

## Murine Hair Follicle Derived Stem Cell Transplantation onto the Cornea Using a Fibrin Carrier

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**[Abstract]** The goal of this protocol is to establish a procedure for cultivating stem cells on a fibrin carrier to allow for eventual transplantation to the eye. The ability to transfer stem cells to a patient is critical for treatment for a variety of disorders and wound repair. We took hair follicle stem cells from the vibrissae of transgenic mice expressing a dual reporter gene under the control of the Tet-on system and the keratin 12 promoter (Meyer-Blazejewska *et al.*, 2011). A clonal growth assay was performed to enrich for stem cells. Once holoclones formed they were transferred onto a fibrin carrier and cultivated to obtain a confluent epithelial cell layer. Limbal stem cell deficient (LSCD) mice were used as the transplant recipient in order to test for successful grafting and eventual differentiation into a corneal epithelial phenotype.

**Keywords:** Holoclones, Clonal growth, Hair follicle stem cells, Transgenic mice, Fibrin carrier

**[Background]** Stem cells are widely used as a therapeutic tool, thus a means for delivery is essential. In fact, many researchers and companies are searching for the best way to deliver cells into the human body to optimize cell survival as well as integration into the host tissue. Injection methods have been widely used in animal models but often result in poor survival and integration. Techniques utilizing biomaterials and surgical devices are currently being employed. One technique that has been utilized to deliver stem cells is fibrin carriers. Fibrin gel is a degradable biopolymer that can adhere to native tissue allowing for cell attachment, migration and proliferation (Ehrbar *et al.*, 2005). Fibrin gels have many advantages including biocompatibility, controlled degradation (Kjaergard *et al.*, 1994; Sidelmann *et al.*, 2000), uniform cell distribution and high cell seeding efficiency (Swartz *et al.*, 2005). Fibrin gels have been utilized for treating skin burns (Pellegrini *et al.*, 1999; Ronfard *et al.*, 2000), junctional epidermolysis bullosa (Hirsch *et al.*, 2017) and corneal damage (Pellegrini *et al.*, 1997; Rama *et al.*, 2010). The method described here uses a fibrin carrier to transplant hair follicle derived stem cells onto the ocular surface of a limbal stem cell-deficient mouse. Cell engraftment and differentiation was assessed for a 5-week period via fluorescent microscopy.

## **Materials and Reagents**

1. TISSEEL [Fibrin Sealant] (Baxter, catalog number: 1501261)
2. Insulin syringe (Fisher Scientific, catalog number: 14-829-1A)  
*Manufacturer: BD, catalog number: 329420.*
3. 6-well plates (Corning, Falcon®, catalog number: 353934)
4. Pipette tips (MidSci, Avant low binding tips)
5. Transfer pipet
6. HFSC discs
7. Ethilon 10-0 nylon sutures (Ethicon, catalog number: 9006G)
8. Microscope slides (Fisher Scientific, catalog number: 12-552-3)
9. Coverglass 22 x 50 (Fisher Scientific, catalog number: 12-548-5E)
10. Thrombin
11. C57BL/6 mice (THE JACKSON LABORATORY, catalog number: 000664)
12. K12<sup>rtTA/rtTA</sup>/tetO-cre/ROSA<sup>mTmG</sup> transgenic mice (see Notes)
13. 0.9% saline (Fisher Scientific, catalog number: 23-535435)
14. BioGlo fluorescein sodium ophthalmic strips (Hub Pharmaceuticals, NDC 17238-900-11)
15. Doxycycline chow (Custom Animal Diets, catalog number: AD3008)
16. Avastin (bevacizumab, Genentech, Inc.)
17. Anti-inflammatory drops—Inflanefran forte (Allergan, NDC 11980-180)
18. 16% paraformaldehyde (Electron Microscopy Sciences, catalog number: 15710)
19. Sodium borohydride (Sigma-Aldrich, catalog number: 71320)
20. DAPI (Thermo Fisher Scientific, Invitrogen™, catalog number: D3571)
21. Sodium chloride (NaCl) (Fisher Scientific, catalog number: BP358-1)
22. Calcium chloride (CaCl<sub>2</sub>) (Acros Organics, catalog number: 349610025)
23. Dulbecco's modified Eagle medium (DMEM) without calcium and magnesium (Thermo Fisher Scientific, Gibco™, catalog number: 21068028)
24. Ham's F12 Nutrient Mix (Thermo Fisher Scientific, Gibco™, catalog number: 11765047)
25. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10082147)
26. Human recombinant epidermal growth factor (Merck, catalog number: GF144)
27. L-Glutamine (Thermo Fisher Scientific, Gibco™, catalog number: 25030081)
28. Human corneal growth supplement (Thermo Fisher Scientific, Gibco™, catalog number: S0095)
29. Penicillin-streptomycin (10,000 U/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15140148)
30. Gibco™ Amphotericin B (Thermo Fisher Scientific, catalog number: 15290026)
31. Sodium phosphate dibasic, anhydrous (Na<sub>2</sub>HPO<sub>4</sub>) (Sigma-Aldrich, catalog number: S7907)
32. Sodium phosphate monobasic, anhydrous (NaH<sub>2</sub>PO<sub>4</sub>) (Sigma-Aldrich, catalog number: S8282)
33. Analytical grade glycerol (Sigma-Aldrich, catalog number: G6279)
34. Mowiol 4-88 (Merck, catalog number: 475904)

