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Advanced Imaging and Tissue Engineering of the Human Limbal Epithelial Stem Cell Niche

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Abstract

The limbal epithelial stem cell niche provides a unique, physically protective environment in which limbal epithelial stem cells reside in close proximity with accessory cell types and their secreted factors. The use of advanced imaging techniques is described to visualize the niche in three dimensions in native human corneal tissue. In addition, a protocol is provided for the isolation and culture of three different cell types, including human limbal epithelial stem cells from the limbal niche of human donor tissue. Finally, the process of incorporating these cells within plastic compressed collagen constructs to form a tissue-engineered corneal limbus is described and how immunohistochemical techniques may be applied to characterize cell phenotype therein.

Keywords

Limbal epithelial stem cell niche; 3View imaging; Limbal epithelial stem cells; Limbal fibroblasts; Corneal stromal stem cells; Immunohistochemistry; Tissue engineering

1 Introduction

Limbal epithelial stem cells (LESC) are responsible for maintenance of the corneal epithelium during both normal homeostasis and in response to injury [1]. They reside within the limbal epithelial stem cell niche, a unique environment that ensures their normal function [2–4].

Using advanced imaging techniques (serial block face scanning electron microscopy (SBFSEM)), imaging of the limbal epithelial stem cell niche within native human cornea in three dimensions (3-D) is now possible. In this technique, ultrathin sections (100 nm) are serially cut from the embedded tissue and the newly exposed tissue surface is subsequently imaged. This process can be repeated many times enabling a complete 3-D reconstruction on a micrometer scale such that cellular interactions and organization can be observed [5].

The limbal epithelial stem cells themselves along with accessory cell types (limbal fibroblasts and corneal stromal stem cells found in the underlying stroma) may be isolated from donor tissue following specific combinations of enzymatic digestion and/or mechanical

disruption of tissue architecture to selectively isolate the different cell populations. Culture conditions for each cell type differ and are designed to promote proliferation/preserve phenotype of each [4, 6].

Following culture of these different cell types, a tissue- engineered limbus may be formed from a collagen hydrogel. Collagen hydrogels can be produced by neutralizing the pH of acid-solubilized type I collagen with heating, allowing fibrillogenesis to occur. Then, by applying a hydrophilic absorber to the top surface of the hydrogel, water can be wicked upwards to produce a thin, mechanically strong, transparent construct (~150 μm thick) that may be utilized as an experimental model of the native limbus. This technique was first proposed by Brown et al. [7] but we have since iteratively improved the method to increase reproducibility and reliability [8, 9]. Collagen constructs produced using this newer methodology are referred to as Real Architecture For 3D Tissue, or RAFT. Most recently, in collaboration with an industrial partner, we have developed a method for producing absorbers with a capability to print different topographies onto the surface of RAFT constructs whilst simultaneously wicking water away [10]. These surface topographies recreate the physical aspects of the LESC niche. LESC can be seeded onto the surface of the constructs and air-lifted to induce stratification to produce a multilayered epithelium, similar to that of native cornea. Furthermore, limbal fibroblasts may be incorporated within the collagen constructs if desired without a loss of cell viability to further support overlying LESC [8].

Once formed, immunohistochemistry (either whole-/flat-mount or sections) may be performed to characterize cell phenotype within and/or on collagen constructs. The transcription factor, p63α, along with markers of corneal differentiation such as cytokeratin (CK) 3 [11] is typically used for expression of putative LESC markers.

2 Materials

Prepare all solutions using deionized water. Prepare and store all reagents at room temperature (unless indicated otherwise). Dispose of all waste materials according to waste disposal regulations. Read all appropriate COSHH and Risk Assessment forms prior to beginning work.

1. Human donor corneal tissue: either whole cornea or corneoscleral rims (*see* Note 1).
2. Dulbecco's phosphate buffered saline (DPBS) 1×, without calcium or magnesium ions (Life Technologies).
3. DMEM with 4.5 g/L glucose and GlutaMAX (Life Technologies). Store at 4 °C.
4. DMEM/F12 (Life Technologies). Store at 4 °C.
5. Fetal bovine serum (FBS). Store at –20 °C.
6. 100× antibiotic, anti-mycotic (anti-anti) (Life Technologies). Store at –20 °C.

[1]Cadaveric donor corneal rims with appropriate research consent are used to isolate hLE, hLF, and hCSSC. Tissue can be fresh postmortem or Optisol-stored tissue. Dispose of human tissue as defined in local policies.

7. 0.5 % Trypsin-EDTA without phenol red (10×) (Life Technologies). Store at −20 °C.
8. 0.05 % Trypsin-EDTA without phenol red (1×): Dilute 5 mL of 0.5 % trypsin-EDTA without phenol red in 45 mL DPBS. Store at 4 °C.
9. Sodium hydroxide (NaOH).
10. Hydrochloric acid (HCl).
11. TT solution: Add 1.36 mg T3 (Sigma-Aldrich) to 2 mL of 0.02 N NaOH. Add 8 mL of DPBS. Store 100 μL T3 stock aliquots at −20 °C. Add 50 mg of transferrin (Sigma-Aldrich) to 6 mL of DPBS. Add 100 μL of T3 stock. Make up to 100 mL with DPBS. Filter sterilize using a 0.22 μm filter. Store at −20 °C.
12. Adenine solution: Add 250 mg adenine powder (Sigma-Aldrich) to 10 mL of 1 M HCl. Make up to 100 mL with water. Filter sterilize using a 0.22 μm filter. Store at −20 °C.
13. Hydrocortisone solution: Dissolve 50 mg of hydrocortisone powder (Sigma-Aldrich) in 10 mL of ethanol. Filter sterilize using a 0.22 μm filter. Add 1 mL of this to 11.5 mL of DPBS and store at −20 °C.
14. Cholera toxin solution: Add 1 mg cholera toxin (Sigma-Aldrich) to 1.18 mL of water. Add 100 μL of this to 10 mL of DMEM/F12 containing 10 % FBS. Filter sterilize using a 0.22 μm filter. Store at −20 °C.
15. Corneal epithelial cell culture medium (CECM): Mix together 250 mL DMEM/F12 (Life Technologies) with 250 mL DMEM. Remove 71.25 mL of this mixture. Add 50 mL of FBS, 5 mL of anti-anti, 5 mL of TT solution, 5 mL of 18.5 mM adenine solution, 0.5 mL of 5 mg/mL hydrocortisone solution, 0.5 mL of 10 μM cholera toxin solution, 250 μL of 10 mg/mL insulin (Sigma-Aldrich). Store at 4 °C. Add 50 μL of 10 μg/mL epidermal growth factor (EGF) (Life Technologies) in DPBS (store at −20 °C) to 50 mL CECM immediately prior to use.
16. hLF medium: Remove 55 mL from a 500 mL bottle of DMEM. Add 50 mL of FBS and 5 mL of anti-anti to give final concentrations of 10 % FBS and 1× anti-anti. Store at 4 °C.
17. 4 % Paraformaldehyde (PFA).
18. Goat serum (Sigma-Aldrich). Store at −20 °C.
19. Triton X-100.
20. Isotype controls. Store at 4 °C.
21. Alexa Fluor secondary antibodies (Life Technologies) (store at 4 °C for short-term storage or at −20 °C for long-term storage).
22. Phalloidin (FITC or TRITC conjugates, Sigma-Aldrich). Store at −20 °C.
23. Vectashield mounting medium (with DAPI or PI, Vector Laboratories). Store at 4 °C.

