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## Ex vivo corneal organ culture model for wound healing studies

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08/1618

Dear *JOVE*,

Please accept our revision of the manuscript entitled: "***Ex vivo* corneal organ culture model for wound healing studies".** We have extensive experience in ocular studies including the use of human and animal corneas for wound healing studies. Here we describe a useful organ culture assay for discovering novel agents that promote regenerative healing or testing the potentially toxic effects of agents. Use of this multi-cellular 3D model is a tremendous cost savings over animal studies and can be used to select the best targets and reagents prior to embarking on *in vivo* studies. Since ocular human tissue is readily available, this assay can be performed with donated human tissue making it translatable to human disease.

Sincerely,

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**TITLE:**

# *Ex Vivo* Corneal Organ Culture Model for Wound Healing Studies

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**KEYWORDS:**

Cornea, Scarring, Fibrosis, Myofibroblast, *Ex vivo*, Organ culture

**SHORT ABSTRACT:**

A protocol for an *ex vivo* corneal organ culture model useful for wound healing studies is described. This model system can be used to assess the effects of agents to promote regenerative healing or drug toxicity in an organized 3D multicellular environment.

**LONG ABSTRACT:**

The cornea has been used extensively as a model system to study wound healing. The ability to generate and utilize primary mammalian cells in two dimensional (2D) and three dimensional (3D) culture has generated a wealth of information not only about corneal biology but also about wound healing, myofibroblast biology, and scarring in general. The goal of the protocol is an assay system for quantifying myofibroblast development, which characterizes scarring. We demonstrate a corneal organ culture *ex vivo* model using pig eyes. In this anterior keratectomy wound, corneas still in the globe are wounded with a circular blade called a trephine. A plug of approximately 1/3 of the anterior cornea is removed including the epithelium, the basement membrane, and the anterior part of the stroma. After wounding, corneas are cut from the globe, mounted on a collagen/agar base, and cultured for two weeks in supplemented-serum free medium with stabilized vitamin C to augment cell proliferation and extracellular matrix secretion by resident fibroblasts. Activation of myofibroblasts in the anterior stroma is evident in the healed cornea. This model can be used to assay wound closure, the development of myofibroblasts and fibrotic markers, and for toxicology studies. In addition, the effects of small molecule inhibitors as well as lipid-mediated siRNA transfection for gene knockdown can be tested in this system.

## INTRODUCTION:

Scarring in the cornea resulting from injury, trauma, or infection can lead to debilitating opacities and permanent vision loss. Thus, there is a critical need to identify pathways that can be targeted for therapeutic intervention. Current treatment options are limited and consist primarily of corneal transplantations, which are not accessible to patients across the world. Both human (**Figure 1**) and animal corneas can be utilized for 2D and 3D cell culture studies[1,2]. Human cadaver corneas not suitable for transplant can be obtained from eye banks or centralized tissue banks (National Disease Research Interchange (NDRI)), and animal eyes can be obtained from an abattoir. Primary corneal epithelial cells, stromal fibroblasts, and more recently, endothelial cells, can be isolated and cultured from these tissues for wound healing and toxicology studies[3-5]. In addition to the importance of understanding the molecular basis of blinding eye disease, the accessibility of tissue and the ability to culture primary cells has made the cornea an important model system for study. The cornea is ideal for testing the effects of agents on scarring as the normal cornea is transparent and certain types of wounds create opacities or fibrotic scars (reviewed in[6]). Several *in vivo* corneal wound healing models have also been extensively utilized for scarring studies[1]. Less utilized has been the *ex vivo* corneal wound healing model[7,8] that is describe in detail here. The goal of this method is to quantify scarring outcomes characterized by fibrotic makers in a 3D multicellular corneal *ex vivo* model system.

