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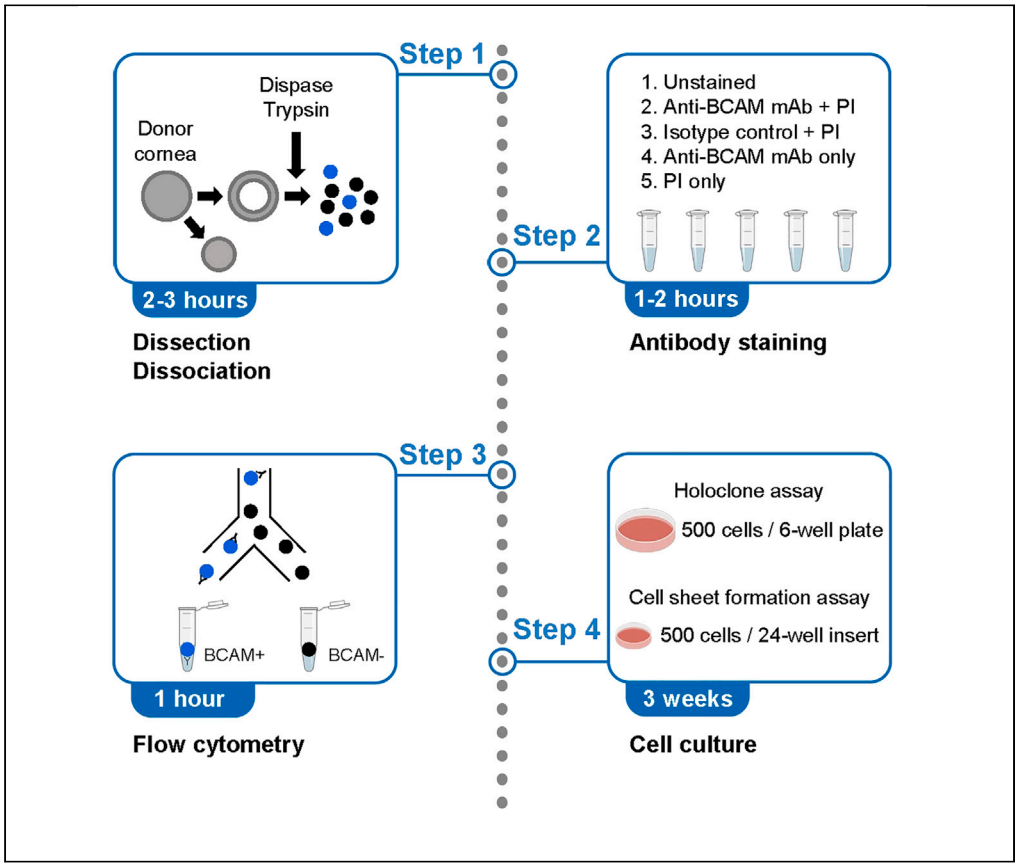
Protocol for isolating human BCAM-positive corneal progenitor cells by flow cytometry and cell sorting



BCAM-positive basal limbal epithelial cells are an early transit-amplifying cell population (TAC) capable of holoclone formation and corneal epithelial differentiation. Here, we present a protocol for isolating BCAM-positive cells from human donor corneas by flow cytometry and cell sorting. We describe steps for cell dissection and dissociation, antibody staining, and flow cytometry. We then detail procedures for culturing the purified BCAM-positive and BCAM-negative cells for holoclone and cell sheet formation assays to study the factors that regulate corneal regeneration.

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

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Highlights

Step-by-step guide for the dissection of human corneas

Isolation of viable human BCAM-positive corneal progenitor cells by flow cytometry

Functional evaluation of BCAM-positive and BCAM-negative cells

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Protocol for isolating human BCAM-positive corneal progenitor cells by flow cytometry and cell sorting

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SUMMARY

BCAM-positive basal limbal epithelial cells are an early transit-amplifying cell population (TAC) capable of holoclone formation and corneal epithelial differentiation. Here, we present a protocol for isolating BCAM-positive cells from human donor corneas by flow cytometry and cell sorting. We describe steps for cell dissection and dissociation, antibody staining, and flow cytometry. We then detail procedures for culturing the purified BCAM-positive and BCAM-negative cells for holoclone and cell sheet formation assays to study the factors that regulate corneal regeneration.

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

BEFORE YOU BEGIN

In the human cornea, transit-amplifying cells (TACs) are derived from limbal stem cells and represent a progenitor subpopulation capable of corneal regeneration and repair. Recently, we discovered that TACs express Basal cell adhesion molecule (BCAM), a transmembrane glycoprotein, which binds to laminins within the adjacent basement membrane.[1] Here, we describe a protocol for isolating BCAM-positive progenitors from the human limbal epithelium. We also provide examples of using the purified BCAM-positive TACs in holoclone and cell sheet formation assays.

We have tested this technique with human donor corneas obtained from the Saving Sight eye bank, Kansas City, MO and the CorneaGen eye bank, Seattle, WA. Isolation of BCAM-positive progenitors was performed within 1 week after the donor's death.

