mori*. Silk is biocompatible, non-immunogenic, and robust under long-term culture conditions, therefore serving as a useful scaffold to control cell alignment, provide a tangible surface for stacking into 3D structures, and form thin films that remain transparent (Abbott, Kimmerling, Cairns, & Kaplan, 2016). Functionalization of silk scaffolds with arginine-glycine-aspartate (RGD) peptide, a binding motif for extracellular matrix (ECM) domains, promotes cell attachment via a number of integrin classes, including integrins $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha v\beta 3$ (Ruoslahti, 1996); these integrins, among many others, are naturally expressed on the naïve keratocyte (Stepp, 2006). In our corneal tissue model, we utilize RGD-functionalized micropatterned films to establish a direct anchor to mediate keratocyte organization similar to the keratocyte network present in the adult cornea, thereby allowing for native ECM deposition between biomimetic silk lamellae (Ghezzi, Marelli, Omenetto, Funderburgh, & Kaplan, 2017). Micropatterned silk films guide keratocyte and fibroblast alignment along microgrooves in conventional 2D cultures (Gil et al., 2010; Gil, Park, Marchant, Omenetto, & Kaplan, 2010; Lawrence, Marchant, Pindrus, Omenetto, & Kaplan, 2009; Wu et al., 2014) and in 3D microenvironments (Chen, Zhang, Kelk, Backman, & Danielson, 2017; Ghezzi et al., 2017), thus serving as a useful means to model keratocyte-ECM interactions in vitro.

Tissue-engineered models aim to reconstruct the in vivo condition to study biological mechanisms in a defined yet physiological microenvironment. This approach allows one to dissect the contribution of specific cell types to physiological and disease processes, which may be difficult to identify in a complex animal model. Using the described corneal tissue model, we have previously reported characterization of activation of pain-related signaling pathways in response to chemical irritation (Wang et al., 2018) and nerve loss following hyperglycemia (Deardorff et al., 2018), two biological events with endpoints

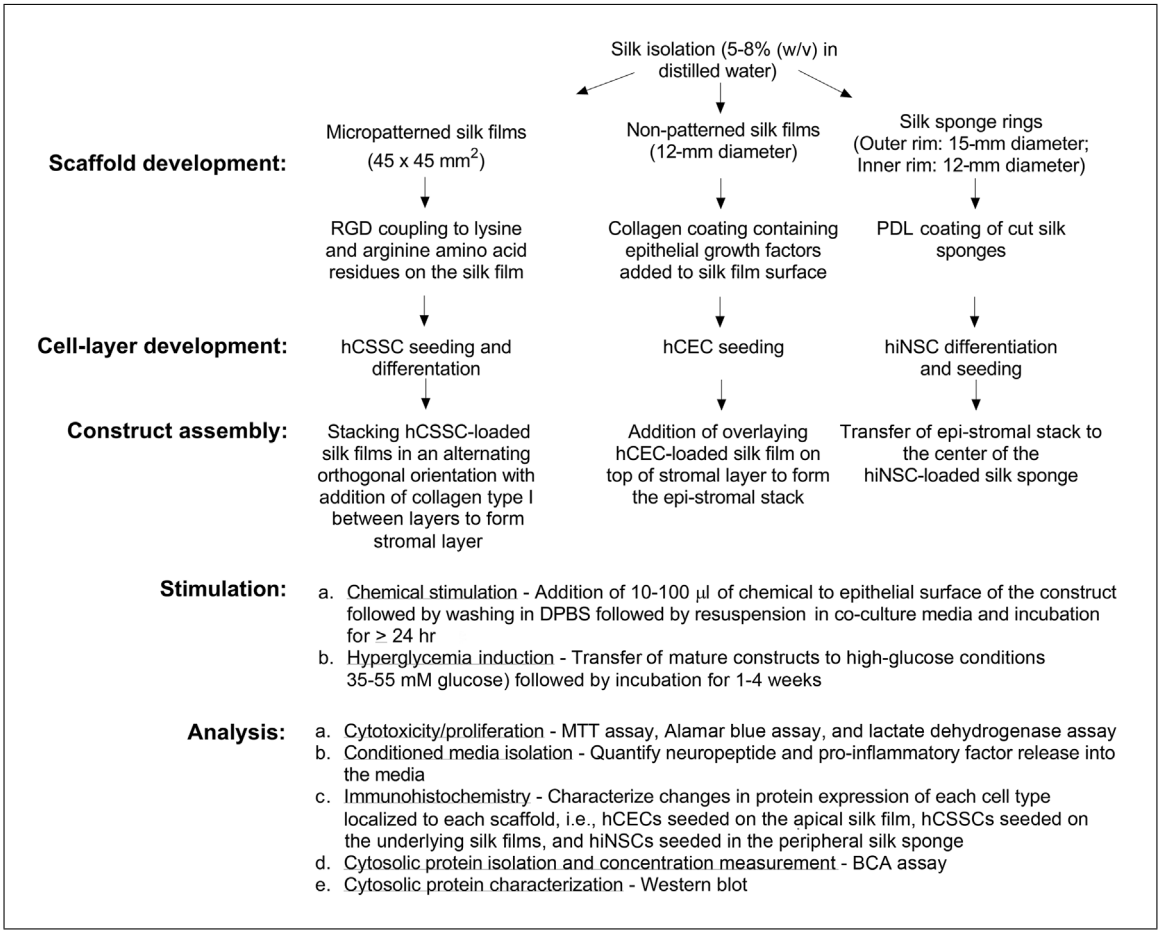

Figure 2 Flowchart of the experimental setup, involving scaffold development; cell culture approaches; and assembly, stimulation, and analysis of the corneal construct.

parallel to in vivo outcomes. A key advantage of this corneal tissue model system is the compartmentalization of individual cell populations into separable scaffolds, i.e., with the epithelium seeded on the anterior silk film, the stroma seeded on posterior silk films, and the sensory nerves seeded in the peripheral silk sponge. While the construct reaches maturation over 4 weeks of culturing, with outgrowth of nerve fibers into the stroma and ECM production by resident keratocytes, the cell bodies remain distinct from one another; this is similar to the human cornea in vivo, with the separation of the epithelium and stroma interfaced by a basement membrane and localization of neuronal somas outside of the cornea. This design provides a technical advantage in allowing for protein analysis of each cell type to determine factors regulated by chemical or mechanical stimuli following stimulation. Characterization of the pain response can involve studying phenotypic changes in the corneal epithelium [e.g., pro-inflammatory factor expression and matrix metalloproteinase (MMP) expression], keratocytes (e.g., fibrotic markers, collagen and proteoglycan secretion, and ECM thickness), and nerve populations (e.g., fiber density and tortuosity) (McKay et al., 2018).

The purpose of this unit is to describe the detailed methodology for preparation, assembly, and maintenance of the 3D corneal tissue model (Fig. 2). The methods are divided into three major categories:

1. Assembly: Basic Protocol 1 describes assembly of the corneal construct using functionalized silk scaffolds and primary human-sourced cells. Support Protocols 1 to 3 detail preparation and functionalization of silk films and sponges for cell seeding.

We also provide information regarding subculture conditions and differentiation approaches for each cell type used in the model (Support Protocols 4 to 6).
2. Application: Basic Protocol 2 describes potential applications of the model, with methods focusing on chemical stimulation of the corneal construct.
3. Characterization: Basic Protocol 3 describes isolation and characterization of the corneal construct in terms of viability as a measure of applied chemical toxicity. Support Protocols 7 to 11 elaborate on methods for further characterizing the biological response to stimuli, including isolation of conditioned medium, fixation and immunohistochemistry (IHC), and protein characterization via western blotting.

BASIC PROTOCOL 1

ASSEMBLY AND MAINTENANCE OF THE CORNEAL TISSUE MODEL

Assembly of the corneal construct requires prior isolation, preparation, and functionalization of silk scaffolds (described in Support Protocols 1 to 3) as well as culturing of primary cells (Support Protocols 4 to 6) and seeding onto the prepared silk biomaterials (Fig. 3). Upon development of each cellular component, the tissue can be assembled and maintained stably at 37°C/5% CO_2 for at least 4 weeks up to 8 weeks, with medium changes every other day. Within 24 hr post-assembly, the corneal construct is robust to manual transfer using forceps to different culture vessels or a bioreactor system (Wang et al., 2018) (Fig. 4). Over time, the construct gains increased rigidity following ECM production within the interlaying stromal layers (Ghezzi et al., 2017; Wang et al., 2017), thus allowing for further manipulation without disrupting the overall tissue structure.

Materials

Subcultured primary hCECs (ATCC, cat. no. ATCC® PCS-700-010™; see Support Protocol 4)
0.05% (w/v) trypsin-EDTA (Gibco, cat. no. 25300054)
Complete corneal epithelial medium (see recipe)
Collagen-coated silk films (see Support Protocol 2)
Subcultured primary hCSSCs (primary cells isolated from limbal rim; see Support Protocol 5)
10% (v/v) fetal bovine serum (FBS; Gibco, cat. no. 16000)
Micropatterned RGD-coated silk films (see Support Protocol 2)
Dulbecco's phosphate-buffered saline (DPBS; Gibco, cat. no. 14190144)
Complete hCSSC proliferation medium (see recipe)
Complete hCSSC differentiation medium (see recipe)
Cultured hiNSCs (see Support Protocol 6)
TrpLE™ Express (Gibco, cat. no. 12604013)
Complete neurobasal medium (see recipe)
Poly-D-lysine (PDL)–coated silk sponges (see Support Protocol 3)
3 mg/ml rat-tail collagen type I (Gibco, cat. no. A1048301) in 20 mM acetic acid
1 M sodium hydroxide (NaOH; Sigma, cat. no. 795429)
10× Dulbecco's modified Eagle's medium (DMEM; Sigma, cat. no. D2429)
Nerve growth factor (NGF)
Complete corneal co-culture medium (see recipe)
Custom waffle-patterned polydimethylsiloxane (PDMS) mold (see Support Protocol 1)

T75 and T175 cell culture flasks
50-ml polypropylene centrifuge tubes
Eppendorf 5810R centrifuge and A-4-81 rotor
6- and 24-well cell culture plates
Light microscope
100-mm petri dish

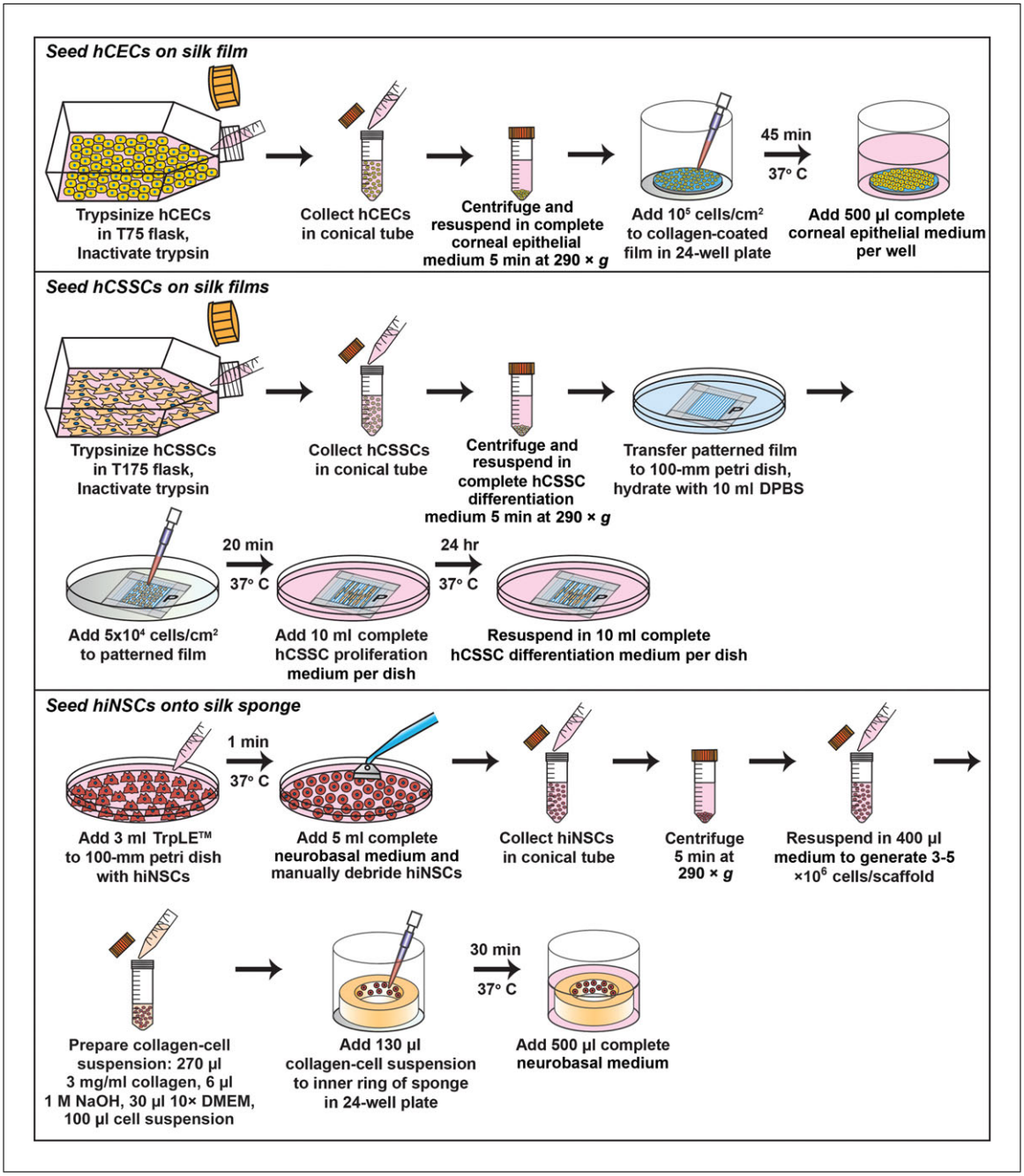

Figure 3 Schematic depicting the protocol for hCEC, hCSSC, and hiNSC seeding onto silk scaffolds (Basic Protocol 1).