Corneal epithelial wounding that does not breach the epithelial basement membrane normally closes within 24–72 h[9]. Soon after wounding, the cells at the edge of the epithelium start spreading and migrating into the epithelial free surface, to reestablish epithelial barrier function. This activity is sequentially followed by activation of corneal basal cell proliferation first and, in a later stage, of precursor cells located at the outer limbal zone to achieve recovery of epithelial cell mass[10,11]. These wounds often heal without scarring. However, a wound that penetrates the basement membrane into the stroma often results in scar formation[1]. After corneal stromal wounding, the stroma is populated with cells of multiple origins including differentiated resident stromal cells as well as bone marrow-derived fibrocytes[12-14]. Fibrotic scarring is characterized by the persistence of myofibroblasts in a healing wound. These pathological myofibroblasts demonstrate increased adhesion through the accumulation of integrins in focal adhesions, contractile α-smooth muscle actin (α-SMA) stress fibers, and local activation of extracellular matrix (ECM)-sequestered latent-transforming growth factor-beta (TGFβ). The differentiation of epithelial-derived cells, known as epithelial to mesenchymal transition (EMT), may also contribute to scar formation[6].

There is a delicate balance between cell differentiation and apoptosis after wounding. Because of the breach in the basement membrane, growth factors such as platelet-derived growth factor (PDGF) and TGFβ from tears and the epithelium bathe the stroma, inducing myofibroblast differentiation, a sustained autocrine loop of TGFβ activation, and the secretion of disorganized fibrotic ECM[15,16]. The persistence of myofibroblasts in the healed wound promotes haze and scarring in the cornea (**Figure 2**). However, in regeneratively healed wound although myofibroblasts develop, they apoptose and thus are absent or significantly reduced in number in the healed tissue (reviewed in[6,10]). Thus, research on fibrotic scarring has focused at least in part

on targeting molecules that prevent excessive myofibroblast development or myofibroblast persistence[17,18]. Because myofibroblast persistence characterizes both scarring and fibrotic disease in all tissues[19], the cornea may be useful as a model system to study general cellular mechanisms of fibrosis.

In our model system, the cornea is wounded with a cylindrical blade called a trephine while still in the globe. Human and pig corneas can be wounded with either a 6 or 7 mm trephine; for rabbit corneas a 6 mm trephine is preferred. The pig cornea is similar in size to the human cornea. Because they are cost effective and readily available in large numbers, pig corneas are routinely used for organ culture. Furthermore, antibodies and siRNAs made to react with human have consistently cross-reacted with pig[7]. After wounding, corneas are cut from the globe with the limbus intact and mounted on an agar/collagen base. The corneas are cultured in serum-free media plus stabilized vitamin C to simulate fibroblast proliferation and ECM deposition[20]. Neither the addition of serum nor growth factors are needed to induce myofibroblast formation[7]. Corneas are routinely fixed and processed for histology after two weeks of culture. For gene knockdown, or to test the effects of an agent on wound healing, the wound can be treated with siRNA in the wound after wounding[7] or a soluble agent can be added to the media, respectively[8].

**PROTOCOL:**

## 1. Organ Culture

### 1.1) Preparations

1.1.1) Prepare agar solution as follows. In a small flask, prepare 1% agar and 1 mg/mL bovine collagen in DMEM-F12 up to 20 mL. Bring to boil on a hot plate. Put the solution into a 50 mL conical tube. Place tube in a water bath on a hot plate to keep the solution from solidifying.

1.1.2) Prepare supplemented serum-free media (SSFM) as per the composition provided in the **Table of Materials**.

Note: The necessary amount of SSFM to be prepared depends on the number of corneas to be processed. Usually 30 mL is enough media for 4 corneas.

### 1.2) Dissection

Note: Perform this step in a dissection hood or a chemical hood. The eyes are shipped with lids still attached in individual bags to protect the globes.

1.2.1) Remove globes from lids with a straight-edge surgical blade on an ethanol-cleaned chopping board. Remove excess fatty tissue from the eye using either a blade or a small scissors (**Figure 3A**, **3B**).

1.2.2) After removing the globe from the lid, hold the globe posteriorly with forceps and immediately dip the eye in phosphate-buffered saline (PBS). Quickly dip it 3x in 10% iodine (in a 100 mL beaker). Quickly dip 2x in PBS (~100 mL in a beaker, change PBS frequently).