Institutional permissions

Human corneas were obtained from consented donors under Institutional Review Board (IRB)-approved protocols.

3T3-J2 cell line maintenance and subculture

Timing: 3–7 days

Murine 3T3-J2 cell line is used as a feeder cell layer for the holoclone and cell sheet formation assays.

1. Prepare culture media for 3T3-J2 cell line. The 3T3-J2 culture media consists of DMEM (high glucose, pyruvate) supplemented with 10% calf serum.
2. Take out cryo-preserved 3T3-J2 cells from the liquid nitrogen tank and revive them by rapidly thawing them in a 37°C water bath for less than a minute, diluting the thawed cells with 9 mL of pre-warmed 3T3-J2 culture media.
3. Centrifuge the cell suspension at 300 g for 5 min at 20°C–25°C.
4. Remove the supernatant and resuspend the cells in 1 mL of 3T3-J2 culture media.
5. Transfer cell suspension into a 10 cm culture dish containing 10 mL of pre-warmed 3T3-J2 culture media and grow cells for 2–4 days until they reach the confluency in a CO_2 incubator at 5%–10% CO_2 at 37°C.
6. Split the cells in 1:10 ratio into a 10 cm culture dish using 1 mL of TrypLE Express Enzyme before the cells reach confluency. Usually, it takes 2–4 days to reach the confluency.

Treatment of 3T3-J2 cells with Mitomycin C (MMC)

Timing: 1–3 days

7. Dissolve 2 mg of Mitomycin C (MMC) powder with 49.5 mL of DMEM (high glucose) and 500 µL of Antibiotic-Antimycotic (100×).
8. Make 2.5 mL of MMC solution aliquots in 15 mL conical tubes and store them at −20°C until use.
9. Add 10 mL of DMEM (high glucose) to 2.5 mL of MMC solution and pre-warm it in a 37°C water bath.
10. Remove the 3T3-J2 culture media from 80%–90% confluent 3T3-J2 cells and rinse with 3 mL of the solution prepared in step 3 twice.
11. Add 6.5 mL of the solution prepared in step 3 to the 3T3-J2 cells and incubate for 2 h at 37°C in a CO_2 incubator.
12. Remove the media and rinse with 5 mL of phosphate-buffered saline (PBS) twice.
13. Add 1 mL of TrypLE Express Enzyme and incubate for 15 min at 37°C in a CO_2 incubator.
14. Add 4 mL of 3T3-J2 culture media and transfer the cells to a 15 mL conical tube.
15. Centrifuge the cell suspension at 300 g for 5 min at 20°C–25°C.
16. Remove the supernatant and resuspend the cells in 1 mL of 3T3-J2 culture media.
17. Count the cell number and seed the cells at 2.6×10^5 cells/well in a 6-well plate for holoclone assay and 5.2×10^4 cells/well in a 24-well plate for cell sheet formation assay.

Note: Use 2 mL of 3T3-J2 culture media for each well in a 6-well plate and 500 µL for each well in a 24-well plate.

18. Incubate the cells at 37°C in a CO_2 incubator until human limbal epithelial cells are seeded.

Note: Use the MMC-treated 3T3-J2 cells within 3 days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Human monoclonal anti-BCAM-VioBright FITC (dilution 1:10)	Miltenyi Biotec	130-104-839 Cat# 130-104-839; RRID:AB_2656519
REA control antibody (S), human IgG1, REAfinity-VioBright FITC (dilution 1:50)	Miltenyi Biotec	130-113-443; RRID:AB_2734084