35. Tris (Fisher Scientific, catalog number: BP152-1)
36. Ketamine/HCl 100 mg/ml (KetaJect; Henry Schein Animal Health, catalog number: 010177)
37. Xylazine AnaSed® 100 mg/ml (Santa Cruz Biotechnology, catalog number: sc-362949Rx)
38. Fibrinogen solution (see Recipes)
39. Thrombin solution (see Recipes)
40. Stem cell media (see Recipes)
41. 0.1 M Phosphate Buffer, pH 7.4 (see Recipes)
42. Mowiol mounting medium (see Recipes)
43. Ketamine/xylazine solution (see Recipes)

## **Equipment**

1. Algerbrush II Corneal rust ring remover (MicroSurgical Technology, catalog number: AM0100)
2. Inverted Fluorescence Microscope (Zeiss Observer Z1 with an apotome attachment) (ZEISS, model: AxioObserver Z1)
3. Suture Tying Forceps (Fine Science Tools, catalog number: 18025-10)
4. Microdissection scissors (Fine Science Tools, catalog number: 15000-00)
5. Epi-fluorescent stereomicroscope (ZEISS, model: Stemi SVII)
6. BSL2 Laminar Flow Hood (Thermo Fisher Scientific, Thermo Scientific™, model: 1300 Series A2, catalog number: 1387)
7. Hemocytometer (Hausser Scientific, catalog number: 3200)
8. CO<sub>2</sub> Incubator (Thermo Fisher Scientific, Thermo Scientific™, model: NAPCO Series 8000 WJ)
9. Dissecting Scope (ZEISS, model: Stemi DV4)
10. Centrifuge (Hettich, model: Rotina 35)

## **Software**

1. AxioVison 4.7

## **Procedure**

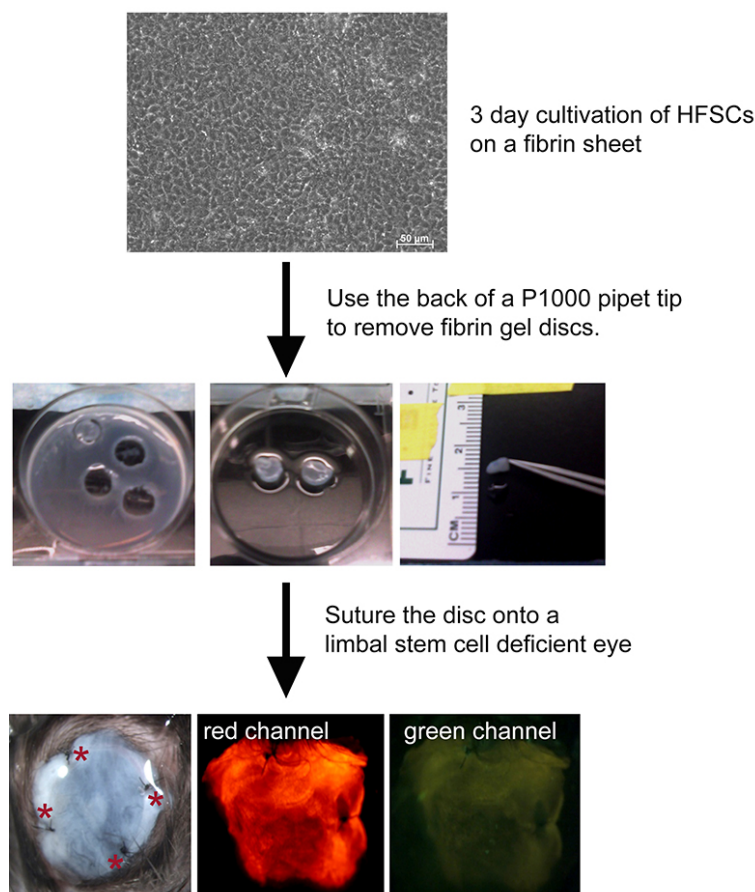
### **A. Preparation of fibrin carrier**