1.2.3) Using a clean towel or tissue, wrap the eye circumferentially with enough pressure to have a taut corneal surface to cut with the trephine.

Note: Take care to prevent the towel or tissue from contacting the cornea.

**1.3) Wounding**

1.3.1) Use a 6 mm trephine to wound the center of the cornea. Penetrate the epithelium and anterior stroma without making a full-thickness wound through the entire cornea.

Note: If the endothelium is penetrated, a loss of pressure and leaking fluid will be seen. In this case the eye should be discarded.

1.3.2) Place the trephine in the center of the cornea, rotate it 180° clockwise and counter-clockwise 5x (each time the direction is changed it will count as one time) while applying light pressure to deepen the wound.

Note: The wound should now be deep enough to allow a tissue flap to be lifted using a pair of forceps. If this is not the case repeat step 1.3.2.

1.3.3) Lift the flap from the edges. At the same time, either with the other hand or with a second person, use a blade, cutting parallel to the globe to cut away the tissue as the forceps continue to lift off the anterior cornea within the wound margin. At the conclusion of this step there should be a circular wound located at the center of the cornea (See **Figure 3C**, **3D**).

**1.4) Cutting and Removing the Cornea from the Globe**

1.4.1) Holding the eye with the tissue, make a small incision 1 mm away from the edge of the cornea with a blade so that the limbus is included in the organ culture.

1.4.2) Using small, sharp scissors access the incision created in the prior step to cut around the globe, keeping a millimeter margin throughout the cornea to keep the limbus intact.

1.4.3) Place the cornea in a 60 mm dish with 1 mL of PBS, wound side down until mounting.

**1.5) Mounting**

1.5.1) Make sure the agar has come to a warm temperature (approximately 25 °C).

1.5.2) With two pairs of forceps, create a cup by holding two sides of the cornea with the endothelial side up. Add the warmed-up agar solution into the cornea using a sterile transfer pipette until it is full.

1.5.3) After the agar hardens (usually about 30-45 s) carefully flip the cornea with the agar into 60 mm plate (**Figure 3E**). Cover with a lid.

**1.6) Incubation**

1.6.1) Add 4 mL of SSFM to the plate, maintaining corneas at an air-liquid interface at the limbal border in 5% $CO_2$ at 37 °C. Refresh media after 24 h and thereafter every other day.

Note: If performing a transfection into the wound, omit the antibiotics until after transfection.

1.6.2) Wet the corneal surface once daily by adding 1 drop of SSFM from the conditioned media in the dish to maintain moisture. For this, take the dish out of the incubator, place it under the hood, remove the dish lid, wet the surface with media from dish using a sterile pipette, cover again and put it back at the incubator.

1.6.3) For gene knockdown, treat the wound with gene-targeting or control siRNA that is complexed to a lipid-mediated carrier as per the supplier's instructions (see below).

1.6.4) Mix 5 µL (50 pmol) of siRNA into 50 µL of reduced-serum minimum essential media (*e.g.*, Opti-MEM). Mix 2 µL of transfection reagent into 50 µL of reduced-serum media. Let this sit for 5 min and then mix them.

1.6.5) Add 200 µL of reduced-serum minimum media to the reagent/siRNA mixture.

1.6.6) Pipette dropwise onto the wound and incubate for 3 h.

1.6.7) Wash out siRNA from corneal surface with media in the dish.Change the incubation media to SSFM + antibiotics (see the **Table of Materials**). Continue incubation as mentioned previously (steps 1.6.1–1.6.2).

**2. Histology: Paraffin Sections and Immunostaining**

**2.1) Preparing the Tissue**

2.1.1) After a two-week incubation, if using some of the tissue for quantitative real time polymerase chain reaction (qRT-PCR) analysis, before fixing, cut the cornea in half through the wound. Place this half or only ¼ (either is enough tissue) into stabilizing RNA-protect reagent.

2.1.2) Using a standard isolation kit, isolate RNA and perform qRT-PCR.