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit monoclonal anti-KRT12 (dilution 1:400)	Abcam	ab185627 Cat# ab185627; RRID:AB_2889825
Mouse monoclonal anti-MUC16 (dilution 1:400)	Abcam	ab1107 Cat# ab1107; RRID:AB_297721
Donkey anti-mouse IgG- Alexa Fluor 488 (dilution 1:400)	Thermo Fisher scientific	A21202 Cat# A21202; RRID:AB_141607
Donkey anti-rabbit IgG- Alexa Fluor 568 (dilution 1:400)	Thermo Fisher scientific	A10042 Cat# A10042; RRID:AB_2534017
Biological samples		
Human corneas	Saving Sight (Kansas City, MO) and CorneaGen (Seattle, WA) eye banks	N/A
Chemicals, peptides, and recombinant proteins		
DMEM, high glucose, pyruvate	Thermo Fisher scientific	11995–065
HyClone iron-supplemented calf serum	Cytiva	SH30072.03
TrypLE™ express enzyme	Thermo Fisher scientific	12605036
DMEM, high glucose	Thermo Fisher scientific	11965–092
Mitomycin C from *Streptomyces caespitosus*	MilliporeSigma	M4287
HyClone phosphate-buffered saline solution	Cytiva	SH30256.01
Fetal bovine serum (FBS)	Thermo Fisher scientific	10438026
DMEM/F-12	Thermo Fisher scientific	11320033
B-27 Supplement (50×), serum free	Thermo Fisher scientific	17504001
Recombinant human KGF (FGF-7)	PeproTech	100–19
Y-27632 dihydrochloride	Tocris	1254
HyClone Penicillin-Streptomycin 100× solution	Cytiva	SV30010
Antibiotic-Antimycotic (100×)	Thermo Fisher scientific	15240062
DMEM, high glucose, pyruvate, no glutamine	Thermo Fisher scientific	10313021
Ham's F-12 Nutrient Mix	Thermo Fisher scientific	11765054
Hydrocortisone hydrogen succinate	MilliporeSigma	BP187
3,3',5-Triiodo-L-thyronine sodium salt	MilliporeSigma	T2752
Cholera toxin (AZIDE-FREE) from *Vibrio cholerae*	List Biological Laboratories	100B
Transferrin, bovine (Holo form), lyophilized	Thermo Fisher scientific	11107047
L-glutamine	Thermo Fisher scientific	25030081
Insulin-Transferrin-Selenium (ITS -G) (100×)	Thermo Fisher scientific	41400045
PluriSTEM Dispase II solution	MilliporeSigma	SCM133
Propidium iodide staining solution	BD Biosciences	556463
10% neutral buffered formalin	Fisher scientific	SF100-4
Rhodamine B	MilliporeSigma	R6626
Hydrogen chloride 5.0 N	VWR Chemicals BDH	BDH7419
iMatrix-511	Nacalai Tesque	892012
16% paraformaldehyde	Electron Microscopy Sciences	15710
Tris buffered saline (10×, pH7.4)	Boston BioProducts, Inc.	BM-300
TissueTek® O.C.T compound	Sakura	4583
Normal donkey serum	Jackson ImmunoResearch Laboratories	017-000-121
Triton™ X-100	MilliporeSigma	X-100
Hoechst 33342	Thermo Fisher scientific	H3570
ProLong Gold Antifade Mountant	Thermo Fisger scientific	P36934
Critical commercial assays		
Hematoxylin and eosin stain Kit	Vector Laboratories	H-3502
Experimental models: Cell lines		
3T3-J2 cell line	Kerafast	EF3003
Software and algorithms		
BD FACSDiva	BD Biosciences	8.0.1
FlowJo	BD Biosciences	10.6.1
NIS-elements AR	Nikon	4.30.01
Adobe Illustrator	Adobe	24.2.1

MATERIALS AND EQUIPMENT

FACS buffer		
Reagent	Final concentration	Amount
PBS	N/A	49 mL
Fetal bovine serum (FBS)	2%	1 mL
Total	N/A	**50 mL**
FACS buffer can be stored at 4°C for 2 weeks.		

Keratinocyte culture medium (KCM)		
Reagent	Final concentration	Amount
DMEM, high glucose, pyruvate, no glutamine	N/A	326.625 mL
Ham's F-12 Nutrient Mix	N/A	108.875 mL
Fetal bovine serum (FBS)	10%	50 mL
Hydrocortisone hydrogen succinate	0.4 μg/mL	500 μL
3,3′,5-Triiodo-L-thyronine sodium salt	2 nM	500 μL
Cholera Toxin (AZIDE-FREE) from Vibrio cholerae	1 nM	500 μL
Transferrin, Bovine (Holo form), lyophilized	2.25 μg/mL	500 μL
L-glutamine	2 mM	5 mL
Insulin-Transferrin-Selenium (ITS -G) (100×)	0.5%	2.5 mL
HyClone Penicillin-Streptomycin 100× solution	1%	5 mL
Total	**N/A**	**500 mL**
KCM can be stored at 4°C for 3 weeks.		

Note: Combine 10 ng/mL keratinocyte growth factor (KGF) and 10 μM Y-27632 dihydrochloride immediately before use.

⚠ CRITICAL: Cholera toxin is harmful if swallowed or in contact with skin. Wear protective clothing, gloves and eye/face protection and handle it in a biosafety cabinet.

Corneal epithelium maintenance medium (CEM)		
Reagent	Final concentration	Amount
DMEM/F-12	N/A	48.4 mL
B-27 Supplement (50×), serum free	2%	1 mL
Keratinocyte growth factor (KGF)	10 ng/mL	50 μL
Y-27632 dihydrochloride	10 μM	50 μL
HyClone Penicillin-Streptomycin 100× solution	1%	500 μL
Total	**N/A**	**50 mL**
CEM can be stored at 4°C for 2 weeks.		

STEP-BY-STEP METHOD DETAILS

Dissection and dissociation of the human limbal epithelial cells

Timing: 2–3 h

This section describes steps to dissect and dissociate the limbal epithelial cells from human donor corneas (Figure 1)[2]

1. Dissection of human donor corneas
   a. Rinse the human donor cornea and remove the iris tissue attached to the back of the cornea in PBS in a 6-well plate.