24. Capture antibody solution: 2 % goat serum in DPBS.
25. Ethanol.
26. Trypan blue.
27. Cell culture inserts with PTFE membrane, 300 mm diameter, 0.4 μm pore (Millipore).
28. 0.22 μm sterile filters (Fisher Scientific).
29. Tissue culture plates and flasks.
30. Glass microscope slides.
31. Coverslips.
32. Hemocytometer.
33. Nail polish.
34. Forceps, small scissors, scalpels.
35. Hot plate.
36. Water bath.
37. Dissecting microscope.
38. Bright-field light microscope.
39. (Confocal) fluorescence microscope.

2.1 Specific Reagents Required for 3View Imaging

1. *0.3 M sodium cacodylate buffer stock*: Dissolve 64 g of sodium cacodylate in 900 mL of water. Adjust the pH to 7.4 and make up to 1 L with distilled water.
2. *10 % aqueous paraformaldehyde*: Add 10 g of paraformaldehyde to 100 mL of distilled water and heat to 65 °C in a fume cupboard. Add 2–5 drops of 1 M NaOH to clarify the solution. Filter through a 0.22 μm filter and allow to cool before use.
3. *Karnovsky's fixative*: Add 100 mL of freshly prepared 10 % paraformaldehyde to 120 mL of 25 % glutaraldehyde (Agar Scientific) and combine with 400 mL of 0.2 M sodium cacodylate buffer. Adjust the pH to 7.4 and make up to 1 L with distilled water. Store at −20 °C.
4. *3 % potassium ferricyanide solution*: Add 3 g of potassium ferricyanide (TAAB Laboratories Equipment Ltd.) to 100 mL of 0.3 M sodium cacodylate buffered to pH 7.4. Store at 4 °C.
5. *2 % aqueous osmium tetroxide solution*: (*see* Note 2).

[2]Particular care must be taken with osmium tetroxide. The vapor pressure and the acute toxicity of osmium are high. Use under a fume cupboard and wear suitable protective clothing and gloves. Used osmium and washes should be stored for safe disposal.

6. *1 % thiocarbohydrazide solution*: Add 0.1 g of thiocarbohydrazide (Sigma-Aldrich) to 10 mL of distilled water and place in a 60 °C oven for 1 h. Agitate every 15 min to facilitate dissolving. Filter through a 0.22 μm filter and leave to cool.
7. *1 % uranyl acetate*: Prepare solution just before use. Dissolve 0.1 g of uranyl acetate (Agar Scientific) in 10 mL of water. Store the solution at 4 °C (*see* Note 3).
8. *Conductive silver epoxy kit*: Prepare a fresh mix of an equal volume of the two components (Agar Scientific) just before attachment of the resin block onto the cryopin.
9. *1 % toluidine blue*: Dissolve 1 g of sodium borate (Sigma-Aldrich) in 100 mL of water. Add 1 g of toluidine blue (Agar Scientific). Gently mix until complete dissolved. Filter through a 0.22 μm filter.
10. *Walton's lead aspartate solution*: Dissolve 0.066 g of lead nitrate in 10 mL of 0.03 M aspartic solution and adjust the pH to 5.5 using 1 N potassium hydroxide. Place the solution in a 60 °C oven for 30 min prior to use.
11. *Durcupan ACM resins*: (Sigma-Aldrich).
    a. Single component A, M epoxy resin.
    b. Single component B, hardener 964.
    c. Single component C, accelerator 960.
    d. Single component D.
12. *Durcupan ACM resin*: Combine 11.4 g of component A, 10 g of component B, 0.3 g of component C, and 0.1 g of component D.
13. Acetone.
14. DePeX mounting media (VWR).
15. Flat embedding molds.
16. Glass knives (Agar Scientific).
17. Diamond knife (dEYEmond, 45 Histo).
18. Single edge stainless steel razor blades (Agar Scientific).
19. Cryopin (Agar Scientific).
20. Sputter coater (Emitech).
21. Scanning electron microscope.
22. 3View ultramicrotome (Gatan).
23. Wacom workstation Cintiq 22HD touch and interactive pen.
24. AMIRA software (Visualization Science Group, FEI).

[3]Uranyl acetate is very toxic and radioactive. Use only in a fume cupboard and wear suitable protective clothing and gloves. Used uranyl acetate including washes should be stored for safe disposal.

2.2 Specific Reagents Required for Isolation and Culture of Human Limbal Epithelial Cells (hLEC)

1. *1.2 IU/mL dispase*: Dissolve 10 IU dispase II (Roche Diagnostics) in 1 mL of DPBS. Store at −20 °C. Immediately prior to use, combine 0.6 mL of 10 IU/mL dispase II with 4.4 mL of CECM without EGF.
2. *3T3-J2 medium*: Remove 55 mL from a bottle of DMEM and add 50 mL of adult bovine serum (Labtech) and 5 mL of anti-anti. Store at 4 °C.
3. *Growth-arrested 3T3-J2 cells*: Maintain 3T3-J2 cells in 3T3-J2 medium. To expand, passage cells one in ten twice per week using 0.05 % trypsin-EDTA with mechanical agitation. Growth arrest 3T3-J2 cells at 70–80 % confluency using 4ug/mL mitomycin-C in 3T3-J2 medium for 2 h at 37 °C in a humidified incubator with 5 % CO_2.

2.3 Specific Reagents Required for Isolation and Culture of Human Limbal Fibroblasts (hLF)

1. *2 mg/mL collagenase*: Dissolve 10 mg collagenase type 1 powder in 5 mL of hLF media. Filter sterilize through a 0.22 μm filter prior to use. Make fresh each time.

2.4 Specific Reagents Required for Isolation and Culture of Human Corneal Stromal Stem Cells (hCSSC)

1. *MCDB-201 stem cell medium*: Add 17.7 g of MCDB-201 powdered stem cell medium (Sigma-Aldrich) to 900 mL of water and stir gently. Adjust the pH to 7.1–7.3 using 1 N NaOH or HCl as needed. Filter sterilize through a 0.22 μm filter prior to use. Store at 4 °C.
2. *hcssc medium*: Mix 300 mL of DMEM-low glucose (Life Technologies), 200 mL of MCDB-201 medium, 10 mL of FBS, 5 mL of AlbuMaxI (Life Technologies), 5 mL of 100× penicillin/streptomycin (CellGro), 1 mL of l-ascorbic acid-2-phosphate sesquimagnesium salt, 0.5 mL of insulin, transferrin, selenium solution (Life Technologies), 0.5 mL of platelet-derived growth factor (R&D Biosystems) together. Add EGF to achieve a final concentration of 10 mg/mL, dexamethasone (Sigma-Aldrich) to 50 mM, cholera toxin to 1 mg/mL, and gentamicin (Life Technologies) to 50 mg/mL. hCSSC medium should be stored protected from light at 4 °C.
3. Fibronectin-collagen (FNC) coating (Athena Enzyme Systems). Store at 4 °C.
4. TrypLE Express (1×) (Life Technologies).
5. Collagenase-L.