Note: Alternatively, the wounded part only can be isolated and tested for gene expression.

2.1.3) Place the other half of the cornea into tissue pathology cassettes and submerge in fixative (10% formalin) for 2–4 days at room temperature (RT).

2.1.4) Paraffin embed this half of the wounded cornea using standard techniques.

Note: Orient the wounded cornea to ensure that the tissue sectioning will produce a cross-section of the cornea.

**2.2) Immunostaining using 3,3'-diaminobenzidine (DAB)**

**2.2.1) Day 1**

2.2.1.1) Label slides properly using a pencil. De-paraffinize the tissue by placing slides into a jar with clearing agent (2 changes, 10 min each).

2.2.1.2) Rehydrate the tissue by transferring the slides into ethanol at decreasing concentrations (100%, 100%, 70%, 50%, $dH_2O$, $dH_20$, 5 min for each change).

2.2.1.3) Perform antigen retrieval by microwaving the slides in a plastic jar with citrate buffer (10 mM, pH 6.4) for 5 min. First cycle 5 min at 50% power. Refill the jar with citrate buffer and repeat. Cool down for 10 min.

2.2.1.4) Wash 3x with PBS, 2 min each. Permeabilize tissue with 1% Triton X-100 in PBS 10 min at RT. Block sections with 3% normal goat serum (NGS) for 1 h at RT in humid chamber.

2.2.1.5) Incubate tissue with primary antibody (1:100 or as the supplier suggests) in 3% NGS overnight at 4 °C (300 µL per slide).

**2.2.2) Day 2**

2.2.2.1) Rinse slides 3x with PBST (PBS plus 1% Tween 20), 2 min each. Place slides in 3% $H_2O_2$ for 10 min to block endogenous peroxidase. Wash 3x with PBST, 2 min each. Incubate sections with HRP secondary antibody (1:250) in 3% NGS for 1 h at RT (300 µL per slide).

2.2.2.2) Wash slides 3x PBST, 2 min each. Treat slides with the DAB kit. Add 300 µL/slide for 3 min. Wash slides with $dH_2O$ 2x (quick dips).

2.2.2.3) Counterstain with Hematoxylin for 20 s. Wash the slide with $dH_2O$ 2x (quick dips). Stain with bluing agent for 20 s. Rinse in $dH_2O$ for 20 s. Dehydrate tissue by placing slides into increasing concentrations of ethanol (50%, 70%, 100%, 100%, all quick dips).

2.2.2.4) Dry slides on a paper towel under the hood for 10–20 min.

2.2.2.5) Mount slides using 1 drop of mounting media, cover with coverslip. Label and store at RT.

2.2.2.6) Image the slides under a microscope and quantify DAB signal with Image J[7] (see section 4).

**2.3) Immunostaining: Fluorescence**

2.3.1) On **Day 1** follow steps described in step 2.2.1. Perform the following steps on **Day 2.**

2.3.2) Rinse slides 3x in PBST for 2 min each. Incubate sections with fluorophore-tagged secondary antibody in 3% NGS (1:200) for 1 h at RT.

2.3.3) Wash 3x in PBST, 2 min each. Mount slides using 1 drop of 4',6-diamidino-2-phenylindole (DAPI) mounting media and cover with a coverslip. Dry on paper towel under the hood for 30 min. Store in the dark at 4 °C until fluorescence imaging.

**2.4) Quantification using Image J**

2.4.1) Download the "Fiji" version of ImageJ, which includes the necessary plugins for DAB staining quantification.

2.4.2) Open an image in Fiji and select **Image → Color → Color Deconvolution**.

2.4.3) Select "H DAB" as the stain and then click **OK**. Three new images will appear. Select the image that contains only DAB staining.

2.4.4) To quantify stromal staining only, use the ImageJ eraser function to remove the epithelium from the DAB image.

2.4.5) Select **Analyze → Measure** (or Ctrl + M) and record the value.