Pipet tip or cell scraper
Sterile forceps
1.5-ml microcentrifuge tube
Cut pipet tip
Sterile 12-mm biopsy punch

NOTE: All biological experiments should be performed in a biological safety level 2 (BSL2)–approved laboratory environment under sterile conditions in a laminar flow hood. All reagents and biologics must be sterilized by autoclaving, filter sterilization (0.2-µm filter), UV light exposure (>30 min), or ethanol sterilization [70% (v/v) ethanol in water] prior to use in cell culture and maintained under aseptic conditions during the duration of the experiment.

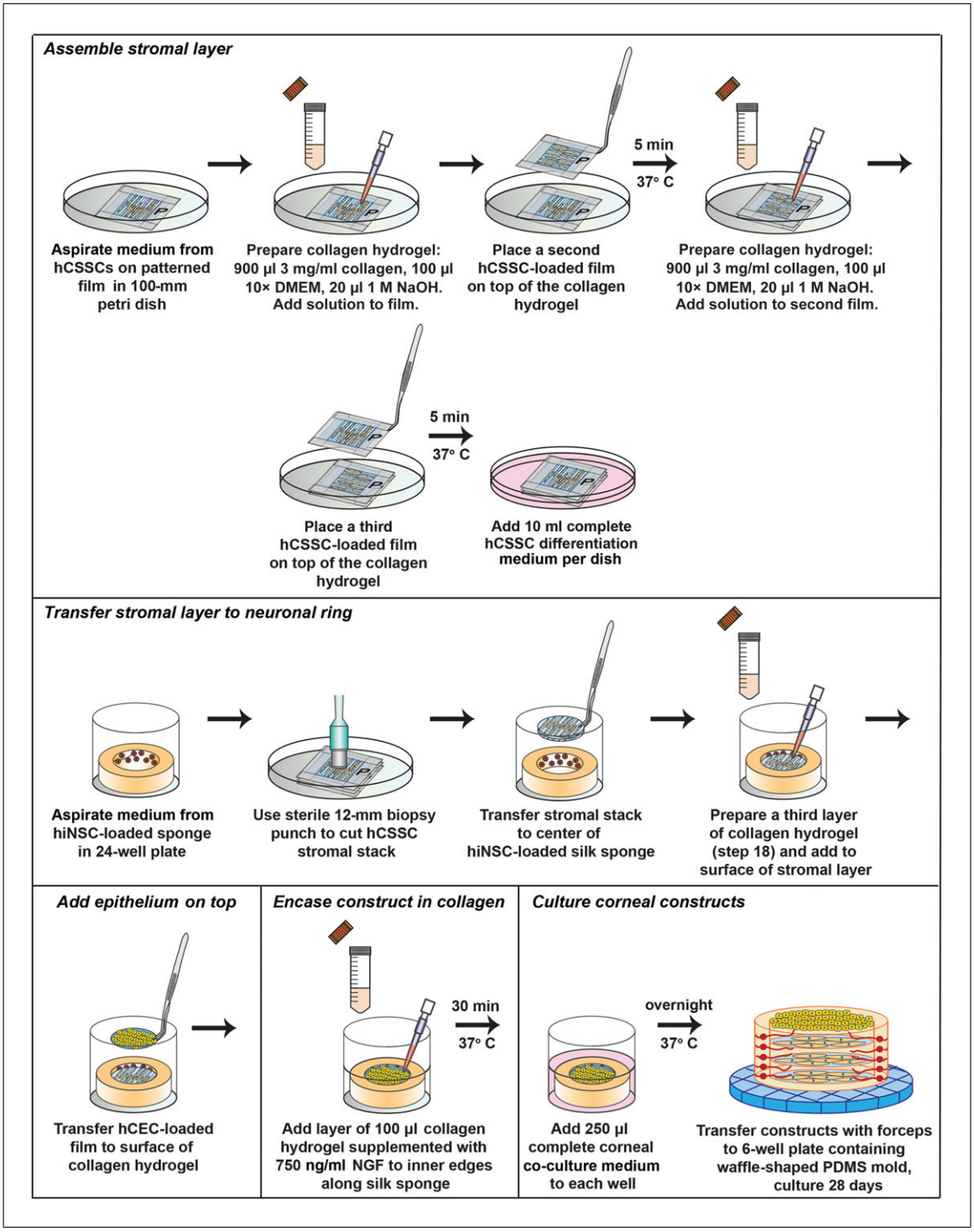

Figure 4 Schematic depicting the protocol for corneal construct assembly from the epithelial and stromal layers (Basic Protocol 1).

NOTE: All culture incubations are performed in a humidified 37°C/5% CO_2 incubator unless otherwise specified, and media should be pre-warmed to 37°C prior to use.

Seed hCECs on silk film

1. Subculture primary hCECs in a T75 cell culture flask as described in Support Protocol 4. Upon ~90% confluence, detach hCECs with 3 ml of 0.05% trypsin-EDTA, inactivate trypsin with two volumes of complete corneal epithelial medium,

transfer to a 50-ml polypropylene centrifuge tube, and centrifuge 5 min at 290 × *g* (1200 rpm in Eppendorf A-4-81 rotor) to pellet cells.

2. Resuspend hCECs in complete corneal epithelial medium to 1.9×10^7 cells/ml and add 1.9×10^6 cells (100 µl) to surface of a collagen-coated silk film in a 24-well cell culture plate (see Support Protocol 2, step 16) for a cell density of 10^5 cells/cm^2. Incubate at 37°C/5% CO_2 for 45 min. Ensure hCEC attachment to silk film by visualization using a light microscope.

   Increase the incubation time up to 4 hr in minimal medium to allow cell attachment, if needed. One hCEC-loaded film is used per construct.

3. Add complete corneal epithelial medium to 500 µl total and incubate at 37°C/5% CO_2 overnight.

   hCECs will appear as flattened cells attached to the film at 24 hr post-seeding.

Seed hCSSCs on silk films

4. Subculture hCSSCs in a T175 cell culture flask as described in Support Protocol 5. Upon 90% confluence, detach hCSSCs with 3 ml of 0.05% trypsin-EDTA, inactivate trypsin with 3 ml of 10% FBS, transfer to a 50-ml polypropylene centrifuge tube, and centrifuge 5 min at 290 × *g* to pellet cells.

5. Prepare micropatterned RGD-functionalized silk films (Support Protocol 2, step 13) for cell seeding by transferring to a 100-mm petri dish. Rehydrate by adding 10 ml DPBS to dish and swirling gently to allow immersion. Aspirate DPBS.

6. Resuspend hCSSCs in complete hCSSC proliferation medium to 2×10^5 cells/ml and add 10^5 cells (500 µl) to each silk film from step 5 for a cell density of 5×10^4 cells/cm^2. Incubate silk film and cells in the 100-mm petri dish for 20 min at 37°C/5% CO_2 in a minimal volume of medium to allow cell attachment to the silk films.

7. Confirm hCSSC attachment to silk film by visualization under a light microscope. Add 10 ml complete hCSSC proliferation medium to 100-mm petri dish and incubate overnight at 37°C/5% CO_2.

8. At 24 hr post-seeding, aspirate medium and immerse hCSSC-loaded silk film in 10 ml complete hCSSC differentiation medium.

   hCSSCs should appear as elongated spindles aligned along the microgrooves of the silk film. hCSSC-loaded silk films may be maintained in proliferation medium if cell density or attachment is low to improve cell proliferation on the silk film prior to construct assembly. Because hCSSC differentiation medium is serum free, cell proliferation is heavily reduced in this medium.

Seed hiNSCs onto silk sponge

9. Culture hiNSCs in a 100-mm petri dish as described in Support Protocol 6. Upon 90% confluence, add 3 ml TrpLE™ Express solution to mediate detachment. Incubate for 1 min at 37°C.

   TrpLE™ is an animal-free replacement for trypsin that does not require resuspension in FBS for inactivation.

10. Add 5 ml complete neurobasal medium and manually remove cells by gently debriding dish surface with a pipet tip or cell scraper.

11. Gently transfer cell solution to a 50-ml polypropylene centrifuge tube. Rinse dish with 5 ml DPBS to remove all remaining cells, transfer to tube, and centrifuge 5 min at 290 × *g*.

12. Aspirate medium and resuspend pellet in 400 µl complete neurobasal medium (equivalent to $3-5 \times 10^6$ cells per scaffold).

13. Aspirate DPBS from PDL-coated silk sponge (Support Protocol 3, step 9) and transfer to a well of a 24-well cell culture plate with sterile forceps.

14. Prepare a collagen-cell suspension in a 1.5-ml microcentrifuge tube using the following recipe:

    270 µl of 3 mg/ml rat-tail collagen type I in 20 mM acetic acid
    6 µl of 1 M NaOH
    30 µl of 10× DMEM
    100 µl cell solution (step 12).

    Add the cell solution to the mixture last, following pH neutralization of the collagen by NaOH. The color of the solution should be light red, rather than orange or yellow, prior to addition of the cell solution.

15. Gently mix collagen-cell suspension by pipetting up and down. Add 130 µl collagen-cell suspension along the inner ring of PDL-coated silk sponge from step 13 using a cut pipet tip.

16. Incubate hiNSC-loaded sponge for 30 min at 37°C/5% CO_2 to allow adhesion to the scaffold. Add 500 µl complete neurobasal medium and maintain at 37°C/5% CO_2.

Assemble stromal layer

17. Aspirate medium from hCSSCs seeded on a silk film (step 8).

18. Quickly prepare a collagen hydrogel in a 1.5-ml microcentrifuge tube using the following recipe:

    900 µl of 3 mg/ml rat-tail collagen type I in 20 mM acetic acid
    100 µl of 10× DMEM
    20 µl of 1 M NaOH.

19. Mix collagen mixture gently by pipetting up and down. Add mixture carefully to the surface of the hCSSC-loaded silk film by pipetting.

    Take care to avoid introducing bubbles into the collagen mixture; any bubbles introduced will be retained within the construct.

    The timing of this step is important to ensure that the hCSSC-loaded silk film adheres to the other silk films. Mix the collagen hydrogel and add it to the surface of the hCSSC-loaded silk film just prior to retrieval of the other hCSSC-loaded silk films.

20. Using sterile forceps, transfer a second hCSSC-loaded silk film (step 8) to the top of the collagen hydrogel, with alignment of the microgrooves orthogonal to the bottom film (step 19). Incubate at 37°C/5% CO_2 for 5 min.

    Use forceps to hold onto the edges of the tape to ensure that the cell surface is undisturbed with transfer.