2.5 Specific Reagents Required for Production of RAFT Constructs

1. RAFT reagent kit (TAP Biosystems). Store at 4 °C.
   a. Type I Collagen.
   b. 10× MEM.

c. Neutralizing solution.

2. Mixing vessel (TAP Biosystems).
3. 24-well plates (Greiner Bio-One).
4. 24-well plate absorbers (TAP Biosystems).
5. Guide plate (TAP Biosystems).
6. Plate heater (TAP Biosystems).

2.6 Specific Reagents Required for Wholemount Immunohisto-chemistry

1. *Blocking buffer*: 5 % goat serum in DPBS, with 0.25 % Triton-X 100.

2.7 Specific Reagents Required for Immunohisto-chemistry on Paraffin Wax Embedded Sections

1. Specimen wrapping paper.
2. Tissue cassettes.
3. Industrial methylated spirit (IMS).
4. Xylene.
5. Paraffin.
6. Plastic molds.
7. Embedding machine.
8. Microtome.
9. Superfrost ++ slides (VWR).
10. Coplin jars.
11. Sodium citrate buffer (pH 6.0): 10 mM sodium citrate containing 0.05 % v/v Tween 20, pH using 1 M hydrochloric acid (this is stable for 3 months at RT).
12. Blocking buffer: 10 % goat serum in DPBS.

3 Methods

3.1 3View Imaging

3.1.1 Tissue Preparation and Resin Embedding

1. Use a scalpel to dissect small limbal biopsies of approximately 2–3 mm under a dissecting microscope in Karnovsky's fixative (*see* Note 4).
2. Place the tissues in fresh Karnovsky's fixative and fix overnight at 4 °C.
3. Wash the tissue 3 × 5 min with ice-cold 0.15 M sodium cacodylate buffer in a fume cupboard.

[4]Do not allow the tissue dry out.

4. Incubate the tissue in equal volumes of 2 % osmium tetroxide solution and 3 % potassium ferricyanide solution for 1 h on ice in a fume cupboard (*see* Note 5).
5. Wash 3 × 5 min in distilled water in a fume cupboard.
6. Transfer the tissues into 1 % thiocarbohydrazide solution for 20 min in a fume cupboard.
7. Wash 3 × 5 min in distilled water in a fume cupboard.
8. Transfer the tissue into 2 % aqueous osmium tetroxide for 30 min in a fume cupboard.
9. Wash 3 × 5 min in distilled water in a fume cupboard.
10. Place the tissues in 1 % aqueous uranyl acetate overnight at 4 °C.
11. The next day, wash the tissues for 3 × 5 min in distilled water.
12. Transfer the tissues into the freshly prepared Walton's lead aspartate solution and place in a 60 °C oven for 30 min.
13. Wash the tissues for 3 × 5 min in distilled water.
14. Dehydrate the tissues through increasing concentration of ethanol:
    a. 5 min in 20 % (×2).
    b. 5 min in 50 % (×2).
    c. 5 min in 70 % (×3).
    d. 5 min in 90 % (×2).
    e. 10 min in 100 % (×3).
15. After dehydration, transfer the tissues into acetone (15 min × 2) prior to infiltration.
16. Infiltrate the tissues with a mixture of Durcupan ACM resin:acetone: 2 h in 25 % resin, 2 h in 50 % resin, 2 h in 75 % resin.
17. Place the tissues into 100 % resin overnight and transfer the tissues the next day into new molds containing 100 % of freshly prepared resin.
18. Place the molds into a 60 °C oven for 48 h.

3.1.2 Specimen Trimming and Mounting

1. Remove the samples from the embedding molds.
2. Trim away excess resin and attach the sample onto a cryopin using the conductive silver epoxy kit.
3. Leave the glue to set overnight.

[5]Use this incubation time to prepare a fresh solution of thiocarbohydrazide.

4. The next day, trim away excess conductive glue and use a glass knife to obtain a flat surface on the resin block.
5. Use a razor blade to trim around the area of interest (this should be approximately 1 × 1 mm).
6. Use a clean diamond knife to cut semi-thin sections of approximately 0.7 μm, collect them onto microscope slides, and dry them on a hotplate.
7. Stain the sections by adding 1 drop of 1 % toluidine blue and return the slides to the hot plate for a further 30 s.
8. Once the edge of the staining drop is dry, gently rinse the slides with distilled water.
9. Mount the slides with DePeX, coverslip and observe using a bright-field light microscope.
10. Once the area of interest has been identified, use a razor blade to trim the resin block to obtain a square surface measuring approximately 0.5 × 0.5 mm (*see* Fig. 1a) (*see* Note 6).
11. Sputter coat the surface of the resin block with a thin layer of gold palladium in order to get a conductive surface. This prevents the accumulation of negative charges at the surface of the specimen softening the resin block.

3.1.3 Sample Loading and Acquisition

1. Carefully load the specimen into the 3View system associated to the scanning electron microscope (*see* Note 7). Figure 1b shows the surface of the resin block once loaded inside the microscope chamber.
2. For imaging of the basal limbal epithelial cell layer of the cornea, the following setting were used:
   - Focus: 5.
   - Magnification: ×6,000.
   - Accelerating voltage: 4 kV.
   - Dwell time: 2 μs.
   - Pressure: 20 Pa.
   - Aperture: 60 μm.
   - Resolution: 4k × 4k.
3. Serially cut ultrathin sections (100 nm) from the resin block to expose a fresh surface to the electron beam. This allows the generation of a new image of the surface of the specimen (*see* Fig. 1c).

[6]It is important to trim at this point, in order to ensure that the specimen surface still fits in the cutting window of the 3View microtome during serial sectioning.
[7]Refer to the instruction of the manufacturer for a detailed description of the loading process.

4. Repeat this automated process 999 times to generate a large data stack of 999 serial images. Serial images are collected as.Dm3 file format in Digital Micrograph.

3.1.4 Data Analysis, Manual Segmentation, and 3D Reconstruction

1. Convert the .Dm3 data file stack into .tiff files using Digital Micrograph.
2. Copy the data into the workstation and load the .tiff file data stack as a complete volume into AMIRA for conversion into voxels (volumetric picture elements).
3. Use the noise reduction median filter and manually segment the area of interest on every single slice using the interactive pen (*see* Fig. 1d).
4. Generate the 3D structure of the segmented surface (*see* Fig. 1e).

3.2 Isolation and Culture of Human Limbal Epithelial Cells (hLEC) Using Dispase Dissociation

3.2.1 Dissection of Corneal Tissue

1. Sterilize instruments in 70 % ethanol for 10 min prior to use.
2. Cut the human donor corneal rim into quarters.
3. Incubate the pieces in 5 mL of 1.2 IU/mL dispase II for 2 h at 37 °C or overnight at 4 °C.
4. Transfer the pieces to a fresh petri dish containing 1 mL of fresh CECM without EGF.
5. Release hLEC from the limbus by scraping the anterior limbal surface with the tips of fine forceps.
6. Triturate to get a single cell suspension and place into a T75 flask containing 1.8×10^6 growth-arrested 3T3-J2 feeder cells.
7. Incubate at 37 °C in a humidified incubator with 5 % CO_2 overnight.