1. Thaw the TISSEEL Fibrin Sealant (2-ml pre-filled syringe) at 37 °C.
2. Prepare diluted fibrin by adding 1 ml of Sealer Protein (fibrinogen) entire contents of the syringe to 1.16 ml of fibrinogen solution (Recipe 1).
3. Prepare thrombin mix by adding 1 ml of thrombin (entire contents of the syringe) to 9 ml of thrombin solution (Recipe 2).
4. Prepare diluted thrombin by adding 1.5 ml of thrombin mix (from Step A3) to 25 ml thrombin solution.

5. To one well of a 6-well plate add 0.75 ml of diluted thrombin (from Step A3). Ensure that the diluted thrombin solution covers the entire well evenly.
6. To the same well add 0.75 ml diluted fibrin. Add dropwise to the well quickly mixing with a pipette tip.
7. Allow gel to set at room temperature. The final concentration of fibrinogen and thrombin is ~10 mg/ml and 3 IU/ml, respectively. The gel will set in approximately 5 min. Once the gel has set proceed to the cultivation of the cells.

#### B. Cultivation on a fibrin carrier

1. Seed hair follicle stem cells derived from a dual reporter mouse at  $1 \times 10^5$  cells/ml onto a fibrin carrier and cultivate for 3 days in stem cell media (Recipe 3) at 37 °C, 5% CO<sub>2</sub>. A total volume of 2 ml is used for each well. Cells can be visualized using phase contrast microscopy (Figure 1)

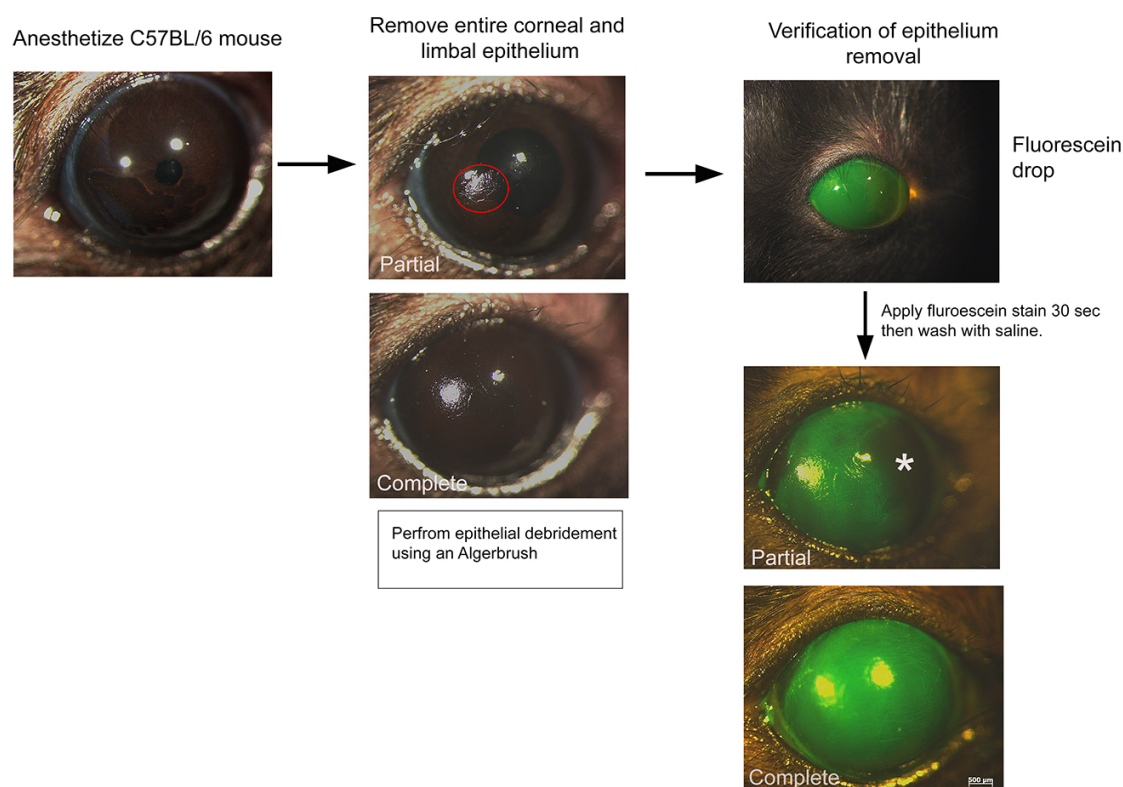


**Figure 1. Scheme showing transplant of a fibrin carrier containing bulge derived hair follicle stem cells.** Discs are prepared from hair follicle stem cells (HFSCs) from a dual reporter mouse cultivated for 3 days on a fibrin sheet by using the back of a pipet tip as a “cookie cutter”. Discs are then sutured to the eye of a limbal stem cell deficient mouse. Four sutures (red