21. Prepare a second collagen hydrogel mixture as before (step 18). Add mixture slowly to the surface of a second hCSSC-loaded silk film by pipetting. Incubate at 37°C/5% CO_2 for 5 min.

22. Transfer a third hCSSC-loaded silk film to the top of the collagen surface, with the microgrooves oriented orthogonal to the second silk film. Incubate at 37°C/5% CO_2 for 5 min. Add 10 ml complete hCSSC differentiation medium.

Transfer stromal layer to neuronal ring

23. Aspirate medium from the hiNSC-loaded silk sponge seeded in a 24-well cell culture plate (step 16).

24. Using a sterile 12-mm biopsy punch, cut hCSSC stromal stack (step 22) and transfer biopsy to the center of the hiNSC-loaded silk sponge.

    Take care to add the stromal layer (three silk films loaded with hCSSCs) to the inner ring of the silk sponge. The stromal layer may fold up like a scroll during the transfer; gently unfold and align the films using the fine forceps tip to create a seamless structure against the rim of the silk sponge. Each film of ~45 × 45 mm^2 (minus the taped edges) should generate four 12-mm biopsies.

25. Prepare a third layer of collagen hydrogel (step 18) and add it to surface of the stromal layer.

Add epithelium on top

26. Immediately retrieve hCEC-loaded silk film (step 3) using sterile forceps. Aspirate medium and carefully transfer hCEC-loaded silk film to the surface of the collagen hydrogel from step 25 using forceps.

    The timing of this step is important to ensure that the hCEC-loaded silk film adheres to the underlying stromal layers. Mix the collagen hydrogel and add it to the surface of the stromal layer (step 25) just prior to hCEC-loaded silk film retrieval.

Encase construct in collagen

27. Add a final layer of 100 µl collagen hydrogel (step 18) supplemented with 750 ng/ml NGF to inner edges of the topmost layer along the silk sponge to encase the construct. Incubate at 37°C/5% CO_2 for 30 min.

    Add NGF to the collagen mixture after neutralization with NaOH to prevent acid-mediated degradation of the growth factor.

    This collagen layer is important in holding the silk films and the silk sponge together to enable robustness of the construct to transfer.

Culture corneal constructs

28. Add 250 µl complete corneal co-culture medium to constructs. Incubate at 37°C/5% CO_2 overnight.

29. At 24 hr post-assembly, using sterile forceps, transfer each construct to a 6-well cell culture plate containing a custom waffle-patterned PDMS mold (see Support Protocol 1, step 6) to maintain an air-liquid interface. Add 2.5 ml complete corneal co-culture medium.

30. Maintain corneal constructs at 37°C/5% CO_2 for 4 weeks, with medium changes every other day.

    Aliquots of NGF should be added to fresh medium immediately prior to use to minimize degradation.

PREPARATION OF POLYDIMETHYLSILOXANE MOLDS

SUPPORT PROTOCOL 1

PDMS molds provide reusable substrates for casting micropatterned silk films (Fig. 5 and Support Protocol 2). They can be cleaned with 70% (v/v) ethanol in water between uses and should be replaced if scratched or damaged. The waffle-patterned PDMS molds are used as stable, nonstick platforms for the corneal constructs (Basic Protocol 1) during cultivation and fit in a 6-well culture plate. They may be autoclaved for sterilization between uses.

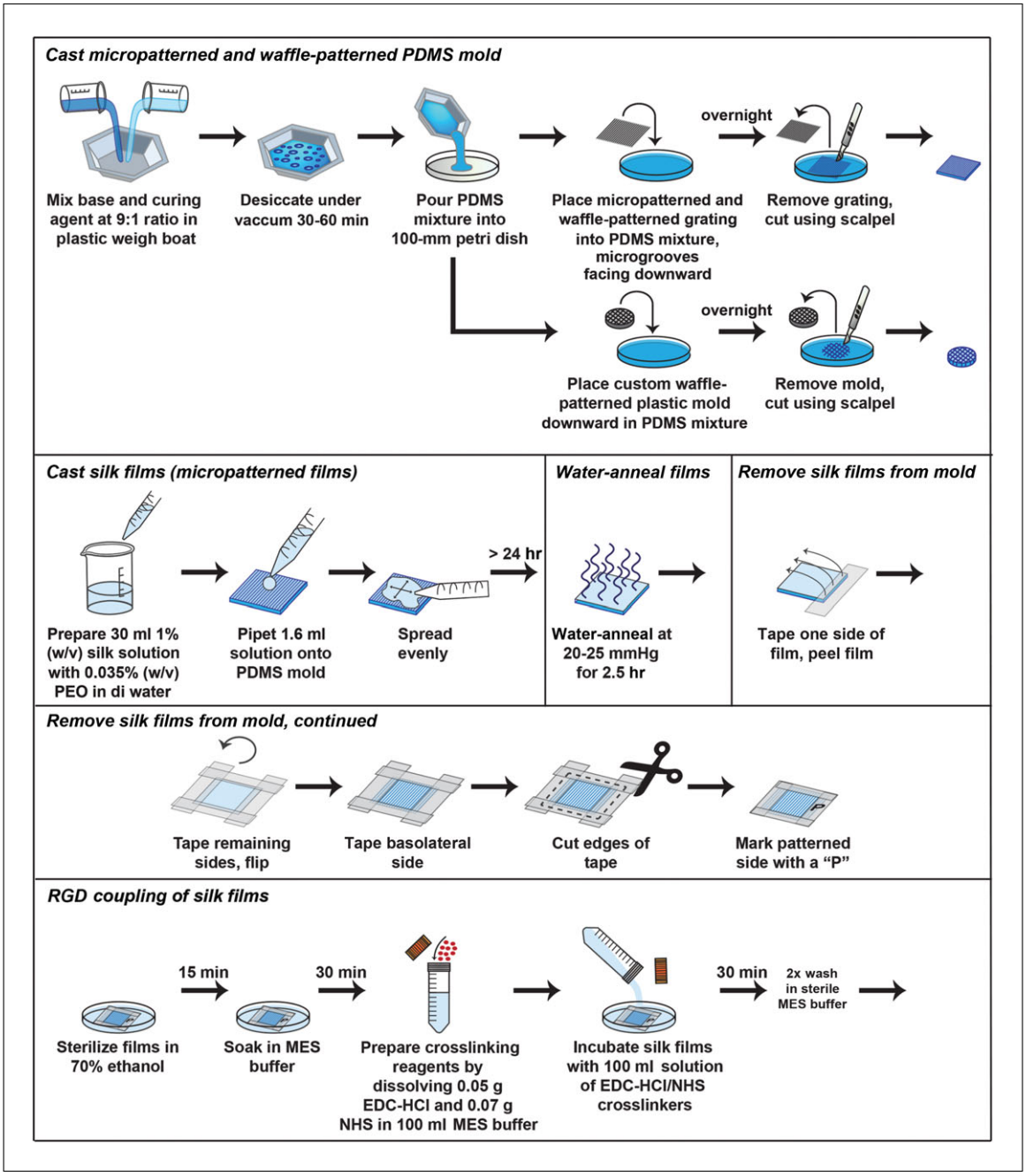

Figure 5 Schematic depicting the protocols for casting PDMS molds (Support Protocol 1) and silk films (Support Protocol 2).

Materials

Base and curing agent: SYLGARD® 184 Silicone Encapsulant Clear (Krayden, cat. no. DC4019862)

Plastic weigh boat
Disposable pipet or glass stirrer
Vacuum desiccator
100-mm petri dishes
Micropatterned diffraction grating with microgrooves, 45 × 45 mm^2 (500-nm depth and 3.5-μm width)
Surgical scalpel

Alcohol-resistant marker
Custom-designed, waffle-patterned plastic Delrin® mold (5-mm height × 5-cm diameter and 16 holes of 1-mm^2 area; McMaster-Carr)
Autoclave

Cast micropatterned PDMS mold

1. Mix base and curing agent at a ratio of 9:1 in a plastic weigh boat and manually stir PDMS mixture with a disposable pipet or glass stirrer for 1 min.
2. Remove bubbles by desiccating mixture under vacuum for 1 hr.
3. Gently pour PDMS mixture into a 100-mm petri dish. Place micropatterned diffraction grating into the mixture, with the microgrooves facing downward. Incubate mixture overnight at room temperature to allow solidification.
4. Remove grating from the surface of the PDMS mold. Cut mold from the dish using a surgical scalpel. Denote micropatterned surface by marking the side of the mold with an alcohol-resistant marker. Store PDMS mold at room temperature until use (see Support Protocol 2).

Cast waffle-patterned PDMS mold

5. Gently pour PDMS mixture into a 100-mm petri dish. Place custom-designed, waffle-patterned plastic Delrin® mold downward in the PDMS mixture. Incubate mixture overnight at room temperature to allow solidification.
6. Remove plastic mold from the surface of the PDMS mold. Cut PDMS mold along the edges of the imprint using a surgical scalpel to generate a waffle-patterned PDMS mold. Autoclave PDMS mold and store at room temperature prior to use in cell culture (see Basic Protocol 1, step 29).

PREPARATION OF SILK FILMS

SUPPORT PROTOCOL 2

Detailed protocols for isolation of silk fibroin and preparation of silk films and sponges have previously been reported by our group (Rockwood et al., 2011). Thus, we refer the reader to the previously published protocols for details regarding general silk biomaterial preparation. We focus the present protocol on detailed steps required for functionalization of silk films specific for construction of the corneal tissue model (Fig. 5 and Basic Protocol 1). EDC-mediated RGD coupling to silk films provides a binding site for hCSSC attachment to the silk substrate (Fig. 6). Alignment of hCSSCs along the microgrooves on the RGD-micropatterned surface can be visualized under a brightfield microscope. Collagen and proteoglycan deposition also aligns along the microgrooves, similar to the corneal stroma in vivo (Wu et al., 2014).

Materials

5% to 8% (w/v) silk solution in distilled water
1% (w/v) polyethylene oxide (PEO; Sigma, cat. no. 189464) in distilled water
45 × 45–mm^2 micropatterned PDMS molds (see Support Protocol 1, step 4)
70% (v/v) ethanol in distilled water
Sterile Dulbecco's phosphate-buffered saline (DPBS; Gibco, cat. no. 14190144)
2-(*N*-Morpholino)ethanesulfonic acid (MES) buffer, pH 6.5 (Thermo Scientific, cat. no. 28390)
1-Ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl; Sigma, cat. no. E6383)
N-Hydroxysuccinimide (NHS; Sigma, cat. no. 130672)
RGD peptide

Sterile phosphate-buffered saline (PBS; Sigma, cat. no. 11666789001)
Epithelial growth factor (EGF) stamping solution (see recipe)

Aluminum foil
Pipet tip or glass spreader
12-mm glass coverslips
24-well cell culture plate
Vacuum desiccator with temperature control
Scotch tape
Scissors
Pencil
Light microscope with 40× objective lens
Sterile petri dish
50-ml filter flasks with 0.2-μm filter
Rocker
UV light source

Cast silk films

1. To prepare micropatterned silk films:

   a. Prepare 30 ml of a 1% (w/v) silk solution (from 5% to 8% stock) containing 0.035% (w/v) PEO (from 1% stock) in distilled water for 18 45 × 45–mm^2 micropatterned PDMS molds. Mix solution gently by pipetting up and down.

      The concentration of the stock silk solution varies based on the efficacy of isolation from silkworm cocoons.

      Add 1.05 ml of 1% PEO to 3.85 ml of 7.8% silk solution in distilled water. Dilute with 25.1 ml distilled water for a final volume of 30 ml.

   b. Pipet 1.6 ml solution onto each PDMS mold. Spread solution evenly using a pipet tip or glass spreader, remove any bubbles gently by pipetting, and incubate mold on a flat surface overnight at room temperature. Cover mold only partially with aluminum foil to allow water evaporation. Allow >24 hr to dry.

2. To prepare non-patterned silk films:

   a. Prepare 30 ml of 1% silk solution containing 0.035% PEO in distilled water as in step 1a. Mix solution gently by pipetting up and down.
   b. Pipet solution onto 12-mm glass coverslips in a 24-well culture plate, with 120 μl per coverslip. Spread solution evenly using a pipet tip or glass spreader, remove any bubbles gently by pipetting, and incubate plate on a flat surface overnight at room temperature. Cover plate only partially to allow water evaporation. Allow >24 hr to dry.