3.2.2 hLEC Culture and Expansion

1. After 1 day, replace the medium with CECM and change the medium three times per week (*see* Note 8).
2. Colonies of hLEC will start to appear between the feeder cells (*see* Fig. 2), easily seen by eye as circles of dense cells. Once confluent (10–14 days), hLEC may be passaged.
3. Wash cells twice with DPBS.
4. Incubate for 3 min in 0.05 % in trypsin-EDTA to selectively detach 3T3-J2 feeder cells (this can be observed using a light microscope).
5. Aspirate and discard the feeder cells.

[8]It may be necessary to top the feeder cells if they begin to detach during culture.

6. Add 2 mL of 0.5 % trypsin-EDTA, return to the incubator for no more than 5 min until hLEC detach when the side of the flask is hit.
7. Add 10 mL of CECM without EGF and centrifuge at 1,000 × *g* for 5 min.
8. Resuspend the hLEC pellet in fresh CECM without EGF and continue culture either on RAFT constructs (*see* Subheading 3.5) or on tissue culture plastic.

3.3 Isolation and Culture of Human Limbal Fibroblasts (hLF)

Air-dry method for hLF isolation and culture.

3.3.1 Dissection of Corneal Tissue

1. Sterilize instruments in 70 % ethanol for 10 min prior to use.
2. Following hLEC isolation using the dispase-dissociation method, dissect the corneal rim into 2–3 mm sections using a scalpel.

3.3.2 Attachment of Tissue to T25 Culture Flask

1. Place 5–6 sections into a T25 flask.
2. Allow to air-dry for 15 min in a biological safety cabinet by placing the flask on its side without the lid.
3. Gently add 6 mL of hLF media.
4. Allow to grow undisturbed in a 37 °C humidified incubator with 5 % CO_2 for 3 weeks.

3.3.3 hLF Culture and Expansion

1. Check for growth of hLF at the explant edge and gently change media without dislodging the tissue from the flask.
2. After a sufficient number of fibroblasts have grown out from the edge of the explant, passage cells into a new T25 flask by rinsing with 2 mL of DPBS and adding 1 mL of 0.05 % trypsin-EDTA for 5 min at 37 °C.
3. Mechanically detach cells by tapping the flask and add 4 mL of hLF media to inactivate trypsin.
4. Centrifuge at 1,000 × *g* for 5 min.
5. Resuspend the cells in hLF cell pellet in 1 mL of hLF media and reseed into a new T25 flask with 5 mL of hLF media.
6. Change media three times per week.
7. Amplify cell stock to use hLF for experimental use at passages 1–6. Figure 3 shows typical hLF morphology.

3.3.4 Collagenase-Dissociation Method for hLF Isolation and Culture

1. Sterilize instruments in 70 % ethanol for 10 min prior to use.

2. Following hLEC isolation using the method described in Subheading 3.2, dissect the corneal rim into 2–3 mm sections using a scalpel.

3.3.5 Enzymatic Dissociation of Corneal Tissue

1. Add dissected sections to 5 mL of collagenase solution in a 35 mm dish.
2. Incubate overnight in a humidified incubator at 37 °C with 5 % CO_2.
3. The next day, transfer the collagenase solution to a 15 mL centrifuge tube and centrifuge at 1,000 × *g* for 5 min.
4. Aspirate and discard the supernatant.
5. Resuspend the cell pellet in 1 mL of hLF media.

3.3.6 hLF Culture and Expansion

1. Add a further 5 mL of hLF media and transfer to a T25 flask.
2. Change hLF media three times per week until 70–80 % confluent.
3. At this confluency, the hLF may be trypsinized.
4. Wash hLF with DPBS twice.
5. Add 1 mL of 0.05 % trypsin-EDTA and incubate for 5 min at 37 °C in a humidified incubator with 5 % CO_2.
6. Mechanically detach cells by tapping the flask and add 4 mL of hLF media to inactivate trypsin.
7. Centrifuge at 1,000 × *g* for 5 min and resuspend the pellet in 1 mL of hLF medium before reseeding into a new T25 flask with 5 mL of hLF media.
8. Change media three times per week.
9. Amplify cell stock to use hLF for experimental use at passages 1–6.

3.4 Isolation and Culture of Human Corneal Stromal Stem Cells (hCSSC)

This method has been previously described by Du et al. [6].

3.4.1 Dissection of Corneal Tissue

1. Sterilize instruments in 70 % ethanol for 10 min prior to use.
2. Place the whole cornea or corneoscleral rim in a single well of a 12-well plate containing DMEM/F12 (supplemented with Gentamicin and pen/strep only).
3. Wash the tissue three times for 10 min each using this media.
4. If there is any trace of the Tenon's capsule (fibrous tissue) (*see* Note 9), remove this using a pair of forceps and scissors.

[9]Tenon's capsule contains fibroblasts, which may contaminate the hCSSC culture if not removed.

5. Dissect the superficial limbus 360° (*see* Fig. 4a, b): cut part of the sclera and cornea with the limbus sandwiched between (*see* Note 10).
6. Cut cornea into two halves to ease dissection, ensuring that the tissue is moistened with hCSSC media whilst dissecting.
7. Cut approximately 100 μm deep to obtain the superficial stroma only.

3.4.2 Enzymatic Digestion of Limbal Stroma

1. Cut the limbal stroma into smaller fragments using scissors and place into collagenase solution in a well of a 6-well plate.
2. Incubate overnight at 37 °C in a 5 % CO_2 humidified incubator. The next day, pipette the mixture up and down to break up any remnants of fibrous tissue that might be remaining in media.
3. Centrifuge mixture in 10 mL of DMEM/F12 (containing Gentamicin and pen/strep) at 1,500 rpm* for 5 min at 4 °C.
4. Remove supernatant leaving approximately 0.5 mL at bottom of tube (*see* Note 11).
5. Add 1 mL of hCSSC media to the tube containing cells and mix gently before adding a further 4 mL of hCSSC media.

3.4.3 hCSSC Culture

1. Coat T25 flask with FNC. Add approximately 1 mL of FNC to cover the surface of the flask, leave for 30 s, then aspirate the excess.
2. Add the hCSSC suspension to the FNC-coated flask.
3. Incubate at 37 °C in a 5 % CO_2 humidified incubator (*see* Note 12) overnight. hCSSC will adhere to the plastic surface of the flask by the next day.
4. Wash cells with DPBS and replace with fresh hCSSC medium (*see* Note 13).
5. Small hCSSC colonies should appear by day 2 or 3 (*see* Fig. 4c). Cells should be passaged as soon as small colonies are visible using 1 mL of TrypLE Express followed by 2 mL of hCSSC media to neutralize.
6. Culture hCSSC at 37 °C in a 5 % CO_2 humidified incubator (*see* Note 14).

3.5 Production of RAFT Constructs

3.5.1 hLF Cell Solution Preparation

1. Trypsinize hLF cells as in Subheading 3.3.

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[10] Remove excess sclera and central cornea using a scalpel.
[11] The pellet is very small so care must be taken not to dislodge/remove it.
[12] hCSSC can be left in hCSSC media for up to 2 days if necessary.
[13] It is difficult to distinguish the hLEC from hCSSC at this stage of culture.
[14] By the second day of culture, hLEC usually begin to grow in small colonies with the characteristic cobblestone morphology. hCSSC grow more sparsely but in close proximity to the epithelial colonies. hCSSC typically look small and square, with four points. Some keratocyte-like cells that are more dendritic in morphology also appear in early passages (Fig. 4c). It is necessary to passage hCSSC as soon as they appear, rather than waiting for confluence since they will become fibroblastic.