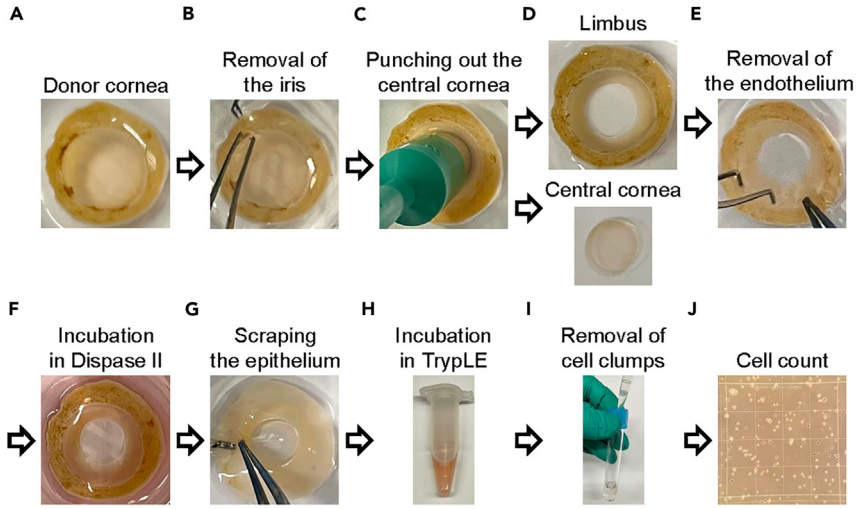

Figure 1. Dissection and dissociation of the human limbal epithelial cells

(A) Donor cornea placed in a 6-well plate. Epithelial side is facing downward and endothelial side is facing upward.
(B) Removal of the iris tissue attached to the back of the cornea by forceps.
(C) Punching out the central cornea by an 8 mm disposable biopsy punch.
(D) Separation of limbus and central cornea.
(E) Mechanical removal of the corneal endothelium by forceps.
(F) Incubation of limbal tissue in 2 mL of PluriSTEM Dispase II Solution.
(G) Scraping off the limbal epithelial cells by forceps.
(H) Incubation of limbal epithelial cells in 400 μL of TrypLE Express Enzyme.
(I) Removal of cell clumps by filtering the cells through 35 μm cell strainer.
(J) Cell count.

   b. Transfer the tissue to a new well of a 6-well plate with PBS and punch out the central cornea by an 8 mm disposable biopsy punch (Integra LifeSciences, Plainsboro, NJ).
   c. Remove corneal endothelium mechanically by using forceps.
2. Dissociation of human limbal epithelial cells
   a. Submerge the limbal tissue in 2 mL of PluriSTEM Dispase II Solution (1 mg/mL) and incubate for 1 h at 37°C in a CO_2 incubator.
   b. Rinse the limbal tissue with PBS twice.
   c. Scrape off the limbal epithelial cells into new PBS by using forceps.
   d. Transfer the limbal epithelial cells with PBS into 1.5 mL microcentrifuge tubes.
   e. Centrifuge the cell suspension at 500 g for 5 min at 20°C–25°C.
   f. Remove the supernatant and resuspend the cells in 400 μL of TrypLE Express Enzyme.
   g. Incubate for 30 min in a 37°C water bath.
   h. Add 600 μL of FACS buffer and centrifuge the cell suspension at 500 g for 5 min at 4°C.
   i. Resuspend the cells with 1 mL of FACS buffer.
   j. Remove the cell clumps by filtering the cells through 35 μm cell strainer (BD Biosciences, San Jose, CA).
   k. Count the cell number.
   l. Centrifuge the cell suspension at 500 g for 5 min at 4°C.
   m. Resuspend the cells at the concentration of 1×10^6 cells/mL.

Antibody staining

Timing: 1–2 h

This section describes steps of staining the limbal epithelial cells with anti-BCAM monoclonal antibody (mAb) and Propidium Iodide (PI) for dead cells removal.

3. Antibody staining.
   a. Separate the cell suspension into 5 tubes as below.

Tube	Condition	Amount
#1	Unstained	50 μL
#2	Anti-BCAM mAb + PI	>50 μL
#3	Isotype control mAb + PI	50 μL
#4	Anti-BCAM mAb only	50 μL
#5	PI only	50 μL

   b. Add VioBright FITC-conjugated anti-BCAM mAb to tubes #2 and #4 (Dilution is 1:10).
   c. Add VioBright FITC-conjugated REA Control Antibody to tube #3 (Dilution is 1:50).
   d. Incubate for 30 min on ice.
   e. Fill each tube up to 1 mL with FACS buffer and centrifuge the cell suspension at 500 g for 5 min at 4°C.
   f. Remove the supernatant, add 1 mL of FACS buffer and centrifuge the cell suspension at 500 g for 5 min at 4°C.
   g. Repeat the above step (3f) once more.
   h. Resuspend the cells with the original amount of cell suspension.
4. Dead cell staining by PI.
   a. Add PI at 1:100 dilution and incubate for 10 min on ice.

   Optional: Add 2.5 μg/mL Alexa Fluor 647-conjugated anti-ABCB5 mAb to detect ABCB5-positive limbal stem cells as well as BCAM-positive progenitors. Refer to Sasamoto et al. for detailed protocol for ABCB5 staining.[3]

   Optional: Other dead cell staining reagents (e.g., DAPI, 7-AAD) can be used instead of PI.

   Note: Use all remaining cell suspension for tube #2 to increase the number of BCAM-positive cells isolated by flow cytometry.