Water-anneal micropatterned films

3. Place dried silk films on PDMS molds from step 1 in a vacuum desiccator with temperature control. Add tap water to vessel at the bottom of the desiccator. Close purge valve and open vacuum valve to create an internal pressure of 20 to 25 mm Hg. Then, close vacuum valve and incubate films for 2.5 hr at room temperature to allow water-annealing.

Remove micropatterned silk films from molds

4. Once the films are dry, place scotch tape on one edge of each film (covering ~1/4 in. of film) and gently pull film off the mold and onto a clean surface (e.g., a sheet of plastic).

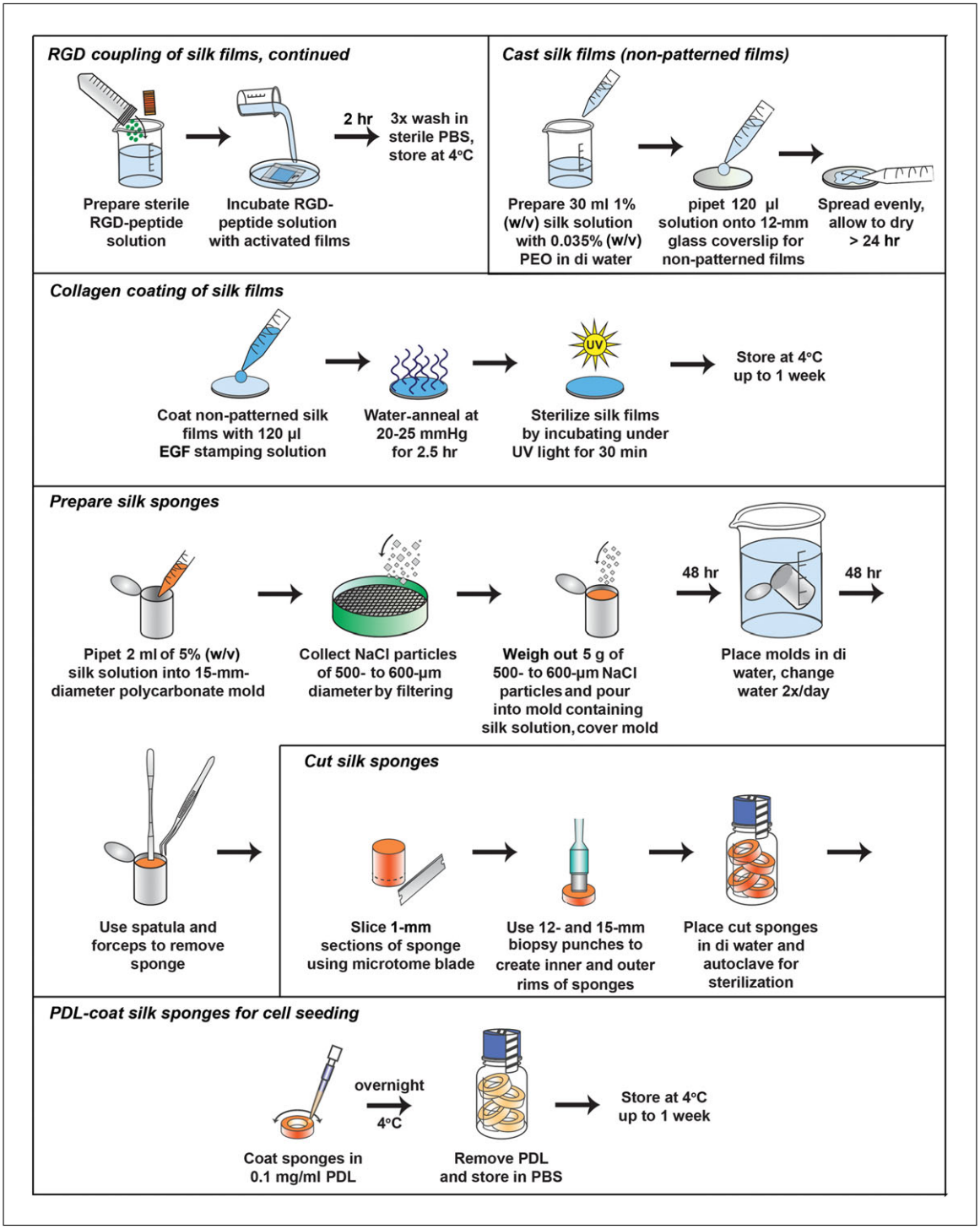

Figure 6 Schematic depicting the protocols for conjugating RGD peptide to silk films (Support Protocol 2) and PDL coating of silk sponges (Support Protocol 3).

5. Gently tape remaining edges of the film onto the clean surface. Unstick tape from the surface and turn film over. Repeat addition of tape on the basolateral side of the film to cover the exposed tape glue.
6. Cut away edges of the tape using scissors, write "P" on tape with a pencil to denote the side that is patterned, and place film in a clean container (e.g., a large petri dish) until use.

7. Check alignment of the grooves using the 40× objective lens of a light microscope. Draw arrows on tape with a pencil to denote the orientation of the grooves. Store silk films at room temperature in a dry place until use.

Perform RGD coupling of micropatterned silk films

8. Sterilize silk films prior to RGD coupling by incubating in 70% ethanol in distilled water in a sterile petri dish for 15 min at room temperature. Gently rinse films five times with sterile DPBS to remove residual ethanol.
9. Soak films in MES buffer for 30 min.
10. Prepare crosslinking reagents by dissolving 0.05 g EDC-HCl and 0.07 g NHS in 100 ml MES buffer. Filter-sterilize into a 50-ml filter flask with a 0.2-µm filter.

    CAUTION: *EDC and NHS are dangerous when inhaled. Weigh these chemicals in a chemical hood or transfer them to a tared tube with a lid before weighing to reduce exposure. Prepare the mixture immediately prior to the reaction due to the labile nature of the reagents.*

11. Incubate silk films from step 9 with 100 ml solution of EDC-HCl/NHS crosslinkers for 30 min at room temperature. Aspirate crosslinkers and wash films with MES buffer twice.
12. Prepare and filter-sterilize 1 mg/ml RGD-peptide solution and incubate it with activated films for 2 hr at room temperature or overnight at 4°C with gentle rocking.
13. Aspirate RGD-peptide solution and wash films with sterile PBS three times. Store silk films in PBS at 4°C.

Perform collagen coating of non-patterned silk films

14. Prior to hCEC seeding, coat non-patterned silk films from step 2 with 120 µl EGF stamping solution to enable cell adhesion.
15. Water-anneal coated films at 20 to 25 mm Hg for 2.5 hr (step 3).
16. Sterilize silk films by incubating under UV light for 30 min on each side. Store ≤1 week at 4°C.

SUPPORT PROTOCOL 3

PREPARATION OF SILK SPONGES

This section describes the preparation of silk sponges (Fig. 6) that are utilized as the primary scaffold for seeding hiNSCs, as described in Basic Protocol 1.

Materials

5% (w/v) silk solution in distilled water
Sodium chloride (NaCl; Sigma, cat. no. S7653)
Distilled water
0.1 mg/ml PDL solution (Corning, cat. no. 354210)

15-mm-diameter polycarbonate molds with lids
500- and 600-µm stainless steel sieves [Fisher Scientific, cat. no. 04-884-1AM (600 µm) and cat. no. 04-884-1AN (500 µm), respectively]
2-L plastic beaker
Spatula
Forceps
Microtome blade
12- and 15-mm diameter biopsy punches
Autoclave

Prepare silk sponges

1. Pipet 2 ml of 5% silk solution into each 15-mm-diameter polycarbonate mold.
2. Collect NaCl particles of 500- to 600-µm diameter by filtering through a 600-µm stainless steel sieve followed by a 500-µm sieve.
3. Weigh out 5 g of the 500- to 600-µm NaCl particles and carefully pour them into a mold containing silk solution (step 1), gently tapping bottom of the mold while adding the salt. Cover molds with lids and store at room temperature for 2 days until solidification.

   Minimize the introduction of bubbles by adding the salt slowly while rotating the mold.
4. After incubation, remove lids and place all molds into distilled water in a 2-L plastic beaker to allow salt to leach from the silk sponge. Change water twice per day for 2 days.
5. To remove the sponge from the mold, insert a spatula at inside periphery of the mold and gently press sponge inward to release it from the mold. Then, use forceps to fully dislodge sponge. Incubate free sponge in distilled water for 1 day, with two water changes.

   Sponges may be stored dry indefinitely at room temperature or for several months at 4°C.

Cut silk sponges

6. To cut the silk sponge into the proper dimensions, slice 1-mm sections using a microtome blade. Utilize commercial 12- and 15-mm-diameter biopsy punches to create a ring with inner and outer rims, respectively.

   Each whole sponge should generate at least four rings for neuronal seeding.
7. Place cut sponges in distilled water and autoclave for sterilization.

Perform PDL coating of silk sponges for cell seeding

8. To PDL-coat the silk sponge prior to seeding, incubate sterile sponge in 0.1 mg/ml PDL solution in distilled water at 4°C overnight. Remove PDL prior to cell seeding.
9. Store sponges in PBS for ≤1 week at 4°C.

SUBCULTURING OF HUMAN CORNEAL EPITHELIAL CELLS

SUPPORT PROTOCOL 4

Primary hCECs can be subcultured up to passage 5 and seeded onto silk films, as described in Basic Protocol 1. Refer to the manufacturer's recommendations for specifics regarding cryopreservation and culture maintenance.

Materials

FNC Coating Mix® (Athena Environmental Sciences, cat. no. 0407; optional)
Complete corneal epithelial medium (see recipe)
Primary hCECs (ATCC, cat. no. ATCC® PCS-700-010™; stored as frozen stock culture)

T75 flask
37°C water bath
500-ml cell culture flask

NOTE: All biological experiments should be performed in a biological safety level 2 (BSL2)–approved laboratory environment under sterile conditions in a laminar flow hood. All reagents and biologics must be sterilized by autoclaving, filter sterilization (0.2-µm filter), UV light exposure (>30 min), or ethanol sterilization [70% (v/v) ethanol

in water] prior to use in cell culture and maintained under aseptic conditions during the duration of the experiment.

NOTE: All culture incubations are performed in a humidified 37°C/5% CO_2 incubator unless otherwise specified, and medium should be pre-warmed to 37°C prior to use.

1. Optional: Add 3 ml FNC Coating Mix® to a T75 flask. Swirl flask to coat the entire surface and then remove mix.

   The fibronectin coating may be used to promote adhesion of hCECs to the flask.

2. Add 15 ml complete corneal epithelial medium to T75 flask.
3. Thaw primary hCECs from a frozen stock culture by immersing bottom of the vial in a 37°C water bath for 1 to 2 min.
4. Immediately transfer hCECs to the T75 flask containing complete corneal epithelial medium from step 2. Incubate at 37°C/5% CO_2 for 24 hr to allow cell adhesion.
5. Aspirate medium from adhered hCECs and add fresh complete corneal epithelial medium. Subculture hCECs upon 90% confluence up to passage 5.

SUPPORT PROTOCOL 5

SUBCULTURING OF HUMAN CORNEAL STROMAL STEM CELLS

Due to the high proliferative capabilities of hCSSCs, they are commonly used as a cell source of keratocytes by following specific differentiation protocols (Syed-Picard et al., 2018; Wu, Du, et al., 2014). As keratocytes tend to proliferate very slowly in culture, the process allows one to obtain sufficient cell numbers using the stem cell progenitor prior to differentiation. In our corneal model, we seed hCSSCs onto silk films, as described in Basic Protocol 1, followed by differentiation into keratocytes. We utilize the term "hCSSCs" throughout the protocol to specify the cell source and to maintain consistency within the field for distinguishing between keratocytes derived from hCSSCs and keratocytes isolated from tissue or obtained via other sources.

Materials

Human corneal tissue
0.5 mg/ml collagenase-I solution (Sigma, cat. no. 10269638001)
Complete hCSSC proliferation medium (see recipe)
Complete hCSSC differentiation medium (see recipe)

Surgical scalpel
15-ml polypropylene centrifuge tube
Eppendorf 5810R centrifuge and A-4-81 rotor
T25, T75, and T175 flasks
Light microscope

Additional reagents and equipment for limbal-rim isolation (Syed-Picard et al., 2018; Wu, Du, et al., 2014)

NOTE: Institutional review board and federal guidelines must be followed regarding obtaining human corneal tissue.