2. Resuspend hLF in 1 mL of hLF medium and perform a cell count.
3. Resuspend hLF at a cell density of 100,000 cells per mL of collagen in an appropriate volume of culture medium, c, depending on the final number of constructs (*see* Note 15) (*see* Table 1).
4. Keep cells on ice until required.

3.5.2 Collagen Solution Preparation

1. Determine the number of collagen constructs required and refer to Table 1 for reagent volumes (*see* Note 16).
2. Add volume × of 10 × MEM to the mixing vessel.
3. Slowly add volume *y* of the collagen solution to the mixing vessel.
4. Swirl the solution gently to mix until a homogenous color is achieved.
5. Add volume *z* of neutralizing solution evenly distributed across the surface of the mixture, swirl the solution again gently to mix.
6. Add volume *c* of hLF cell suspension (from Subheading 3.5.1) (*see* Note 17), evenly distributed across the surface of the mixture and swirl again gently to mix.
7. Leave the collagen solution on ice for 30 min to allow any bubbles that might have formed to disperse (*see* Note 18).

3.5.3 Hydrogel Formation

1. Allow the plate heater to reach 37 °C.
2. Using a 5 mL pipette, transfer 2.4 mL of collagen/hLF mix to each well of a 24-well plate (*see* Note 19).
3. Place the 24-well plate on the plate heater and close the lid (*see* Note 20).
4. Allow 30 min for fibrillogenesis to occur and hydrogels to form.
5. Whilst gelling is occurring, UV sterilize the desired number of absorbers.

3.5.4 Production of the RAFT 3D Constructs

1. Populate the guide plate with the correct number and configuration of sterile absorbers (ridged or plain) to match the hydrogels (*see* Fig. 5a) (*see* Note 21).
2. Open the lid of the plate heater and remove the lid of the cell culture plate.

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[15]Cell densities have been optimized for this specific application; the final cell density in collagen construct must be determined by the end user depending on the application.
[16]The reagent volumes include an excess to allow for solution lost when pipetting and transferring between different vessels.
[17]If hLF is not required in the RAFT construct, add hLF culture media alone.
[18]Keep all reagents ice-cold and perform all processes on ice as far as possible after neutralization. Gelling will commence if the solution is left to warm.
[19]When dispensing the viscous collagen solution with serological pipettes, reverse pipette to increase accuracy.
[20]It is possible to carry out the fibrillogenesis process in a 37 °C cell culture incubator.
[21]If bioengineered limbal crypts in the RAFT construct are required, use ridged absorbers. If a flat surface is required, use plain absorbers. The process is identical for both.

3. Place the guide plate on top of the cell culture plate aligning the absorbers with the hydrogels and leave for 30 min (*see* Fig. 5b) (*see* Note 22).
4. Remove the guide plate and accompanying absorbers by lifting it straight up.
5. Discard the absorbers.
6. Add 500 μL of hLF medium to the surface of the RAFT constructs and replace the lid of the well plate.
7. Place the culture plate in a humidified incubator at 37 °C with CO_2 in air until addition of hLEC to the surface. An example of an acellular RAFT construct is shown in Fig. 5c. (A schematic to describe the hydrogel formation and RAFT production process is shown in Fig. 5d).

3.5.5 Seeding of hLE onto the Surface of RAFT Constructs

1. Selectively trypsinize and discard 3T3-J2 feeder cells as in Subheading 3.2.
2. Trypsinize hLEC as in Subheading 3.2.
3. Count hLEC using a hemocytometer and dilute cell suspension to the correct density to add 560,000 cells to each RAFT construct in a 24-well plate (*see* Note 15).
4. Add a total of 1.5 mL of CECM (without EGF) (*see* Note 23) to each well and return the plate to the incubator at 37 °C and 5 % CO_2 in air.

3.5.6 Submerged Culture to Expand hLE on the Surface of RAFT

1. After 24 h, aspirate the CECM and any unattached cells and add 1.5 mL of fresh CECM.
2. Change medium three times per week.
3. Maintain RAFT constructs for 2 weeks in submerged culture.

3.5.7 Airlifting the Culture to Achieve a Stratified Epithelium on the Surface of RAFT

1. Remove the RAFT constructs from wells and place onto an insert in a 6-well culture plate using forceps and a flat spatula.
2. Add 800 μL of CECM to the well below the insert adding a drop of medium to the surface of the constructs and fill any empty wells with DPBS (*see* Note 24).
3. Change the medium three times per week, each time adding a drop of CECM to the surface of the constructs.
4. Maintain the RAFT constructs for 1 week in airlifting culture.

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[22]Take care not to apply too much pressure to the hydrogels. Allow the absorbers to gently fall onto the surface of the gels. It is possible to carry out this process directly in the cell culture hood without the use of the plate heater.
[23]Omission of EGF when initially cell seeding hLE cells aids attachment to the surface of RAFT constructs.
[24]Adding DPBS to the empty wells helps to maintain a humid atmosphere and addition of a drop of medium to the surface of the constructs prevents drying of the cells on the construct.

3.6 Wholemount Immunohisto-chemistry

3.6.1 Fixing

1. Fix RAFT constructs for 30 min using 4 % PFA (*see* Note 25).
2. Following fixation, wash the construct three times with DPBS (*see* Note 26) and, if required, store at 4 °C in DPBS, up to a maximum of 2 weeks.

3.6.2 Blocking

1. Prevent nonspecific binding by blocking with blocking buffer (*see* Note 27) for 1 h.
2. Wash once with DPBS (*see* Note 26).

3.6.3 Capture Antibody

1. Apply capture antibody in capture antibody solution at the required dilution (*see* Table 2) (*see* Note 28).
2. For the isotype negative control (*see* Note 29), apply the isotype control in capture antibody solution at the same concentration used for the capture antibody (*see* Table 2).
3. Incubate samples overnight at 4 °C.
4. Following the overnight incubation, wash samples with DPBS for 3 × 5 min (*see* Note 26).

3.6.4 Detection Antibody

1. Apply Alexa Fluor secondary antibody (1:500) (*see* Note 30) and phalloidin (1:1,000) (*see* Note 31) in DPBS.
2. Incubate samples for 1 h at RT in the dark.
3. Wash samples with DPBS for 3 × 5 min (*see* Note 26).

3.6.5 Mounting

1. Transfer the samples to glass slides and remove any excess liquid carefully by blotting with a tissue.

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[25]Fixation reagents and incubation times may vary dependent upon the antibody used. Therefore, consult the relevant data sheet if antibodies other than those given in Table 2 are used before performing this step.
[26]For all wash steps, be careful to expel liquid around the construct, and not directly on top. This prevents epithelial detachment.
[27]For cell surface antigens Triton-X-100 detergent should be omitted from the blocking solution as the detergent may destroy cell membrane structure.
[28]For capture antibodies other than those given in Table 2, the user should optimize the dilution.
[29]The isotype control should be sourced from the same species as the host antibody, and should match the same antibody subtype. The purpose of the isotype control is to enable the end user to assess the level of background staining, thereby helping to differentiate between nonspecific and true antigen- antibody binding.
[30]Secondary antibody selection is important to allow for antibody- antibody binding. The secondary antibody should be raised in the same species used for the blocking serum, but directed against the immunoglobulins of the primary antibody species; i.e., the appropriate secondary antibody to use in a protocol using goat serum and a rabbit primary antibody would be termed “goat anti-rabbit.”
[31]Phalloidin binds to the cells’ actin filaments, allowing for visualization of the cells’ cytoskeleton. Therefore, this reagent is a useful tool to aid localization of the target antigen within the cell.