   ⚠ CRITICAL: PI is a potential carcinogen. Wear protective clothing, gloves and eye/face protection.

Flow cytometry

Timing: 1 h

This section describes the steps for isolating BCAM-positive and negative- cells from the limbal epithelial cell suspensions.

5. Set up the flow cytometry machine.

   Note: FACSAria II cell sorter (BD Biosciences) is used for the current protocol.

6. Gate settings (Figure 2).
   a. Adjust the scale of forward scatter (FSC)-A and side scatter (SSC)-A using tube #1.
   b. Run all the remaining samples for the analyses.
   c. Adjust settings for spectral compensation using tubes #4 and #5.
   d. Remove cellular debris based on the FSC-A and SSC-A.
   e. Remove doublets based on SSC-W and SSC-H.
   f. Remove doublets based on FSC-W and FSC-H.
   g. Determine the viable cell gating by comparing the tubes #2 and #4.

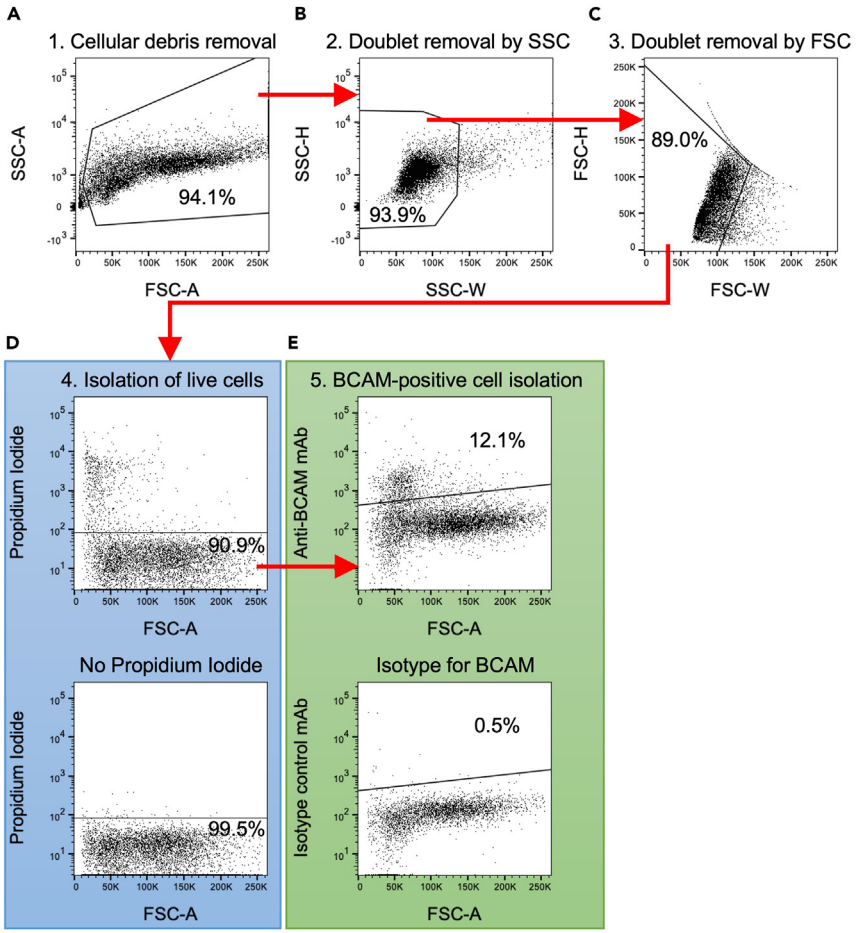

Figure 2. Gate settings for the flow cytometry

(A) Cellular debris removal based on FSC-A and SSC-A.

(B) Doublet removal based on SSC-W and SSC-H.

(C) Doublet removal based on FSC-W and SSC-H.

(D) Isolation of live cells as Propidium Iodide (PI)-negative cells. Determine the viable cell gating by comparing the tubes #2 (Anti-BCAM mAb + PI) and #4 (Anti-BCAM mAb only).

(E) BCAM-positive cell isolation. Determine the BCAM-positive and -negative cell gating by comparing the tubes #2 (Anti-BCAM mAb + PI) and #3 (Isotype control mAb + PI). FSC, forward scatter; A, area; SSC, side scatter; W, width; H, height.

h. Determine the BCAM-positive and -negative cell gating by comparing the tubes #2 and #3.

7. Sort BCAM-positive and -negative cells from tube #2.

Note: Use 1.5 mL microcentrifuge tubes with 300 μL of KCM supplemented with 10 ng/mL KGF and 10 μM Y-27632 for cell collection.

Note: Sort the same number of BCAM-positive and -negative cells.

Cell culture

Timing: 3 weeks

This section describes steps to culture the purified BCAM-positive and -negative cells for holoclone and cell sheet formation assays.