**REPRESENTATIVE RESULTS:**

Immunohistochemistry is the primary assay utilized to analyze the success of the *ex vivo* wound healing experiment. **Figure 4** depicts the epithelium and anterior stroma in control tissue (**Figure 4A**, **4B**). Six hours after wounding, the epithelium was absent (**Figure 4C**, **4D**). Six days after wounding as expected, the epithelium had regrown (**Figure 4E**, **4F**). This tissue was immunostained for alpha-smooth muscle actin (α-SMA), the expression of which characterizes myofibroblasts. There is a dramatic increase in α-SMA immunostaining in the stroma as detected by colorimetric DAB substrate. There was also an increase in epithelial reactivity that may suggest EMT transition[7] (see the Discussion). Disorganization in the epithelium and stroma was evident. A wounding experiment at lower magnification and with fluorescent immunostaining instead of DAB is shown (**Figure 4G**, **4H**). The wound margin is visible as well as a gradient of active myofibroblasts from the anterior to posterior stroma.

Although fibrotic markers are expressed by one week (**Figure 4**), to obtain consistent and reliable development of fibrotic markers, a two-week time point was chosen. In **Figure 5** an assay using a one-time application of control or gene-targeting siRNA to be tested for promoting regenerative healing is demonstrated. In this case, the targeting siRNA was for USP10, a deubiquitinase. Pathological myofibroblasts demonstrated increased adhesion through the accumulation of αv-integrins in focal adhesions[21]. Our previous studies showed that αvβ1 and αvβ5 are important fibrotic integrins in corneal stromal healing[7]. Integrins bind ECM outside the cell and together they are internalized. The internalized integrin is ubiquitinated and sent for degradation in the lysosome or the ubiquitin tag is removed by a deubiquitinase (DUB) and the integrin is recycled to the cell surface. We discovered that an increase in the gene expression of the DUB (USP10) increased the rate of ubiquitin removal from the integrin subunits β1 and β5 leading to a resultant accumulation of αv/β1/β5, on the cell surface, with subsequent TGFβ activation and induction of fibrotic markers[7]. Knockdown of USP10 in corneal organ culture prevented the appearance of fibrotic markers[7]. An example of these results is shown in **Figure 5**. As above, α-SMA is utilized as a marker for myofibroblasts. Another indicator of scarring is Fibronectin-EDA (FN-EDA), a splice variant of FN that contains an RGD, αv integrin binding domain[22-24]. It is also termed cellular FN (c-FN). It serves as a key fibrotic marker since FN-EDA is not in circulating plasma but instead is only expressed and secreted by cells under fibrotic conditions[25]. In **Figure 5A–5C** immunostaining for α-SMA is shown. Compared to unwounded (**Figure 5A**), wounding plus control siRNA (**Figure 5B**) showed a dramatic increase in α-SMA protein expression, whereas addition of USP10 siRNA[7] dramatically reduced expression in the stroma and epithelium. Similarly, compared to unwounded (**Figure 5D**), wounding plus control siRNA (**Figure 5E**) demonstrated a dramatic increase in fibronectin-EDA protein expression compared to treatment with USP10 siRNA (**Figure 5F**). Immunohistology for the target protein (in this case, USP10) was used to demonstrate successful knockdown[7]. In addition, performing qRT-PCR can assure gene knockdown in the tissue or also to assay for other fibrotic markers[7]. Image J can be used to quantify signal in the stroma only or total signal (**Figure 5G**). At least 3 corneas for each condition being tested should be used to quantify immunostaining to generate statistical significance as we have shown here and previously published[7]. Other proteins that have been routinely utilized for fibrotic markers are collagen III expression and an increase in integrin expression[1,26].

In **Figure 6**, the use of *ex vivo* cornea culture is demonstrated as a toxicology assay. In this experiment, corneas were either left unwounded (**Figure 6A**), wounded (**Figure 6B**), or wounded and treated with 10 µM Spautin-1[27], which was added to the cell culture media for increasing periods of time before wash out (**Figure 6C–6F**). Spautin-1 is a drug that non-specifically targets USP10[27]. Because of our success with USP10 siRNA, Spautin treatment was tested for effectiveness in preventing scarring. Unlike the siRNA, Spautin at this concentration was toxic to the tissue. Increasing time with Spautin-1 in culture prevented re-epithelialization and resulted in qualitative cell death, disorganized matrix and stromal vacuoles suggesting that Spautin-1 does not promote healing at the concentration assayed. Standard histological assays can be employed to quantify cell proliferation or apoptosis.