2. Apply one or two drops of Vectashield mounting medium to each sample and fix in place with a coverslip (*see* Note 32).
3. Seal using nail polish, carefully brushing the polish around the perimeter of the coverslip.
4. Leave to dry for 30 min before visualizing using a confocal fluorescence microscope. An example of RAFT wholemount staining is given in Fig. 6.

3.7 Immunohisto-chemistry on Paraffin Wax Embedded Sections

3.7.1 Fixing

1. Fix RAFT constructs for 30 min using 4 % PFA (*see* Note 25).
2. Following fixation, wash the construct three times with DPBS and, if required, store at 4 °C in DPBS, up to a maximum of 2 weeks.

3.7.2 Processing

1. Carefully wrap RAFT constructs in specimen wrapping paper using forceps and place into a tissue cassette.
2. Leave in formalin until transfer to the tissue processor, which processes the tissue using gentle agitation, as follows:
   - 70 % IMS for 5 min.
   - 90 % IMS for 30 min.
   - 100 % IMS for 1 h (×2).
   - 100 % IMS for 1.5 h (×2).
   - 100 % IMS for 2 h, xylene for 2 h (×2).
   - paraffin for 2 h (×2).
3. The tissue cassette should be left in paraffin until required.

3.7.3 Embedding

1. Open the tissue cassette and remove the RAFT construct.
2. Embed in paraffin wax and leave to cool for at least 1 h before sectioning (*see* Note 33).

3.7.4 Sectioning

1. Trim excess paraffin from the sides of the tissue cassette to ensure that the block fits in the microtome (*see* Note 34).

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[32]Mounting medium prevents rapid photo-bleaching of fluorophores and provides an optimal refractive index enabling sharper images to be obtained. Excess liquid may alter the refractive index resulting in poorer quality images. Removal of excess liquid is advised.

[33]Ensure a margin of at least 2 mm of paraffin on all sides of the gel to give it a good cutting support. If cracks are observed in the wax, re-melt the paraffin and repeat the embedding process, as the block will crack further during sectioning making obtaining sections impossible.

2. Cut 5 μm sections and float onto Superfrost ++ slides.
3. Allow excess water to drain away and transfer the slide to the hot plate for 15 min to ensure paraffin wax has melted and section is firmly stuck to the glass slide.
4. Store at RT until staining.

3.7.5 Dewaxing

1. Place labeled slides into a Coplin jar.
2. Dewax in xylene for 3 × 5 min.

3.7.6 Rehydrating

1. In a Coplin jar, perform the following steps:
   - 100 % IMS for 5 min.
   - 90 % IMS for 5 min.
   - 70 % IMS for 5 min.
   - Water for 5 min.
2. Wash slides in DPBS for 5 min at 95 °C in a water bath.

3.7.7 Antigen Retrieval

1. Perform antigen retrieval with sodium citrate buffer at 95 °C in a water bath for 20 min.
2. Wash for 3 × 5 min with DPBS.

3.7.8 Blocking

1. Prevent nonspecific binding by blocking in blocking buffer for 1 h.

3.7.9 Capture Antibody

1. Apply the capture antibody in capture antibody solution at the required dilution (*see* Table 2) (*see* Note 30).
2. To prepare an isotype negative control, add the isotype control in capture antibody solution at the same concentration used for the capture antibody (*see* Table 2) (*see* Note 29).
3. Incubate samples overnight at 4 °C.
4. Following the overnight incubation, wash samples with DPBS for 3 × 5 min.

[34]Cool each block facedown on an ice block for at least 10 min prior to sectioning to reduce friction between the blade and block. This also reduces the chance of cracking the block.

3.7.10 Detection Antibody

1. Apply Alexa Fluor secondary antibody (1:500) (*see* Note 30) and phalloidin (1:1,000) (*see* Note 31) in DPBS.
2. Incubate samples for 1 h at RT in the dark.
3. Wash samples with DPBS for 3 × 5 min.

3.7.11 Mounting

1. Apply one or two drops of Vectashield to each sample and fix in place with a coverslip (*see* Note 32).
2. Seal using nail polish, carefully brushing the polish around the perimeter of the coverslip.
3. Leave to dry for 30 min before visualizing using a fluorescence microscope.

An example of RAFT section staining is given in Fig. 7.

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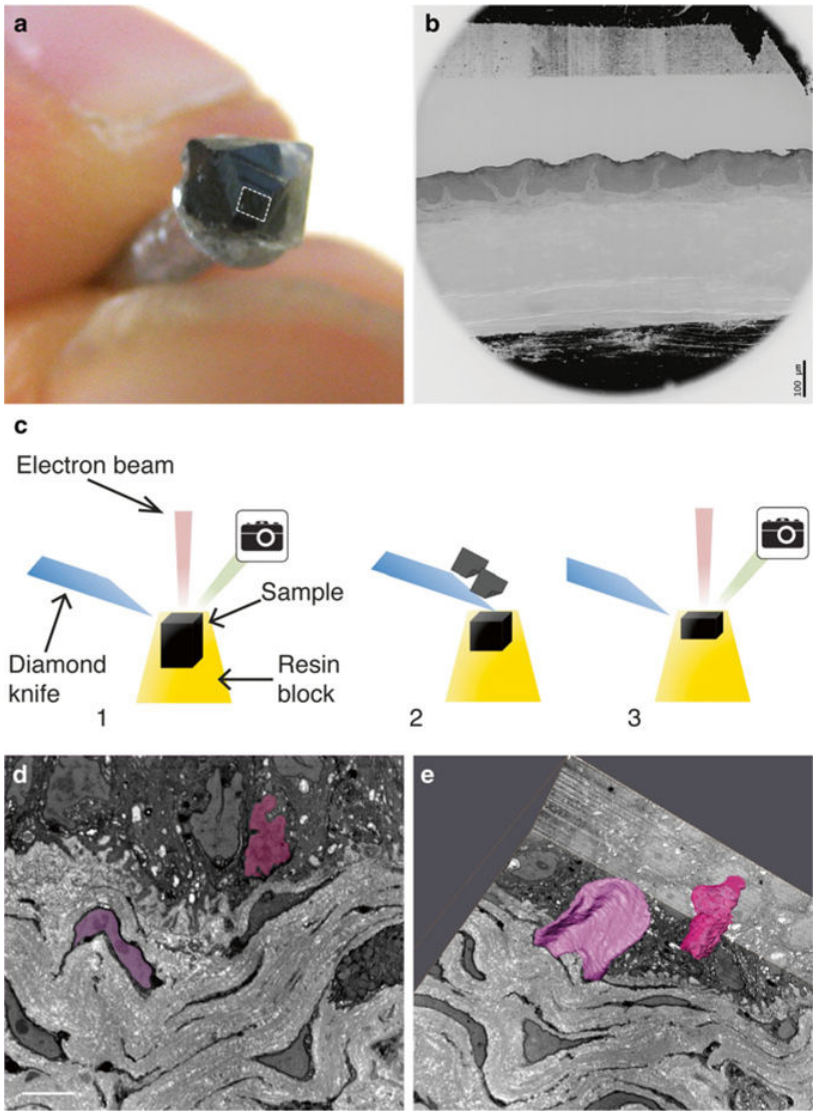

Fig. 1.