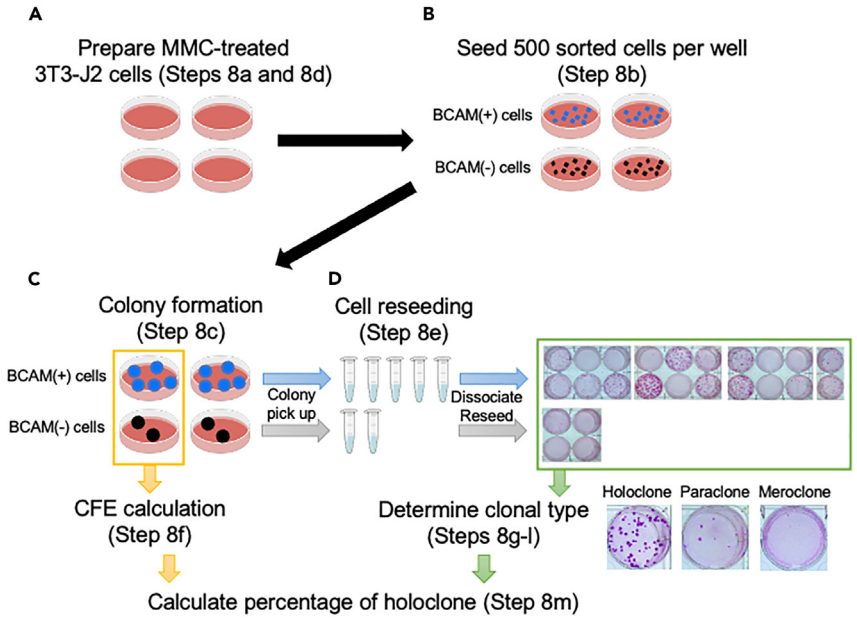

Figure 3. Holoclone assay

(A) Preparation of Mitomycin C (MMC)-treated 3T3-J2 cells.

(B) Seeding of 500 sorted BCAM-positive and -negative cells on the MMC-treated 3T3-J2 cells.

(C) Initial colony forming assay.

(D) Cell reseeding and holoclone assay.

8. Holoclone assay (Figure 3).[4–6]
   a. Remove the 3T3-J2 culture media from 6-well plates with MMC-treated 3T3-J2 cells and add 2 mL of KCM supplemented with 10 ng/mL KGF and 10 μM Y-27632.
   b. Seed sorted BCAM-positive and -negative cells on the MMC-treated 3T3-J2 cells at 500 cells per well on a 6-well plate.

   Note: Seed the cells into at least two wells of a 6-well plate. One for initial colony forming assay (CFA) to calculate colony-forming efficiency (CFE) and the other for reseeding for holoclone formation.

   c. Incubate the cells for 10 days at 37°C in a CO_2 incubator.

   Note: Change the media every 2–3 days.

   d. Prepare MMC-treated 3T3-J2 cells at day 7–9 in 6-well plates.

   Note: Prepare enough number of 6-well plates for cell reseeding for holoclone assay.

   e. Cell reseeding at day 10.
      i. Remove the media from MMC-treated 3T3-J2 cells (step 8d) and add 2 mL of KCM media.
      ii. Remove the 3T3-J2 cells by gentle pipetting from one of the wells from step 8c.
      iii. Replace the media to CEM.
      iv. Pick up the colonies under microscope and transfer each colony to a single 1.5 mL micro-centrifuge tube with 50 μL of PBS supplemented with 0.5 μL of B-27 Supplement.
      v. Add 300 μL of TrypLE Express Enzyme to each tube and incubate for 15 min in a 37°C water bath.
      vi. Vortex for 5–10 s and pipette the cells 10 times.

vii. Seed 110 μL of cell suspension to each well prepared in step 8e-i.
viii. Incubate the cells for 10 days at 37°C in a CO_2 incubator.

Note: Change the media every 2–3 days.

Note: Add 1–2 more days for incubation if the colonies are small.

f. Calculate CFE from initial CFA wells.
   i. Fix the cells in one of the wells from step 8c with 10% formalin for 1 h at 20°C–25°C.
   ii. Rinse the cells with distilled water three times and incubate for 1 h at 20°C–25°C in the last distilled water.
   iii. Remove the distilled water, add 2 mL of 2% Rhodamine and incubate for 72 h at 20°C–25°C.
   iv. Remove the Rhodamine and rinse with 0.2 M hydrogen chloride three times.
   v. Dry and take images of stained colonies.
   vi. Calculate CFE as a ratio of the colony numbers per well to the seeded cell numbers (500 cells).
g. After 10 days culture of the cells from step 8e, fix the cells with 10% formalin for 1 h at 20°C–25°C.
h. Rinse the cells with distilled water three times and incubate for 1 h at 20°C–25°C in the last distilled water.
i. Remove the distilled water, add 2 mL of 2% Rhodamine and incubate for 72 h at 20°C–25°C.
j. Remove the Rhodamine and rinse with 0.2 M hydrogen chloride three times.
k. Dry and take images of stained colonies.
l. Determine clonal type by the protocol shown in Pellegrini et al.[4]

Clonal type	% Of terminal clone
Holoclone	0%–5%
Paraclone	5%–95%
Meroclone	95%–100%

m. Calculate the percentage of holoclones by multiplying the CFE (%) (step 8f-vi) by the percentage of wells containing a holoclone among the seeded wells (step 8L).