NOTE: All biological experiments should be performed in a biological safety level 2 (BSL2)–approved laboratory environment under sterile conditions in a laminar flow hood. All reagents and biologics must be sterilized by autoclaving, filter sterilization (0.2-µm filter), UV light exposure (>30 min), or ethanol sterilization [70% (v/v) ethanol in water] prior to use in cell culture and maintained under aseptic conditions during the duration of the experiment.

NOTE: All culture incubations are performed in a humidified 37°C/5% CO_2 incubator unless otherwise specified, and medium should be pre-warmed to 37°C prior to use.

1. Obtain human corneal tissue according to institutional guidelines and isolate limbal rim using a surgical scalpel based on previous protocols (Syed-Picard et al., 2018; Wu, Du, Mann, Funderburgh, & Wagner, 2014).
2. Digest corneal tissue in a 15-ml polypropylene centrifuge tube using 1 ml of 0.5 mg/ml collagenase-I solution overnight at 37°C, followed by centrifugation for 5 min at 453 × *g* (1500 rpm in Eppendorf A-4-81 rotor). Aspirate supernatant and resuspend cell pellet in 2 ml complete hCSSC proliferation medium.
3. Plate cell solution in a T25 flask containing 5 ml complete hCSSC proliferation medium, gently swirl, and incubate at 37°C/5% CO_2 overnight.
4. Check cell attachment and cell number using a light microscope. Maintain hCSSCs in complete hCSSC proliferation medium until 90% confluence and then transfer to a T75 flask. Subculture hCSSCs up to passage 5 in a T175 flask.
5. To promote differentiation of the hCSSCs into keratocytes, substitute the complete hCSSC proliferation medium with complete hCSSC differentiation medium.

   Expression of the keratocyte markers keratocan and aldehyde dehydrogenase 3a by hCSSCs can be verified by IHC, western blot, or RT-PCR to confirm proper differentiation. Downregulation of the stem cell markers ABCG2 and Nestin and upregulation of keratocan and aldehyde dehydrogenase 3a by hCSSCs have been shown to occur by 1 week of culturing in complete differentiation medium (Basu et al., 2014).

SUBCULTURING OF HUMAN INDUCED NEURAL STEM CELLS

SUPPORT PROTOCOL 6

hiNSCs are used as the neural stem cell source in our corneal tissue model. They are highly proliferative, allowing one to obtain sufficient cell numbers prior to sensory nerve differentiation. Differentiated hiNSCs are seeded onto silk sponges, as described in Basic Protocol 1, and are allowed to mature and innervate into the corneal construct over 4 weeks.

Materials

0.1% (w/v) gelatin (in distilled water; Sigma, cat. no. ES006B)
Mouse embryonic fibroblasts (MEFs; ATCC, cat. no. ATCC® SCRC-1008™)
DMEM containing 10% (v/v) FBS and 1× antibiotic-antimycotic
10 µg/ml mitomycin dissolved in DMEM containing 10% FBS and 1× antibiotic-antimycotic
hiNSCs (stored as frozen stock culture)
Complete neurobasal medium (see recipe)
Complete knockout medium (see recipe) with or without 20 ng/ml fibroblast growth factor-2 (FGF-2)
DPBS (Gibco, cat. no. 14190144)
TrpLE™ Express (Gibco, cat. no. 12604013)
Complete sensory nerve medium (see recipe)

100-mm petri dishes
37°C water bath
15- and 50-ml polypropylene centrifuge tubes
Eppendorf 5810R centrifuge and A-4-81 rotor
Vortex
Plastic cell strainer

NOTE: All biological experiments should be performed in a biological safety level 2 (BSL2)–approved laboratory environment under sterile conditions in a laminar flow hood. All reagents and biologics must be sterilized by autoclaving, filter sterilization (0.2-µm filter), UV light exposure (>30 min), or ethanol sterilization [70% (v/v) ethanol in water] prior to use in cell culture and maintained under aseptic conditions during the duration of the experiment.

NOTE: All culture incubations are performed in a humidified 37°C/5% CO_2 incubator unless otherwise specified, and medium should be pre-warmed to 37°C prior to use.

Subculture hiNSCs

1. Add 3 ml of 0.1% gelatin to 100-mm petri dishes, swirl to coat entire surface, and then aspirate gelatin solution. Culture MEFs in DMEM containing 10% FBS and 1× antibiotic-antimycotic on gelatin-coated petri dishes.
2. Upon 100% confluence, inactivate MEFs by treatment with 10 µg/ml mitomycin C dissolved in DMEM containing 10% FBS and 1× antibiotic-antimycotic for 2 to 3 hr at 37°C/5% CO_2. Aspirate solution, wash cells twice with medium, and resuspend in fresh medium until hiNSC seeding.

   CAUTION: *Mitomycin C is a carcinogen and must be handled with care. All waste should be disposed of in the appropriate biological and chemical waste streams.*

3. Retrieve hiNSCs from a frozen stock culture and thaw by immersing bottom of the vial in a 37°C water bath for 1 to 2 min. Add thawed cells to 10 ml complete neurobasal medium in a 15-ml polypropylene centrifuge tube. Centrifuge 5 min at 290 × *g* (1200 rpm in Eppendorf A-4-81 rotor). Aspirate supernatant and resuspend cells in complete knockout medium.
4. Aspirate medium from the inactivated MEFs from step 2 and carefully transfer hiNSC cell solution to the MEF feeder layer. Swirl dish gently to distribute the hiNSCs evenly over the feeder layer. Add 9 ml additional complete knockout medium with 20 ng/ml FGF-2 to dish and maintain at 37°C/5% CO_2 until multiple hiNSC colonies form on surface of the feeder layer.
5. Once the cells are confluent, aspirate medium and wash cell layer gently with DPBS.
6. Add 3 ml TrpLE™ Express and incubate for 1 min at 37°C.
7. Transfer detached cells to a 50-ml polypropylene centrifuge tube. Gently add a small volume of DPBS to colonies remaining on dish that appear as white, dome-shaped deposits and use a pipet tip to mechanical remove cells. Transfer cell suspension to a separate 50-ml tube. Centrifuge both tubes for 2 to 3 min at 453 × *g* (1500 rpm in Eppendorf A-4-81 rotor).
8. Resuspend colony pellet in 8 ml complete knockout medium with 20 ng/ml FGF-2.
9. Aspirate medium from a fresh dish of inactivated MEFs (step 2). Add 9 ml complete knockout medium with 20 ng/ml FGF-2 and then transfer resuspended colony pellet to the MEF feeder layer and incubate at 37°C/5% CO_2. Maintain hiNSCs on the MEF feeder layer until multiple colonies form.
10. To obtain a single-cell suspension, add 12 ml complete knockout medium, vortex briefly, and strain cells using a plastic cell strainer.
11. Add filtered single-cell suspension to a gelatin-coated 10-mm petri dish and incubate at 37°C/5% CO_2 for 24 hr. Verify cell attachment and maintain cells in complete knockout medium until differentiation.

Differentiate hiNSCs

12. Differentiate hiNSCs into a sensory neuronal phenotype by incubating with complete sensory nerve medium containing a number of growth factors and small molecule inhibitors.

13. Aspirate complete knockout medium and substitute with complete sensory nerve medium.

14. Maintain hiNSCs in complete sensory nerve medium on a gelatin-coated 10-mm petri fish for 10 days at 37°C/5% CO_2, with medium changes every other day.

    Expression of nociceptor markers, e.g., transient receptor potential cation channel (TRP) receptors TRPV1, TRPA1, and TRPM8, may be used to confirm successful sensory nerve differentiation.

CHEMICAL STIMULATION

BASIC PROTOCOL 2

The corneal construct (Basic Protocol 1) may serve as an alternative to animal models for evaluating chemical toxicity or biological responses to acute or chronic stimuli during pharmacological screening. Prior to application of this model to evaluate toxicological responses, we recommend assessing the dose-dependent cytotoxicity of the chemical of interest using 2D monocultures. These experiments should provide a baseline for assessing the chemical of interest in the 3D corneal construct. The presence of the silk scaffold and ECM generated over the length of cultivation likely promotes increased resistance to chemical-induced toxicity, similar to the cornea in vivo, in contrast to direct cell contact, as in 2D cultures. We have assessed the response of the corneal tissue model to different microenvironments and chemical stimulants, with results summarized in Table 1.

Materials

Assembled corneal constructs (see Basic Protocol 1)
Complete corneal co-culture medium (see recipe)
Chemical stimulant (e.g., capsaicin or sodium lauryl sulfate)
DPBS (Gibco, cat. no. 14190144)

Table 1 Evaluating the Effects of the Microenvironment (Elevated Glucose or Dynamic Perfusion) and Different Chemical Stimulants (Capsaicin or Sodium Lauryl Sulfate) on Cell Viability and Gene and Protein Expression Using the Corneal Tissue Model

Microenvironment or chemical	Specific condition applied	Response[a]	Reference
Elevated glucose	35-55 mM glucose	Concentration-dependent loss of hCECs and hCSSCs, nerve degeneration,↑ IL-1β	Deardorff et al. (2018)
Dynamic perfusion	Intraocular pressure of 15-20 mm Hg and tear flow of 50 µl/min	↑ *CRCP*, ↑ *SCN*, ↑ *KERA*, ↑ *LUM*	Wang et al. (2018)
Capsaicin	0.005% (w/v) in DMEM	↑ SP, ↑ CGRP	Wang et al. (2018)
Sodium lauryl sulfate	10% (v/v) in PBS	↑ SP, ↑ Tissue permeability	Wang et al. (2018)

[a] *Protein names:* IL-1β, interleukin-1 beta; SP, substance P; CGRP, calcitonin gene–related peptide. *Gene names*: *CRCP*, accessory protein for CGRP; *SCN*, sodium channel; *KERA*, keratocan (cornea-specific proteoglycan); *LUM*, lumican (proteoglycan abundant in the stroma).

6-well cell culture plates
0.2-µm filter and syringe
15-ml polypropylene centrifuge tube
Sterile forceps

Additional reagents and equipment for characterization of biological response (see Basic Protocol 3 and Support Protocols 7 to 11)

NOTE: All biological experiments should be performed in a biological safety level 2 (BSL2)–approved laboratory environment under sterile conditions in a laminar flow hood. All reagents and biologics must be sterilized by autoclaving, filter sterilization (0.2-µm filter), UV light exposure (>30 min), or ethanol sterilization [70% (v/v) ethanol in water] prior to use in cell culture and maintained under aseptic conditions during the duration of the experiment.

NOTE: All culture incubations are performed in a humidified 37°C/5% CO_2 incubator unless otherwise specified, and medium should be pre-warmed to 37°C prior to use.

1. Maintain assembled corneal construct in a 6-well cell culture plate in complete corneal co-culture medium.
2. Prepare sterile chemical stimulant by filtering through a 0.2-µm filter via syringe into a 15-ml polypropylene centrifuge tube.
3. Apply 10 µl chemical stimulant to top epithelial surface of the construct and incubate for 10 min at 37°C/5% CO_2.

   The volume of chemical stimulant added may be increased to cover the entire surface of the construct. Furthermore, the length of incubation may be decreased or increased. To evaluate the effects of the chemical on the neurons independent of the epithelial and stromal layers, the chemical stimulant may be injected into the silk sponge directly, thereby minimizing direct contact with the other cell types.

4. Submerge construct in 2 ml DPBS and then aspirate DPBS. Repeat wash four additional times.
5. Using sterile forceps, transfer construct to a clean 6-well cell culture plate and incubate in 2 ml complete corneal co-culture medium for ≥24 hr.
6. Characterize biological response as described in Basic Protocol 3 and Support Protocols 7 to 11.

BASIC PROTOCOL 3

ASSESSING VIABILITY BY MTT ASSAY

In order to determine the biological response to chemical stimuli (Basic Protocol 2), characterization of the corneal construct may be performed on the conditioned medium, the whole construct, or protein isolates from separate layers. Determining cell viability within the corneal construct using the MTT assay provides an optimal method to evaluate cell toxicity or proliferation in response to chemical stimulants. Limitations of this approach include the destructive nature of the assay and the requirement for use of the entire construct for analysis. A similar approach as described here may also be applied to evaluation of total double-stranded DNA via a Picogreen® assay. Further characterization of the protein content by enzyme-linked immunosorbent assay (ELISA), IHC, and western blot is described in Support Protocols 7, 8, and 11, respectively.

Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Invitrogen, cat. no. V13154)

PBS (Sigma, cat. no. 11666789001)
Stimulated corneal constructs (see Basic Protocol 2)
DPBS (Gibco, cat. no. 14190144)
Phenol red–free complete corneal co-culture medium (see recipe)
Dimethylsulfoxide (DMSO; Sigma, cat. no. D8418)

12-well cell culture plate
Forceps
Aluminum foil
1.5-ml microcentrifuge tubes
Vortex
Hand-held homogenizer (optional)
96-well plate
Spectrophotometer

NOTE: All biological experiments should be performed in a biological safety level 2 (BSL2)–approved laboratory environment under sterile conditions in a laminar flow hood. All reagents and biologics must be sterilized by autoclaving, filter sterilization (0.2-µm filter), UV light exposure (>30 min), or ethanol sterilization [70% (v/v) ethanol in water] prior to use in cell culture and maintained under aseptic conditions during the duration of the experiment.

NOTE: All culture incubations are performed in a humidified 37°C/5% CO_2 incubator unless otherwise specified, and medium should be pre-warmed to 37°C prior to use.

Incubate construct with MTT reagent

1. Prepare MTT stock solution by dissolving 5 mg MTT in 1 ml PBS.
2. To prepare for incubation with MTT, transfer stimulated corneal construct to a 12-well cell culture plate using forceps. Aspirate phenol red–containing medium, gently wash construct with DPBS twice, and add 500 µl phenol red–free complete corneal co-culture medium.
3. Add 10 µl MTT solution from step 1 to each construct submerged in medium. Protect from light by covering plate with aluminum foil. Incubate constructs at 37°C/5% CO_2 for 2 hr.

Isolate cell layers of construct

4. Using forceps, transfer constructs to fresh wells containing 500 µl PBS.
5. Begin gently separating layers of each construct by using forceps to remove the topmost silk film, containing the epithelium. Transfer this film to a 1.5-ml microcentrifuge tube containing 100 µl DMSO.

   As an alternative to DMSO, sodium dodecyl sulfate (SDS) may be used to lyse the cells by adding 100 µl of 10% (w/v) SDS-HCl solution [1 g SDS dissolved in 10 ml of 0.01 M hydrochloric acid (HCl)] and incubating for ≤4 hr at 37°C.

6. Repeat separation using forceps (step 5) to remove the stromal layers (three silk films with interlaying hCSSCs and collagen) from the silk sponge seeded with hiNSCs. Transfer stromal layers to separate 1.5-ml microcentrifuge tubes containing 100 µl DMSO.

   The silk sponge retains medium in its pores. The presence of excess solution within the scaffold itself may limit the accuracy of the quantification of cells within the sponge, depending on the residual volume retained within the scaffold prior to cell lysis. As the assay is colorimetric, variations in total volume should be kept consistent to ensure

accurate measurement between samples. This limitation must be considered when comparing cell viability between the epithelial, stromal, and neuronal layers.

7. Vortex tubes briefly to mix the contents. Optional: In addition, using a hand-held homogenizer, degrade scaffolds by homogenizing for 30 s on ice three times.

   Homogenization may be performed if the scaffolds do not submerge well into the DMSO solution.

8. Incubate tubes for 10 min at 37°C/5% CO_2 to solubilize the formazan salt.

9. Transfer 100 µl of each DMSO-cell lysis solution to a 96-well plate. Measure absorbance at 540 nm using a spectrophotometer. Follow MTT manufacturer's instructions for quantification.

SUPPORT PROTOCOL 7

PROTEIN ANALYSIS OF CONDITIONED MEDIUM

Changes in secreted protein concentrations in response to stimuli (Basic Protocol 2) may be evaluated by ELISA for common neuropeptides and pro-inflammatory factors. Depending on the protein of interest, stock media may contain basal levels of the protein, thus requiring special consideration in data analysis or experimental setup, such as utilization of complete media in standard-curve preparation. The co-culture medium formulation applied in these studies is serum free, therefore removing many of the common serum contaminants from the media. Common secreted markers for sensory nerve activation or tissue inflammation are included in Table 2.

Materials

Stimulated corneal constructs (see Basic Protocol 2)
Dry ice (optional)
PBS (Sigma, cat. no. 11666789001; optional)

15-ml polypropylene centrifuge tubes
ELISA kit (e.g., for Substance P, CGRP, IL-1β, TNF-α, or MMP-9)

Table 2 Common Secreted Proteins Related to Nerve Activation or Tissue Inflammation Present in Conditioned Medium Isolated from Corneal Constructs

Protein[a]	Major cell source	Subclass	Concentration detected in conditioned medium[b]	Reference
SP	Sensory neurons (hiNSCs)	Neuropeptide	100-4000 pg/ml	Wang et al. (2018)
CGRP	Sensory neurons (hiNSCs)	Neuropeptide	300-800 pg/ml	Wang et al. (2018)
IL-1β	Epithelium (hCECs)	Pro-inflammatory cytokine	5-50 pg/ml	Deardorff et al. (2018), Wang et al. (2018)
TNF-α	Epithelium (hCECs)	Pro-inflammatory cytokine	20-350 pg/ml	Deardorff et al. (2018), Wang et al. (2018)
MMP-9	Epithelium (hCECs)	Matrix metalloproteinase	2000-5000 pg/ml	Deardorff et al. (2018)

[a]SP, substance P; CGRP, calcitonin gene–related peptide; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor-alpha; MMP-9, matrix metalloproteinase-9.
[b]The relative concentration ranges for each protein detected in conditioned medium in response to various chemical stimuli have been previously reported by our group (Deardorff et al., 2018; Wang et al., 2018).

−80°C freezer (optional)
Lyophilizer (optional)
Rocker

1. Collect 2 ml conditioned medium from stimulated corneal constructs in 15-ml polypropylene centrifuge tubes on ice. Add fresh medium to constructs and continue cultivation as needed.
2. Depending on the protein of interest, perform protein analysis via ELISA kit on the medium without further concentration or dilution. If concentration is necessary, place samples in dry ice or incubate overnight at −80°C to freeze the contents, followed by lyophilization and resuspension in 1 ml PBS.
3. Follow ELISA kit manufacturer's protocol for analysis.

IMMUNOHISTOCHEMISTRY OF CORNEAL CONSTRUCTS

SUPPORT PROTOCOL 8

Assessing changes in cell density and morphology after stimulation (Basic Protocol 2) requires fixation of the tissue and incubation with antibodies labeled with stable fluorophores prior to confocal microscopy. Whole-mount analysis is ideal, thereby removing the risk of tissue damage caused by traditional paraffin embedding and sectioning.

Materials

Stimulated corneal constructs (see Basic Protocol 2)
Sterile DPBS (Gibco, cat. no. 14190144)
4% (w/v) paraformaldehyde (PFA) in PBS (Santa Cruz Biotechnology, cat. no. sc-281692)
Permeabilization reagent: 0.1% (v/v) Triton-X 100 (Sigma, cat. no. X100) in DPBS
Blocking buffer: 5% (w/v) bovine serum albumin (BSA; Sigma, cat. no. 05470) in PBS (Sigma, cat. no. 11666789001)
Primary antibody (e.g., anti-BIII-tubulin)
1% (w/v) BSA (Sigma, cat. no. 05470) in PBS (Sigma, cat. no. 11666789001)
1× Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBST; see recipe)
Secondary antibody

6-well cell culture plate
Forceps
Mechanical rocker
Aluminum foil
Confocal microscope
ImageJ with NeuroJ (Meijering et al., 2004) or Simple Neurite Tracer (Longair, Baker, & Armstrong, 2011) plugin

Isolate corneal constructs

1. Aspirate culture medium from stimulated corneal constructs and gently transfer to a clean 6-well cell culture plate using forceps. Add 2 ml sterile DPBS to each construct. Aspirate DPBS and repeat wash twice more.

   At this point, using a sterile surgical scalpel, the constructs may be cut into smaller pieces for other applications, including western blot and RT-PCR analysis.

Fix, permeabilize, and block

2. Add 1 ml of 4% PFA in PBS to each construct and incubate for 1 hr at room temperature or overnight at 4°C.

   CAUTION: *This step must be completed in a chemical hood to minimize exposure to PFA fumes.*

The construct may be stored at 4°C in DPBS until further use. Long-term stability is dependent on the protein of interest.

3. Aspirate PFA and wash constructs with DPBS three times to remove residual fixative.
4. Permeabilize cells for antibody probing using 1 ml permeabilization reagent by static incubation for 15 min at room temperature. Aspirate reagent and wash constructs with DPBS three times.

   This step is dependent on the protein of interest. Permeabilization is needed for cytosolic antigens, requiring antibody perfusion into the cell. Probing for membrane proteins may not require permeabilization prior to antibody probing.

5. Block nonspecific binding sites with 2 ml blocking buffer for 1 hr at room temperature with gentle rocking.

   This step may be increased to overnight at 4°C, if necessary.

   The concentration of BSA may be decreased to 2% (w/v) BSA in PBS depending on the primary antibody. Refer to the manufacturer's recommendation for the antibody.

Incubate with antibodies

6. Prepare primary antibody solution according to the manufacturer's recommendation using 1% BSA in PBS as the diluent. Aspirate blocking buffer and incubate 1 ml primary antibody solution with constructs overnight at 4°C with gentle rocking.

   Commonly used concentrations include 1:50 to 1:200 primary antibody/1% (w/v) BSA in PBS. The total volume of antibody solution added to each construct is dependent on the proportion of construct used for IHC analysis. The construct may be transferred to a 12- or 24-well plate to reduce the volume of antibody solution required.

7. Wash constructs gently with 1× TBST three times.
8. Prepare secondary antibody solution according to the manufacturer's recommendation. Incubate 1 ml secondary antibody solution with constructs for 2 hr at room temperature or overnight at 4°C in the dark (cover with aluminum foil) with gentle rocking. Aspirate secondary antibody and wash constructs gently with 1× TBST three times.

   Commonly used concentrations include 1:1000 to 1:5000 secondary antibody/PBS. Secondary antibodies conjugated to a fluorophore are light sensitive; thus, this step should be performed in the dark, and samples should be covered with foil during incubation to reduce photobleaching.

Confocal microscopy

9. Perform imaging of whole mounts using a confocal microscope.

   The average thickness of the corneal constructs may vary depending on ECM production by hCSSCs, length of incubation, and collagen hydrogel thickness. Our studies have measured thicknesses of roughly 500 to 1000 μm after a 4- to 6-week cultivation period.

Measure nerve fiber length

10. To quantify nerve fiber length, utilize the ImageJ plugin NeuroJ (Meijering et al., 2004) for 2D images or Simple Neurite Tracer (Longair, Baker, & Armstrong, 2011) for 3D stacks of the regions of interest.

    Follow the available manuals for converting the tiff images to 8-bit black-and-white images and for automated analysis. Average $n > 3$ regions of interest per construct for technical replicates and $n > 3$ different constructs for biological replicates.

    The average nerve fiber length can vary depending on the region of the scaffold imaged, i.e., focusing on the sponge region versus the stromal region. Thus, comparison between

treatments and controls should be consistently quantified based on similar regions of interest.

ISOLATION OF TOTAL PROTEIN CONTENT FROM EACH CELL LAYER

SUPPORT PROTOCOL 9

The amount and type of protein in each layer of the corneal construct can be determined by separating the silk scaffolds containing the appropriate cell populations, i.e., hCECs seeded on the apical silk film, hCSSCs on the underlying silk films, and hiNSCs on the peripheral silk sponge. This separation is advantageous in allowing for biochemical analysis of each cell type to determine variances in cell-specific responses to stimulation while maintaining a complex tissue microenvironment during the experiment. Protein isolation can be carried out similarly to isolation from tissue explants, starting with using forceps to separate the layers.