**FIGURE lEGENDS:**

**Figure 1. Cross-section of a human eye with an expanded view of the cornea.** In primates and chickens, histologically there are five distinct layers: epithelium, Bowman's membrane, stroma, Decement's membrane, and endothelium[28,29]. In all other mammals, Bowman's membrane is not visible histologically. At the transmission electron microscopic level, a basement membrane is observed separating the corneal epithelium and stroma in all corneas including those with a Bowman's membrane. An intact Bowman's membrane or basement membrane separating the epithelium from the stroma is a necessary to prevent scarring in all mammals. Image reprinted with permission from AllAboutVision.com (http://www.allaboutvision.com/resources/cornea.htm).

**Figure 2. Diagram of the cellular events that lead to corneal scarring.** This diagram depicts the basic events that unfold in the anterior cornea after wounding. (**A**) Depiction of the epithelium, basement membrane, stroma, and the quiescent cells embedded in the stroma, *i.e*, keratocytes. (**B**) The red triangle depicts a wound, which can be mechanical, an ulcer, virus, or persistent infection. (**C**) After wounding, in which the Bowman's or basement membrane is breached, the cells around the wound apoptose. (**D** and **E**) An influx of cells repopulate the wound from resident keratocytes or bone marrow-derived fibroblasts and transition into activated fibroblasts or directly into myofibroblasts. (**F**) These adherent pathological myofibroblasts create an autocrine loop of TGFβ activation and secretion of disorganized fibrotic matrix that promotes corneal haze and scar formation. In a regeneratively healed wound, myofibroblasts appear but have apoptosed in the healed tissue[6,29,30].

**Figure 3. Receiving and processing corneas for organ culture.** (**A**) Pig eyes are received with lids to protect the cornea during shipping. (**B**) Image of the globe after tissue is removed. (**C**) Image of a 6 mm trephine. (**D**) Wounding of the central cornea with a trephine. (**E**) Image of the mounted cornea after removal from the globe.

**Figure 4. Corneal tissue after wounding.** Immunohistological analysis of unwounded (control) or wounded corneas. Pig corneas were either left unwounded (control) or wounded. Tissue sections were immunostained with antibody to alpha-smooth muscle actin (α-SMA) to identify myofibroblasts. (**A** and **B**) Control, unwounded. (**C** and **D**) Wounded and fixed at 6 h post-wounding. Epithelium is removed (arrow). (**E** and **F**) Wounded and fixed at 6 days post-wounding. Stroma has filled-in and the epithelium has regrown (arrow head). Representative activated myofibroblasts are denoted with asterisks (*). (**G**, **H**) Low magnification images of α-SMA immunostaining 2 weeks after wounding: α-SMA (red), DAPI (blue). Images were captured using an upright fluorescence/brightfield microscope with a CCD camera. In **A**, **C**, **E** scale bar = 100 μm; **B**, **D**, **F** scale bar = 50 μm; **G**, **H** scale bar = 200 μm.

**Figure 5. Testing regenerative healing agents.** Pig corneas were either (**A** and **D**) unwounded (control), (**B** and **E**) wounded and treated with control siRNA, or (**C** and **F**) wounded and treated with USP10 siRNA. Immunostaining for (**A**–**C**) α-SMA or (**D**–**F**) Fibronectin-EDA. After treatment with USP10 siRNA, α-SMA was reduced by 2.2 ± 0.6 fold ***$p < 0.001$ and FN-EDA 3.3 ± 1.2 fold

**p < 0.01. Images were captured using an upright fluorescence/brightfield microscope with a CCD camera. Scale bar = 50 µm. (**G**) Corneal stromal staining as quantified by Image J. Statistical significance was calculated by one-way ANOVA with Bonferroni's test. Figure has been adapted with permission from Gillespie *et al.*[7].