9. Cell sheet formation assay (Figure 4).
   a. Place 24-well inserts onto the 24-well plates.
   b. Coat 24-well insert with iMatrix-511 at 0.5 μg/cm^2 in 200 μL of PBS by incubation at 37°C for 1 h.
   c. Remove the 3T3-J2 culture media from 24-well plates with MMC-treated 3T3-J2 cells and add 1.25 mL of KCM supplemented with 10 ng/mL KGF and 10 μM Y-27632.
   d. Remove PBS with iMatrix-511 from the inserts and transfer the inserts to 24-well plates with MMC-treated 3T3-J2 cells prepared in Step 9c.
   e. Add 250 μL of KCM supplemented with 10 ng/mL KGF and 10 μM Y-27632 to the upper part of the 24-well inserts.
   f. Seed sorted BCAM-positive and -negative cells on the insert at 500 cells per well.
   g. Incubate the cells for 15 days at 37°C in a CO_2 incubator.

Note: Change the media every 2–3 days.

   h. Remove the media from top of the insert (airlifting) at day 15.
   i. Incubate the cells for 5 more days at 37°C in a CO_2 incubator.

Note: Change the media under the insert every 2–3 days.

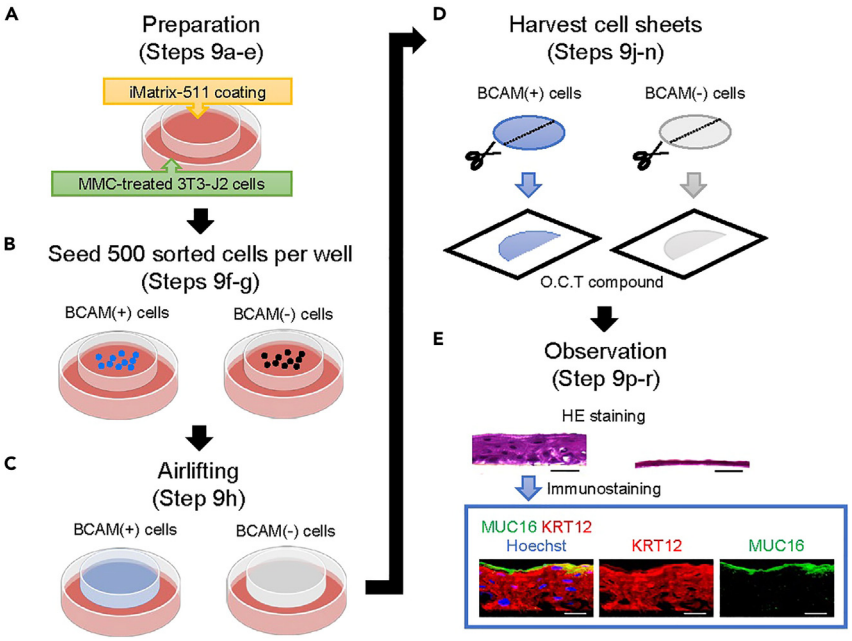

Figure 4. Cell sheet formation assay

(A) Preparation of Mitomycin C (MMC)-treated 3T3-J2 cells and iMatrix-511 coating.

(B) Seeding of 500 sorted BCAM-positive and -negative cells on the insert.

(C) Removal of the media from top of the insert (airlifting).

(D) Embedding cell sheets cut in half into O.C.T. compound.

(E) Observation of cell sheets by Hematoxylin and eosin (HE) staining, immunofluorescence or immunohistochemistry. Example shows HE staining of cell sheet derived from BCAM-positive cells and -negative cells (upper panel) and immunofluorescence of mature corneal epithelium markers, Keratin 12 (KRT12) and Mucin 16 (MUC16) in the cell sheet derived from BCAM-positive cells (lower panel). Nuclei stained with Hoechst 33342 (blue). Scale bar, 20 μm.

j. Rinse the insert with PBS once.

k. Cut apart the bottom part of the insert with cell sheet from the anchor of the insert and cut the cell sheet in half.

l. Soak the cell sheets in 4% paraformaldehyde and incubate for 15 min at 20°C–25°C.

m. Rinse the cell sheet with Tris Buffered Saline (TBS) for 10 min at 20°C–25°C twice.

n. Submerge the cell sheets into O.C.T compound and freeze them in −80°C.

o. Make sections of the cell sheet by cryostat.

p. Proceed to Hematoxylin and eosin (HE) staining, immunofluorescence or immunohistochemistry.

q. Use Hematoxylin and Eosin Stain Kit (Vector Laboratories, Burlingame, CA) for HE staining.

r. Perform immunostaining of Keratin 12 (KRT12) and Mucin 16 (MUC16).

   i. Rinse the section with TBS for 10 min at 20°C–25°C.

   ii. Permeabilize and block the section with blocking buffer (TBS containing 5% normal donkey serum and 0.3% Triton X-100) for 30 min at 20°C–25°C.

   iii. Dilute primary antibodies (rabbit anti-KRT12 mAb and mouse anti-MUC16 mAb) with staining buffer (TBS containing 1% normal donkey serum and 0.3% Triton X-100) at 1:400.