asterisks) are placed. Cells fluoresce red but not green as they do not yet express the corneal epithelial differentiation marker, Krt12.

### C. Total limbal stem cell deficiency (Figure 2)

1. Anesthetize 6- to 8-week old C57BL/6 mice with ketamine (90 mg/kg) and xylazine (13 mg/kg) (Recipe 6) via intraperitoneal injection using a 28-G insulin syringe.
2. Generate total limbal stem cell deficiency by removing the entire corneal and limbal epithelium using an Algerbrush II corneal rust ring remover with a 0.5 mm burr (Note 6). Hair follicle stem cell transplantation (see below) should immediately follow the removal of epithelial cells.
3. Verify complete removal of corneal and limbal epithelium using fluorescein stain.
  - a. Dip the tip of a fluorescein sodium ophthalmic strip in 0.9% saline for 20 times.
  - b. Using a transfer pipet, add a drop of the fluorescein stain onto the eye. Let sit 30 sec.
  - c. Extensively wash in 0.9% saline.
  - d. Image using a fluorescent stereomicroscope.



**Figure 2. Scheme of limbal stem cell deficiency.** An algerbrush is used to remove the entire corneal and limbal epithelium. The red circle depicts partial debridement. Fluorescein stain can be used to verify the complete removal of the epithelium. Note the absence of fluorescein stain in the partial removal (asterisk).

D. Hair follicle stem cell transplantation

1. Prepare ~7 mm fibrin gel containing HFSC discs by using the back of a P1000 pipet tip (see Figure 1). Place the discs into PBS (Note 5) using forceps (Figure 1).
2. Suture an approximately 7 mm fibrin carrier containing HFSC onto the surface of a limbal stem cell-deficient mouse eye with the stem cells facing the recipient's basement membrane. A suture was placed superiorly, inferiorly, nasally and temporally (Figure 1).
3. Following surgical recovery, administer anti-inflammatory eye drops for the first five days followed by Avastin eye drops for the next 7 days. Eye drops should be administered once a day.

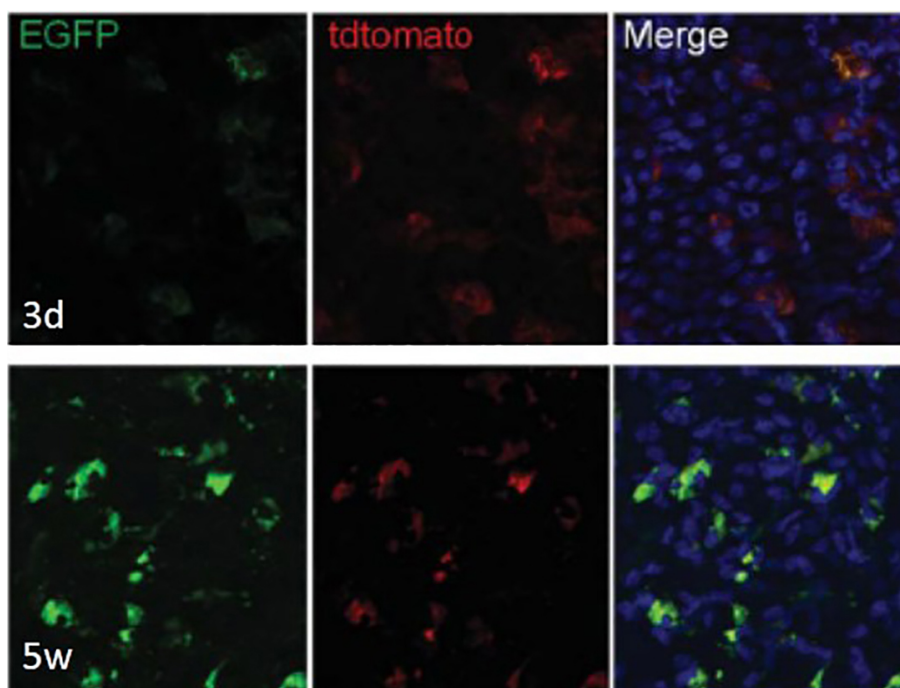
E. Conversion to corneal epithelial cells