Serial block face scanning electron microscopy. (**a**) Resin block attached to the cryopin, ready to be loaded into the SEM. The *dashed white line* outlines the surface of the resin block measuring approximately 0.5 × 0.5 mm. (**b**) Low magnification scanning electron micrograph of the resin block in **a**. (**c**) Schematic showing the principle of the SBFSEM (3View). *1*: Imaging of the surface of the resin block. *2*: A diamond knife inside microscope chamber cuts an ultrathin section away from the specimen. *3*: The freshly exposed edge is imaged. (**d**) Manual segmentation of the area of interest. The nuclei of a stromal cell and epithelial cell are outlined. (**e**) 3D reconstruction of the areas manually segmented in **d**. Scale bars: **b**: 100 μm and **d**: 5 μm

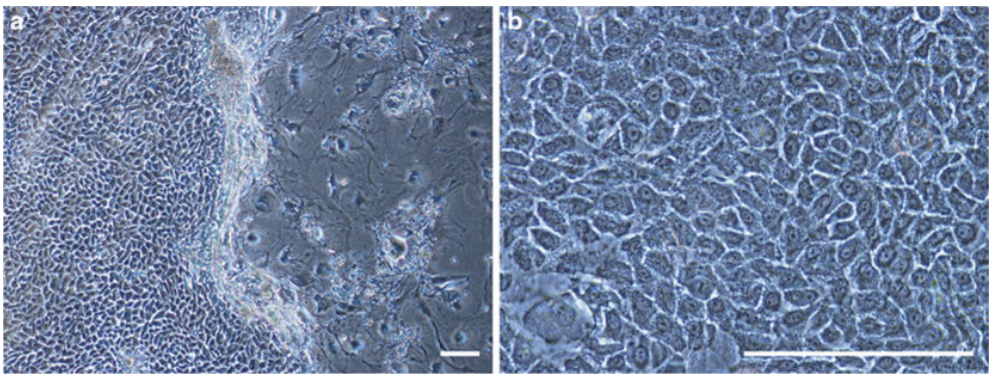

Fig. 2.

hLEC cultures. (**a**) hLEC form colonies (*left*) between the feeder cells (*right*). (**b**) hLEC display typical cobblestone morphology, with scant cytoplasm. Scale bars: 200 μm

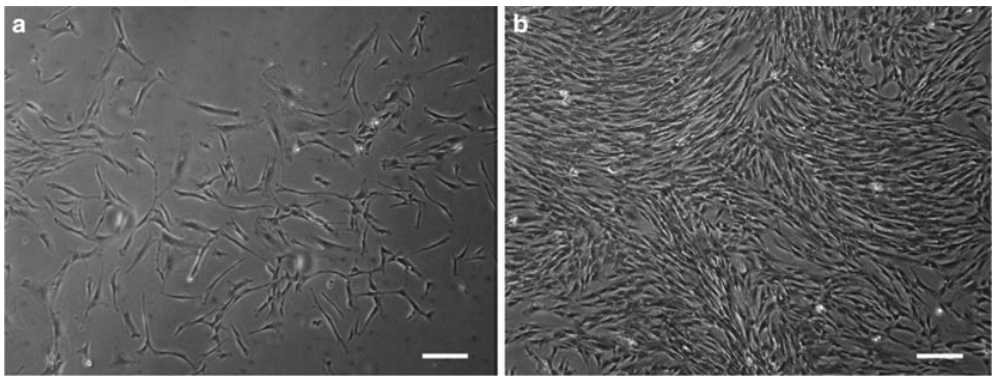

Fig. 3.

hLF cultures. (**a**) hLF in culture at sub-confluency. (**b**) A confluent hLF layer, ready for trypsinization. hLF appear dendritic. Scale bars: 200 μm

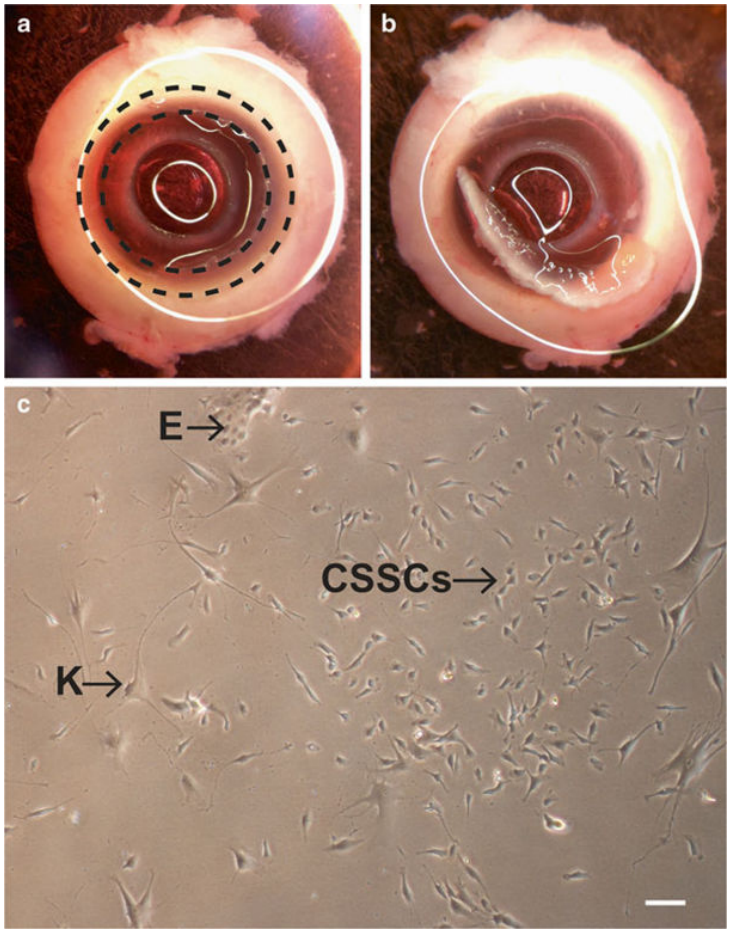

Fig. 4.

hCSSC isolation and culture using enzymatic dissociation. (**a**) *Black dotted lines* illustrate the limbal region before dissection. (**b**) Upper half of superficial limbal rim is dissected. (**c**) hCSSC in culture (passage 1). CSSCs appear small and square in sparsely arranged colonies. Some epithelial colonies (E) and keratocyte- like cells (K) remain visible in early passages. Scale bar: 100 μm