Materials

Stimulated corneal constructs (see Basic Protocol 2)
PBS (Sigma, cat. no. 11666789001)
1× M-PER™ lysis buffer (M-PER™ Mammalian Protein Extraction Reagent, Thermo Fisher, cat. no. 78501) plus 1 × protease inhibitor cocktail (Sigma, cat. no. P8340) or 1× RIPA lysis buffer (see recipe), 4°C

Vortex
2-ml microcentrifuge tubes
Forceps
Hand-held homogenizer with blunt attachment
Refrigerated microcentrifuge

1. Aspirate medium from stimulated corneal constructs and gently wash each in 2 ml PBS three times.
2. Vortex cold 1× M-PER™ lysis buffer plus 1× protease inhibitor cocktail or cold 1× RIPA lysis buffer, vortex to mix, and add 200 µl lysis buffer to 2-ml microcentrifuge tubes on ice.
3. Gently separate topmost silk film and epithelium using forceps and transfer to a labeled tube containing lysis buffer on ice.
4. Gently separate stromal layers (three silk films and interlaying hCSSCs/ECM) from the silk sponge using forceps and transfer to another labeled tube on ice.
5. Transfer remaining silk sponge containing differentiated hiNSCs to another labeled tube on ice.
6. Using a hand-held homogenizer with a blunt attachment, homogenize each cellular fraction for 30 s each time for a total of three times, with 1 min in between. Maintain tubes on ice during homogenization. Then, vortex briefly to mix and incubate samples on ice for 20 min to ensure complete cell lysis.

   Ensure that samples do not overheat by keeping the tubes on ice at all times. Additionally, ensure that samples are submerged in lysis buffer and add additional lysis buffer if needed so that all cells are adequately lysed.

7. Centrifuge samples for 15 min at 14,000 rpm, 4°C, to pellet insoluble debris. Transfer supernatants to clean, labeled 2-ml microcentrifuge tubes and store at −20°C until further use (see Support Protocol 10).

   Aliquot protein samples (25 µl per fraction) to minimize freeze-thaw cycles and maintain protein integrity.

SUPPORT PROTOCOL 10

CONCENTRATION MEASUREMENT OF ISOLATED PROTEIN

A standard BCA assay can be applied to determine the relative protein concentration following cell lysis and protein isolation from each layer of the tissue construct, as described in Support Protocol 9.

Materials

BSA (Sigma, cat. no. 05470)
Lysis buffer: 1× M-PER™ lysis buffer (M-PER™ Mammalian Protein Extraction Reagent, Thermo Fisher, cat. no. 78501) plus 1× protease inhibitor cocktail (Sigma, cat. no. P8340) or 1× RIPA lysis buffer (see recipe)
BCA protein assay (Thermo Scientific, cat. no. 23250), including:
Reagent A, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate, and sodium hydroxide
Reagent B, containing cupric sulfate

1.5-ml microcentrifuge tubes
96-well plate
Spectrophotometer

Additional reagents and equipment for total protein isolation from cellular layers of human corneal constructs (see Support Protocol 9)

1. Isolate total protein from each cellular layer of human corneal constructs, as described in Support Protocol 9.
2. Prepare a BSA standard curve by dissolving 0.002 g BSA in 1 ml lysis buffer and performing sequential two-fold dilutions in lysis buffer in 1.5-ml microcentrifuge tubes to generate a total of seven standards ranging from 2000 to 15.63 µg/ml.

   Briefly vortex each sample in between dilutions to mix. Change pipet tips between samples.
3. Prepare working reagent by mixing Reagent A and Reagent B from BCA protein assay at a ratio of 50:1.

   Perform measurements with at least two technical replicates. Prepare a total of 5.6 ml working reagent for six biological replicates, seven standards, and one blank.
4. Add 200 µl working reagent to each well of a 96-well plate. Add 10 µl of each standard, unknown sample, and blank to appropriate wells. Pipet solution up and down to mix.
5. Incubate samples at 37°C/5% CO_2 for 30 min. Measure absorbance at 562 nm using a spectrophotometer.
6. Plot standard concentration relative to absorbance based on a linear correlation to generate a standard curve. Extrapolate unknown concentrations based on standard curve.

   Technical replicates should be consistent between standards and samples, with little variability.

SUPPORT PROTOCOL 11

WESTERN BLOT ANALYSIS OF PROTEIN ISOLATED FROM CORNEAL CONSTRUCTS

Protein analysis of the corneal constructs can provide useful information regarding signal transduction pathways affected by chemical stimulation (Basic Protocol 2). By isolating each cellular layer independently, one can determine the specific effects on the epithelium, stroma, and neuronal cell populations. Key phenotypic protein markers are listed in

Table 3 Phenotypic Markers of Each Cell Type Present in the Corneal Construct

Cell type	Protein marker
Epithelial cells (hCECs)	Keratin 3, Keratin 12
Keratocytes (hCSSCs)	Keratocan, Lumican, Aldehyde dehydrogenase 3a, Decorin, Collagen types I and V
Sensory neurons (hiNSCs)	BIII-tubulin, TRPV1, TRPA1, TRPM8[a]

[a]TRPV1, transient receptor potential cation channel subfamily V member 1; TRPA1, transient receptor potential cation channel subfamily A member 1; TRPM8, transient receptor potential cation channel subfamily M member 8.

Table 3 for each cell type. Standard loading controls include glyceraldehyde dehydrogenase (GAPDH), β-actin, and β-tubulin.

Materials

Protein isolates from corneal constructs (see Support Protocol 9)
4× SDS–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (see recipe)
1× Tris-glycine running buffer (see recipe)
1× Tris-glycine transfer buffer (see recipe)
5% (v/v) BSA (Sigma, cat. no. 05470) in 1× TBST (see recipe)
Primary and secondary antibody solutions
1× TBST (see recipe)

Vortex
Microcentrifuge
60°C heating block
Polyacrylamide gel (4%−20% Tris-glycine gradient gel, Invitrogen, cat. no. XP0420A)
SDS-PAGE chamber with power source
Filter paper
Nitrocellulose membrane
Transfer box with power source
Forceps
Rocker
Aluminum foil
Gel imager

Perform SDS-PAGE

1. Prepare samples for SDS-PAGE by combining 21 µl protein isolate from each corneal construct (in 2-ml microcentrifuge tube) with 7 µl of 4× SDS-PAGE loading buffer, with a final protein concentration between 20 and 50 µg/ml (see Support Protocol 10).
2. Vortex samples briefly and centrifuge 30 s at 14,000 rpm to sediment solution.
3. Heat samples on a 60°C heating block for 10 min, followed by centrifugation for 30 s at 14,000 rpm.
4. Load samples onto a polyacrylamide gel. Add 1× Tris-glycine running buffer to SDS-PAGE chamber.
5. Run gel at 120 V for 1.5 hr or until the bromophenol band reaches the bottom of the gel.

Transfer protein to nitrocellulose membrane

6. Remove gel and soak in 1× Tris-glycine transfer buffer briefly. Prepare western blot setup by generating a transfer sandwich as follows, from bottom to top: filter paper, gel, nitrocellulose membrane, and filter paper.
7. Set up transfer sandwich in a transfer box with 1× Tris-glycine transfer buffer. Perform transfer at 100 V for 1 hr at 4°C.

Block and incubate with antibodies

8. Following transfer, carefully remove nitrocellulose membrane using forceps and block in 5% BSA in 1× TBST in a clean container (e.g., a small petri dish) for 1 hr at room temperature with rocking.
9. Incubate with primary antibody solution (1:1000) overnight at 4°C with rocking. After incubation, wash membrane three times with 1× TBST and incubate with secondary antibody solution (1:10,000) for 1 to 2 hr at room temperature with rocking and covered with aluminum foil.
10. Wash membrane three times with 1× TBST for 5 min each with rocking.
11. Image fluorescence (for fluorescent secondary antibodies) or light emissions (for horseradish peroxidase–coupled secondary antibodies) from probed membrane using a gel imager.

REAGENTS AND SOLUTIONS

Complete corneal co-culture medium

1 mM ascorbic acid-2-phosphate
10 ng/ml basic FGF-2
0.1 ng/ml transforming growth factor-β3 (TGF-β3)
100 µg/ml penicillin
100 µg/ml streptomycin
5 µg/ml gentamicin
50 ng/ml NGF
Advanced DMEM (with or without phenol red; Gibco, cat. no. 12491015) to 500 ml
Filter-sterilize using 0.2-µm filter flask
Store ≤1 month at 4°C and minimize heating to reduce growth-factor degradation

Growth factors should be dissolved and aliquoted upon receipt to minimize freeze-thaw cycles.

Complete corneal epithelial medium

Corneal epithelial cell growth kit (ATCC® PCS-700-040™): apo-transferrin (5 µg/ml), epinephrine (1.0 µM), extract P (0.4%), hydrocortisone (100 ng/ml), L-glutamine (6 mM), rh insulin (5 µg/ml), and CE Growth Factor (proprietary formulation, ATCC)
1× antibiotic-antimycotic
Corneal epithelial medium (ATCC, cat. no. ATCC® PCS-700-030™) to 500 ml
Filter-sterilize using 0.2-µm filter flask
Store ≤1 month at 4°C and minimize heating to reduce growth-factor degradation

Complete hCSSC differentiation medium

1 mM ascorbic acid-2-phosphate
10 ng/ml basic FGF-2
0.1 ng/ml transforming growth factor-β3 (TGF-β3)
100 µg/ml penicillin
100 µg/ml streptomycin

5 µg/ml gentamicin
Advanced DMEM (Gibco, cat. no. 12491015) to 500 ml
Filter-sterilize using 0.2-µm filter flask
Store ≤1 month at 4°C and minimize heating to reduce growth-factor degradation

Growth factors should be dissolved and aliquoted upon receipt to minimize freeze-thaw cycles.

Complete hCSSC proliferation medium

Low-glucose DMEM
17.7 g/l MCDB-201, pH 6.67 (200 ml total volume)
2% (v/v) FBS
1% (v/v) AlbuMAX™ (lipid-rich BSA; Gibco, cat. no. 11021029)
0.1 mM ascorbic acid-2-phosphate
5 mg/ml transferrin
5 mg/ml insulin
5 ng/ml selenous acid
100 µg/ml penicillin
100 µg/ml streptomycin
5 µg/ml gentamicin
10 ng/ml EGF
10^{-8} M dexamethasone
100 ng/ml cholera toxin
Low-glucose DMEM to 500 ml
Filter-sterilize using 0.2-µm filter flask
Store ≤1 month at 4°C and minimize heating to reduce growth-factor degradation

Growth factors should be dissolved and aliquoted upon receipt to minimize freeze-thaw cycles.

Complete knockout medium

20% (v/v) knockout serum replacement (Gibco, cat. no. 10828010)
0.1 mM β-mercaptoethanol
1% (v/v) GlutaMAX™ (Gibco, cat. no. 35050061)
1× antibiotic-antimycotic
Knockout medium (Gibco, cat. no. 12618013) to 500 ml
Filter-sterilize using 0.2-µm filter flask
Store ≤1 month at 4°C and minimize heating to reduce growth-factor degradation

Complete neurobasal medium

1% (v/v) GlutaMAX™ (Gibco, cat. no. 35050061)
1× antibiotic-antimycotic
2% (v/v) B-27™ supplement (Gibco, cat. no. 17504044)
Neurobasal medium (Gibco, cat. no. 21103049) to 500 ml
Filter-sterilize using 0.2-µm filter flask
Store ≤1 month at 4°C and minimize heating to reduce growth-factor degradation

Complete sensory nerve medium

1× GlutaMAX™ (Gibco, cat. no. 35050061)
1× antibiotic-antimycotic
25 ng/ml NGF
25 ng/ml brain-derived neurotrophic factor (BDNF)
25 ng/ml glial cell–derived neurotrophic factor (GDNF)
10 µM DAPT (CAS # 208255-80-5)

10 µM SU-5402 (CAS # 215543-92-3)
3 µM CHIR99021 (CAS # 252917-06-9)
Neurobasal medium (Gibco, cat. no. 21103049) to 500 ml
Filter-sterilize using 0.2-µm filter flask
Store ≤1 week at 4°C and minimize heating to reduce growth-factor degradation

Growth factors should be dissolved and aliquoted upon receipt to minimize freeze-thaw cycles.