1. Mice are fed doxycycline chow (1 g/kg) *ad libitum* until sample collection at 3 days, 1 week, 2 weeks, 3 weeks and 5 weeks.
2. Euthanize mice and enucleate the eyes at the sample collection time points and fix in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (Recipe 4) at 4 °C overnight followed by PBS washes.
3. Dissect the cornea using microdissection scissors.
4. Treat the corneas with 0.2% sodium borohydride (Note 7) for 30 min at room temperature followed by washing in 0.1 M phosphate buffer, pH 7.4.
5. Stain corneas with 4'6-diamidino-2-phenylindole (DAPI, 1 µg/ml), mount onto a microscope slide using cover glass and Mowiol medium (Recipe 5) and visualize using a Zeiss Observer Z1 microscope with an apotome attachment.

**Data analysis**

AxioVison 4.7. Z-stack images in the red (membrane tomato red), green (membrane EGFP) and blue (DAPI) channels were taken for each collection time point. Conversion of red to green was noted at each of the time points (Figure 3).





**Figure 3. Engraftment and differentiation of the hair follicle bulge-derived stem cells into corneal epithelial cells.** Single color images taken from a Z-stack depicting the cellular localization of membrane tomato red and membrane EGFP at 3 days and 5 weeks post-transplantation. Magnification x200. Reprinted with permission from Meyer-Blazejewska *et al.*, 2011.

## Notes

1. Preparation of the fibrin gel and cultivation of the HFSC should be performed in a class II biological safety cabinet.
2. Various dimensions of the fibrin carrier can be made as long as the ratio of diluted thrombin and diluted fibrin remain 1:1.
3. All cell counts were performed using a hemocytometer.
4. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. Genetically modified mouse lines *Krt12rtTA* (Chikama *et al.*, 2005), *TetO-cre* (Perl *et al.*, 2002) and *Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J(ROSA<sup>mTmG</sup>)* (Muzumdar *et al.*, 2007) have been previously described. Compound transgenic mice were generated by breeding individual mouse lines to create *K12<sup>rtTA/rtTA</sup>/tetO-cre/ROSA<sup>mTmG</sup>*. This dual reporter mouse model uses the keratin 12 promoter (corneal epithelium-specific) to drive the expression of the Tet-on system. In conjunction with doxycycline and cre, the membrane tomato red hair follicle stem cells will turn green if they have differentiated into corneal epithelial cells.

5. When creating and transferring the fibrin gel discs be sure to handle them with care as to avoid damaging the cells. Additionally, be sure the discs are placed in PBS with the HFSC facing upwards.
6. When removing the epithelium with the Algerbrush care should be taken to avoid damaging the basement membrane. This can be done by holding the Algerbrush at a 45° angle and moving it in a circular, sweeping motion. Use only gentle pressure as the epithelium is relatively easy to remove.
7. Care should be taken when handling sodium borohydride as it poses physical and health hazards.

## **Recipes**

1. Fibrinogen solution  
10% NaCl  
100 mM CaCl<sub>2</sub>  
0.9% NaCl  
ddH<sub>2</sub>O
2. Thrombin solution  
10% NaCl  
100 mM CaCl<sub>2</sub>  
ddH<sub>2</sub>O
3. Stem cell media  
3 parts DMEM/High glucose without Ca<sup>2+</sup> or Mg<sup>2+</sup>  
1 part Ham's F12  
10% FBS  
10 ng/ml EGF  
500 mg/L L-glutamine  
0.4 mM calcium chloride  
1x human corneal growth supplement  
10,000 U/ml penicillin  
10,000 µg/ml streptomycin  
25 µg/ml amphotericin B
4. 0.1 M Phosphate Buffer, pH 7.4  
76 mM Na<sub>2</sub>HPO<sub>4</sub> (anhydrous)  
27 mM NaH<sub>2</sub>PO<sub>4</sub> (anhydrous)
5. Mowiol mounting medium  
24 g analytical grade glycerol  
9.6 g Mowiol 4-88  
24 ml ddH<sub>2</sub>O



48 ml 0.2 M Tris-HCl buffer, pH 8.5

6. Ketamine/xylazine solution

2 ml of ketamine (100 mg/ml)

0.25 ml xylazine (100 mg/ml)

7.75 ml PBS

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