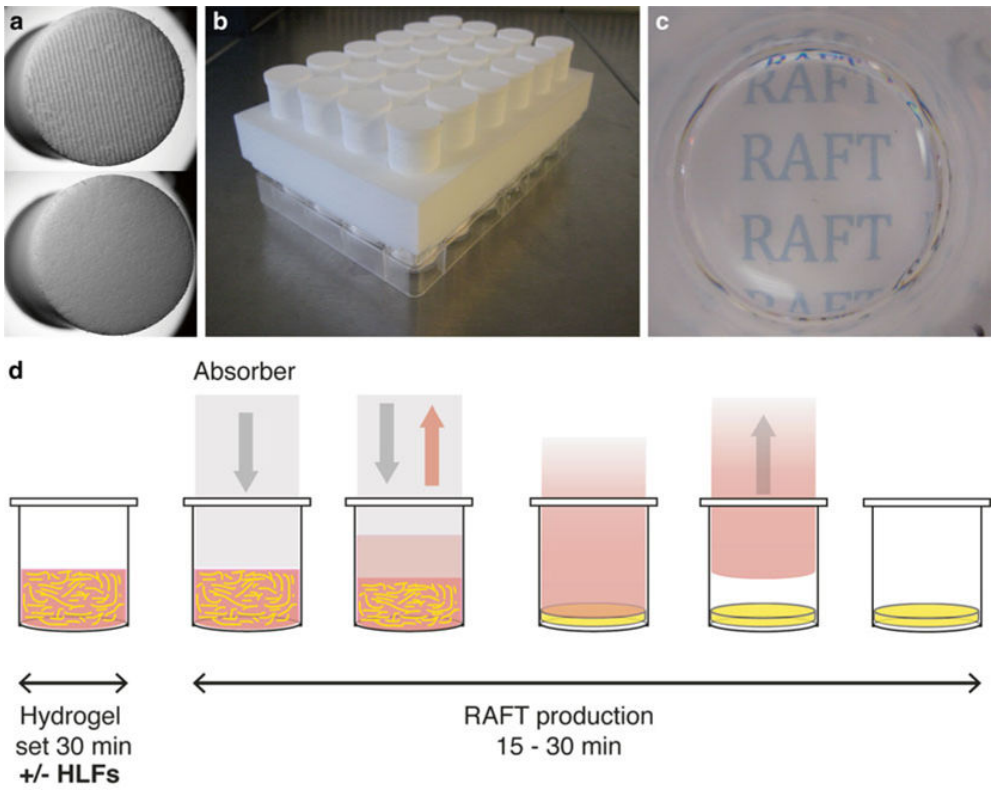

Fig. 5.

RAFT production process. (**a**) Base of absorbers showing ridged and plain topographies. (**b**) Guide plate populated with absorbers on top of 24-well plate. (**c**) Acellular RAFT construct in the base of a 24-well plate. (**d**) Schematic summarizing the RAFT production process

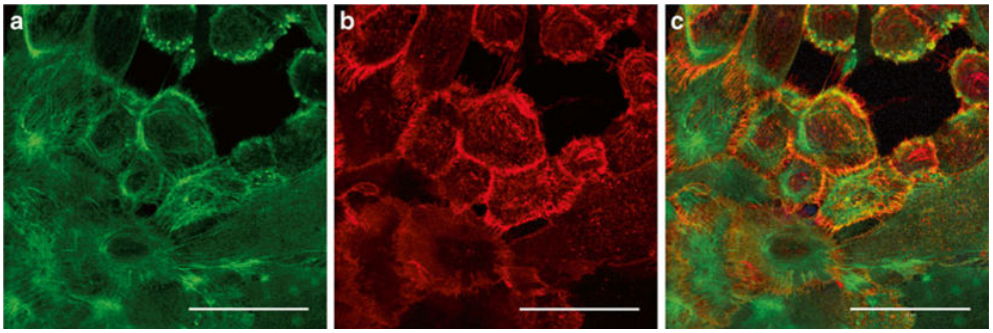

Fig. 6.

Example of a wholemount-stained RAFT construct. (**a**) hLEC on RAFT stained for β1-integrin, putative stem cell marker. (**b**) hLEC on RAFT stained for phalloidin-FITC. (**c**) Merge. Scale bars: 50 μm

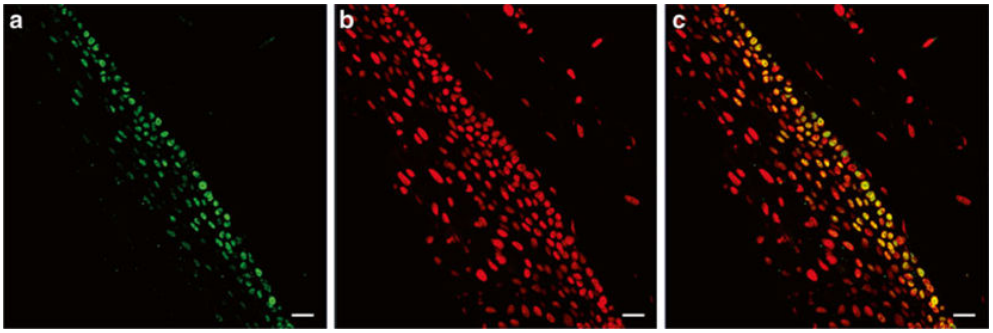

Fig. 7.

Example of a stained RAFT section. (**a**) hLEC on RAFT stained for p63α, putative stem cell marker. (**b**) PI counter-staining of hLEC (*left*) nuclei on RAFT and entrapped hLF (*right*) nuclei. (**c**) Merge showing that only basal hLEC express p63α. Scale bars: 20 μm

Table 1

Reagent volume guide for RAFT production in 24-well Greiner plates (all volumes are in mL)

	10× MEM	Collagen	Neutralizing solution	Cells or medium		
Number of constructs	***x***	***y***	***z***	***c***	**Total volume**	**Excess**
1	0.4	3.5	0.261	0.180	4.4	2
2	0.7	5.4	0.404	0.279	6.8	2
3	0.9	7.4	0.546	0.377	9.2	2
4	1.2	9.3	0.689	0.476	11.6	2
5	1.4	11.2	0.832	0.574	14.0	2
6	1.6	13.1	0.974	0.672	16.4	2
7	1.9	15.0	1.117	0.771	18.8	2
8	2.1	17.0	1.259	0.869	21.2	2
9	2.4	18.9	1.402	0.968	23.6	2
10	2.6	20.8	1.544	1.066	26.0	2
11	2.8	22.7	1.687	1.164	28.4	2
12	3.1	24.6	1.830	1.263	30.8	2
13	3.3	26.6	1.972	1.361	33.2	2
14	3.6	28.5	2.115	1.460	35.6	2
15	3.8	30.4	2.257	1.558	38.0	2
16	4.0	32.3	2.400	1.656	40.4	2
17	4.3	34.2	2.542	1.755	42.8	2
18	4.5	36.2	2.685	1.853	45.2	2
19	4.8	38.1	2.827	1.952	47.6	2
20	5.0	40.0	2.970	2.050	50.0	2
21	5.2	41.9	3.113	2.148	52.4	2
22	5.5	43.8	3.255	2.247	54.8	2
23	5.7	45.8	3.398	2.345	57.2	2
24	6.0	47.7	3.540	2.444	59.6	2

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Table 2

Antibody and isotype controls supplier information and dilutions for immunohistochemistry

Antigen	Capture Ab manufacturer	Dilution for wholemount	Dilution for wax sections	Isotype control manufacturer
p63α	Cell Signaling Technology	1:50	1:100	New England Biolabs
Pax-6	Covance	1:100	1:100	
Ki67	Millipore	1:100	1:100	
CK3	Millipore	1:200	1:500	Cambridge Bioscience