   iv. Remove blocking buffer, add diluted primary antibodies and incubate 8–12 h at 4°C.

   v. Rinse the section with TBS for 10 min at 20°C–25°C twice.

   vi. Dilute secondary antibodies (Donkey anti-Mouse IgG- Alexa Fluor 488 and Donkey anti-Rabbit IgG- Alexa Fluor 568) with staining buffer at 1:400.

   vii. Add diluted secondary antibodies and incubate for 1 h at 20°C–25°C in the dark.

   viii. Dilute Hoechst 33342 with staining buffer at 1:400.

ix. Remove secondary antibodies and add diluted Hoechst 33342 and incubate for 10 min at 20°C–25°C in the dark.
x. Rinse the section with TBS for 10 min at 20°C–25°C twice in the dark.
xi. Seal the slides with ProLong Gold Antifade Mountant.
xii. Take images of slides by C2+ confocal microscope (Nikon, Tokyo, Japan) and analyze by NIS-Elements AR v4.30.01 (Nikon).

EXPECTED OUTCOMES

This protocol describes the purification of the BCAM-positive progenitors with high holoclone formation and corneal sheet generation capabilities from human donor corneas. This protocol also describes the robust methods of holoclone formation assay and corneal sheet formation assay.

With the current protocol, the expected yield is > 2,000 viable BCAM-positive cells from a single human donor limbus. In addition to isolating the cells for cell cultures, the obtained cells can be used for RNA extraction. We confirmed the high expression level of *BCAM* RNA in BCAM-positive cells by quantitative reverse transcription PCR (qRT-PCR).[1]

BCAM-positive cells possess higher holoclone-forming capacity than BCAM-negative cells (5.06% ± 2.65% vs. 0.13% ± 0.19%, $p = 0.0028$),[1] showing that BCAM-positive cells have cell-intrinsic self-renewal capacity with high proliferative phenotype.

Stratified cell sheet can be formed by BCAM-positive cells, but not by BCAM-negative cells (Figure 4E). We showed that siRNA-based BCAM knockdown attenuated the cell sheet thickness formed.[1] Thus, BCAM is functionally required for corneal epithelial cell sheet formation. In addition, the cell sheet obtained from BCAM-positive cells showed high expression of corneal differentiation markers such as KRT12 and MUC16 (Figure 4E).

LIMITATIONS

This protocol is only applicable to human donor corneas. We have not tested this protocol on other species, such as mice, rat and rabbit. Note that the anti-BCAM mAb used in the current study is specific to human BCAM protein.

The yield of BCAM-positive cells is highly variable because of the high variability of human donor corneas. If a large number of BCAM-positive cells are needed for the subsequent experiments, using a pair of corneas from a single donor is recommended.

We have not tested the blocking effect of anti-BCAM mAb bound to the BCAM-positive cells. It may block the function of BCAM-positive cells at a certain level.

TROUBLESHOOTING

Problem 1

Low yield of limbal epithelial cells from human donor corneas (Step 2).

Potential solution

Low yield of limbal epithelial cells is mainly due to the relatively bad preservation condition of donor corneas. Means et al. showed that preserving corneas in Optisol-GS increases the corneal epithelial damage daily, especially after day 6.[7] Therefore, using the corneas within 1 week after death is recommended.

Problem 2

Low yield of BCAM-positive cells isolated by flow cytometric cell sorting (Step 7).

Potential solution

It is difficult to anticipate the yield of BCAM-positive cells as it is highly variable among donors. To obtain more BCAM-positive cells, it is recommended to prepare cell suspensions stained with anti-BCAM mAb plus PI using a pair of corneas from a single donor.

Problem 3

Small colonies in colony forming assay (CFA) and holoclone assay (Step 8).

Potential solution

The growth rate of colonies differs by donors. Add a few more days to let the colonies grow, if needed.

Problem 4

Merged colonies in colony forming assay (CFA) and holoclone assay (Step 8).

Potential solution

The growth rate of colonies differs by donor. Monitor the colony size frequently, especially after day 8 and fix the cells before the colonies start to merge.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Natasha Y. Frank (nyfrank@bwh.harvard.edu).

Materials availability

This study did not generate any unique reagents.

Data and code availability

This study did not generate unique datasets or code.

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AUTHOR CONTRIBUTIONS

Y.S., M.H.F., and N.Y.F. designed the study. Y.S., P.C.Y., M.H.F., and N.Y.F. wrote the manuscript. Y.S., P.C.Y., J.T., M.H.F., and N.Y.F. performed experiments and analyzed and compiled the data. All authors read and edited the manuscript.

DECLARATION OF INTERESTS

M.H.F. and N.Y.F. are inventors or co-inventors of US and international patents assigned to Brigham and Women's Hospital, Boston Children's Hospital, the Massachusetts Eye and Ear Infirmary, and/or the VA Boston Healthcare System, Boston, MA, licensed to Rheacell GmbH & Co. KG (Heidelberg, Germany). M.H.F. serves as a scientific advisor and holds equity in Rheacell GmbH & Co. KG.

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