EGF stamping solution

2 mg/ml rat-tail collagen
200 ng/ml NGF
200 ng/ml EGF
100 ng/ml hepatic growth factor
100 ng/ml keratinocyte growth factor
PBS to 100 µl
Add to 0.5-ml microcentrifuge tube
Mix by gently pipetting up and down
Store ≤1 week at 4°C

Growth factors should be dissolved and aliquoted upon receipt to minimize freeze-thaw cycles.

RIPA lysis buffer, 1×

150 mM NaCl
1% (v/v) Nonidet P-40 (NP-40)
0.5% (w/v) sodium deoxycholate
0.1% (w/v) SDS
25 mM Tris·HCl, pH 7.4
1× protease inhibitor cocktail (Sigma, cat. no. P8340)
H_2O to 10 ml
Store ≤1 week at 4°C

SDS-PAGE loading buffer, 4×

20 mM Tris·HCl, pH 6.8
0.4 M dithiothreitol (DTT)
0.4% (w/v) bromophenol blue
40% (v/v) glycerol
H_2O to 10 ml
Aliquot and store ≤6 months at −20°C

TBST, 1×

137 mM NaCl
2.7 mM KCl
19 mM Tris base
H_2O to 1 L
Adjust final pH to 7.4 by adding HCl, as needed
Add 1 ml Tween-20 (Sigma, cat. no. P1379) to 0.1% (v/v)
Store ≤1 month at room temperature

Tris-glycine running buffer, 1×

30 g Tris base (0.25 M final)
144 g glycine (1.92 M final)
10 g SDS [10% (w/v) final]
H_2O to 1 L

Dissolve while stirring to make 10× buffer
Store ≤6 months at room temperature or 4°C
Dilute to 1× immediately prior to use

Tris-glycine transfer buffer, 1×

24.2 g Tris base (0.2 M final)
150.1 g glycine (2 M final)
H_2O to 1 L
Dissolve while stirring to make 20× buffer
Store ≤6 months at room temperature or 4°C
Dilute to 1× immediately prior to use

COMMENTARY

Background Information

Advantages of the application of this 3D co-culture approach over conventional 2D conventional methods include higher physiological relevance due to the presence of a self-assembled ECM, co-culture conditions that collectively influence individual cell responses, the separation of functional units in distinct scaffolds for analysis of each cell type, and sustained cultivation for weeks or even months to permit both acute and chronic assessments of treatments (versus only acute assessments). These factors may significantly improve measurement of the response to chemical toxins or microenvironmental changes, such as prolonged exposure to high glucose.

The corneal tissue model described has relative dimensions consistent with the average diameter of the human cornea proper, e.g., 12-mm diameter for the inside rim of the sponge, similar to the corneal diameter of the human eye (Rufer, Schroder, & Erb, 2005). In terms of human corneal thickness, the average may range from 0.5 to 0.6 mm, with significant variability from person to person (Doughty & Zaman, 2000). It is widely accepted that dissection and maintenance of cadaveric corneal tissue in culture medium result in significant swelling that correlates to loss in transparency of the tissue (Meek, Leonard, Connon, Dennis, & Khan, 2003) with an average thickness of >1 mm ex vivo (Thuret, Manissolle, Campos-Guyotat, Guyotat, & Gain, 2005). Likewise, the corneal tissue model is assembled with a thickness of 1 mm; however, relatively high tissue transparency is maintained in this system. De-swelling of the construct may be performed using standard dextran-containing medium prior to in vivo transplantation, if needed (Borderie, Baudrimont, Lopez, Carvajal, & Laroche, 1997; Wolf et al., 2009).

Although our model represents a recent advance in developing a functional corneal system, further studies are warranted to characterize the predictability of the construct as an in vitro platform to screen the safety and efficacy of pharmaceuticals and chemicals applied to the human cornea. Given the diversity within the human population and the various animal models used in the study of corneal pain, identifying reliable pain biomarkers remains a common goal within the field (McKay et al., 2018). The application and further development of this human-focused in vitro model may aid in bridging this gap.

Critical Parameters and Troubleshooting

Preparation of silk scaffolds (Support Protocols 2 and 3) requires strict adherence to standard silk isolation and processing to ensure quality control. The isolated silk solution should be used within 1 month following dilution and stored at 4°C. Alternatively, lyophilized silk is much more stable for long periods of time and can be maintained at room temperature. Maintenance of the corneal constructs (Basic Protocol 1) requires proper culture conditions at 37°C/5% CO_2 and aseptic approaches to minimize contamination. Given that the model contains primary cells, only low passages of hCECs and hCSSCs (passages less than 4 to 5) should be utilized in order to allow sustainability over long culture times (≥4 weeks). The chosen endpoint assessments will depend on the research question, with multiple methods available to determine morphological, biochemical, and functional changes in the tissue structure following exposure to chemical irritants. Table 4 summarizes common troubleshooting issues that may arise during assembly, maintenance, or characterization of the corneal constructs.

Table 4 Troubleshooting Guide for Corneal Construct Assembly and Characterization

Problem	Possible cause	Solution
hCECs do not attach to silk film and are floating or adhered to cell culture plate	• Lack of collagen coating on film	• Always prepare fresh EGF stamping solution and growth factors prior to coating of film.
	• Older passage of hCECs	• Use hCEC passages <5; these cells are particularly sensitive to aging and thawing.
	• Addition of medium to well prior to cell adhesion	• Visualize proper cell adherence to film by microscopy prior to addition of medium to well.
Lack of confluent hCEC layer	• Older passage of hCECs	• Utilize only hCEC passages <5; these cells are particularly sensitive to aging and thawing.
	• Absence of epithelial growth factors in medium	• Poor cell proliferation
	• Prepare fresh medium containing standard epithelial growth factors, e.g., EGF, BPE.	• Substitute corneal epithelial cell line, e.g., HCE-TJ, or limbal epithelial stem cell source to improve epithelial barrier integrity.
Poor hiNSC proliferation on MEF feeder plate	• Older passage of MEFs	• Utilize only MEF passages <5.
	• Lack of confluent feeder layer	• Seed MEFs at high density and allow for 100% confluence prior to inactivation.
	• Absence of fresh FGF in medium	• Prepare fresh medium containing FGF immediately prior to use.
hCSSCs do not attach to RGD-coated films and are floating or adhered to cell culture plate	• Low pH of films may cause cell death	• Ensure that MES buffer pH is neutral (pH 6.5-7). Wash films in PBS prior to use in cell culture.
	• Lack of RGD functionalization of films	• Utilize freshly dissolved RGD peptide for crosslinking to films.
	• Addition of medium to petri dish prior to cell adhesion	• Visualize proper cell adherence to films by microscopy prior to addition of medium to petri dish.
Frequent bacterial or fungal contamination	• Contamination of scaffolds prior to seeding	• Autoclave silk sponges after cutting and immediately prior to use in cell culture. Sterilize silk films under UV light (30 min each side) and immerse in 70% (v/v) ethanol in distilled water prior to RGD coupling. Filter-sterilize (0.2 μm) crosslinking solutions and RGD peptide prior to incubation with sterile films. Autoclave waffle-shaped PDMS mold prior to inclusion in system.

continued

Table 4 Troubleshooting Guide for Corneal Construct Assembly and Characterization, *continued*

Problem	Possible cause	Solution
	• Lack of aseptic technique during cell culture	• Adopt standard aseptic approaches during construct assembly and maintenance.
Low total protein isolated	• Inadequate cell lysis	• Ensure that isolated cell layer is submerged in lysis buffer.
	• Protein degradation	• Include protease inhibitors in lysis buffer and maintain samples on ice during homogenization and incubation steps. Aliquot samples to reduce freeze-thaw cycles.
	• Cell loss during cultivation	• Monitor medium color between medium changes; slight change should be noticeable, indicating active metabolism. Ensure that cells properly attach to scaffolds prior to assembly of construct.
Unusually high total protein isolated	• Inadequate removal of medium prior to construct lysis	• Be sure to gently wash or immerse construct in PBS prior to isolation.
	• Bacterial or fungal contamination	• Monitor medium color between medium changes; severe change in color to yellow and cloudiness of medium are common indicators of contamination. Visually inspect constructs for contamination.
Poor IHC images	• High silk autofluorescence in green channel	• Choose secondary antibodies with emission wavelengths outside of green-channel range, e.g., λ_{em} = 480-638 nm.
	• Lack of antibody binding	• Incubate primary and secondary antibodies with construct overnight at 4°C with rocking to allow perfusion into tissue.

Understanding Results

Assembly of the corneal tissue model (Basic Protocol 1) is expected to yield intact corneal constructs that are stable for at least 4 weeks up to 8 weeks for applications in drug screening or biological studies (Basic Protocol 2). Cultures beyond 8 weeks may be possible but are limited by the passage number of the primary hCECs and hCSSCs. This timeframe is consistent with self-assembled stromal models that rely on de novo ECM deposition by human corneal fibroblasts and can be maintained for 4 to 11 weeks (Guo et al., 2007; Ren et al., 2008), followed by analysis of the biochemical responses to various chemical or environmental stimuli (McKay, Hjortdal, Priyadarsini, & Karamichos, 2017; McKay, Hjortdal, Sejersen, & Karamichos, 2017).

A total of 12 constructs are usually assembled per experiment, allowing for sufficient technical replicates for each characterization. Assembly of 12 constructs requires at least nine RGD-coated silk films seeded with hCSSCs based on isolation of four stacks per three films. Confirmation of phenotypic marker expression for each cell type should be performed using IHC (Support Protocol 8) or western blot (Support Protocol 11) approaches to verify that successful differentiation has been achieved (refer to Table 3). Furthermore, the morphology of each cell type can be validated by microscopy following seeding on

Table 5 Relative Timeframes for Completion of Each Step in the Assembly, Stimulation, and Characterization of the Corneal Tissue Model

Stage	Objective	Active time	Total time[a]
Scaffold preparation	PDMS molds	30 min	1 day, with overnight incubation
	Silk isolation	3-4 hr	5-6 days
	Patterned, RGD-conjugated silk films	3 hr	2.5 days, with 48 hr incubation
	Non-patterned silk films with EGF stamping	2-3 hr	2.5 days, with 48 hr incubation
	Silk sponges	2 hr	5 days
Cell culture	hCEC culture	2 hr	1 week of culture from frozen stock + 2 days on silk film = ~9 days
	hCSSC culture	2 hr	2-3 days of culture from frozen stock + 2 days on silk film = ~5 days
	hiNSC culture	5 hr	1 week to culture MEF feeder plate + 0.5 day to inactivate + 1-2 weeks of hiNSC culture on MEFs + 10 days of sensory differentiation = ~3-5 weeks
Cultivation	Assembly of construct	2−3 hr	0.5 day
	Maintenance of construct	12 hr	4 weeks, with medium changes every other day
Chemical stimulation	Topical application of chemical to construct	1 hr	1 day, with overnight incubation
Characterization	MTT toxicity assay	1.5 hr	4 hr
	Conditioned medium isolation and ELISA	3 hr	1.5 days, with overnight incubation
	IHC	2 hr + imaging	2.5 days
	Cell lysis and protein isolation	30 min	2 hr
	Measurement of protein concentration by BCA assay	30 min	1 hr
	Western blot	1 hr	1.5 days, with overnight incubation

[a]The amount of time may vary significantly depending on the user and the total number of constructs generated. These timeframes are based on 12 constructs per experiment.

silk material and after 4 weeks of incubation to verify stratification of the epithelial barrier and keratocyte quiescence. Primary epithelial cells have been found to proliferate slowly on silk films in the co-culture medium, thus requiring a high cell number/density at seeding. Furthermore, multi-layer stratification of the epithelial layer may require utilization of limbal epithelial stem cells with high proliferative capabilities, which may be derived from pluripotent stem cells (Mikhailova et al., 2016; Zhang et al., 2017).

Time Considerations

Table 5 describes relative time considerations for construction and application of the corneal tissue model.

Acknowledgments

The authors acknowledge funding support from the NIH (R01EY020856, P41EB002520; D.K.). The authors declare no conflicts of interest.

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Key References

Rockwood et al. (2011). See above.

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Details the development and characterization of stable hiNSC lines.

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Describes the growth factor and inhibitor cocktail needed to promote sensory nerve differentiation from stem cells.

Wang et al. (2018). See above.

Details the development and application of the corneal tissue model.

McKay et al. (2018). See above.

Details the biological mechanisms and clinical features of corneal pain and the rationale and methodology for developing in vitro models to study this condition.