**Figure 6. Testing agents for effects on reepithelialization: toxicology studies.** Immunostaining for α-SMA. Pig corneas were (**A**) unwounded, and (**B**) wounded. (**C**–**F**) Cornea were wounded and incubated with 10 µM Spautin-1. The inhibitor was washed out and replaced by media after (**C**) 2 days, (**D**) 4 days, (**E**) 6 days, (**F**) 14 days. All media changes during the incubation period included Spautin as indicated. All corneas were fixed and embedded in paraffin after 2 weeks in culture. Images were captured using an upright fluorescence/brightfield microscope with a CCD camera. Scale bar=50 µm.

**Discussion:**

This protocol describes a model for studying wound healing in a natural stratified 3D environment. Use of organ culture as an intermediate between cell culture and *in vivo* studies significantly reduces costs as well as reducing procedures on live animals. Other 3D models have been of great benefit to the field including self-synthesizing collagen gels made from primary human corneal fibroblasts[2] or these same cells embedded in gels made from animal-derived collagens[31]. The organ culture model system is particularly useful for testing putative healing agents since the wound is localized and thus there is a clear margin between wounded and non-wounded tissue in the same cornea (**Figure 4**). In addition, a mechanical trephine wound allows direct access to the stroma, which is excellent for administration of siRNAs into the wound (**Figure 5**). Although it is not shown here, viral transduction into corneal tissue in organ culture has also been demonstrated[32-34]. Another permutation of this assay would be to infect corneas with a reporter construct of interest and image gene expression in real time after wounding. In terms of translation to *in vivo* studies, this same procedure can be accomplished in rabbits[35] and thus organ culture on human, pig, or rabbit corneas can be compared to *in vivo* results. In our experience, the data that we obtained using the organ culture model with siRNA treatment have translated into similar findings *in vivo* (unpublished data). Since the organ culture corneas lack a functional limbal vasculature, tears, and aqueous humor, each investigator must assess if this will be a useful model for their studies. Resident activation of immune cells has been demonstrated, but the exact parallel to *in vivo* studies is not yet clear[1].

A key step in the protocol is not to penetrate the cornea by wounding too deeply. This will be obvious as the anterior chamber fluid will leak in this case. If this occurs, the globe should be discarded. To produce an even mechanical wound, grip the lip of the demarcated tissue within the trephined area with a forcep and then move the surgical blade parallel to the corneal surface to cut the tissue away within the boundaries of the trephine wound. The corneal surface should not dry out thus we recommend that after making the wound and cutting out the cornea from the globe, place the cornea face down in PBS until the agar mixture is at the correct temperature. Making sure that the agar is not too hot will avoid endothelial damage.

A limitation of this model is that the use of a trephine to produce a wound is uneven and cannot be reproduced identically from cornea to cornea compared to laser-induced wounds[36]. However, naturally occurring wounds are not all equivalent in depth and a large body of data suggest that any breach in the basement membrane generates myofibroblast development and haze in the stroma, whereas regeneration of the basement membrane leads to diminished scarring[37-39]. This pig corneal organ culture model employs a severe wound in which the basement membrane is removed within the area of the trephine. Development of myofibroblasts and fibrotic markers in the corneal stroma have been consistently and reproducibility achieved using this model system. Some epithelial staining is usually evident that darkens in the wounded and regrown epithelium. This has been demonstrated in other corneal organ culture reports[40] but is absent in most corneas from *in vivo* mouse and rabbit studies[41,42]. However, a study performed in an *in vivo* canine model demonstrated strong epithelial α-SMA staining in the epithelium after wounding[43]. The siRNA that promoted regenerative healing in our studies also significantly reduced this epithelial immunostaining, suggesting that the epithelium may be undergoing EMT (fibrotic scarring) when it regrows in organ culture. In addition, omission of a primary antibody in the staining protocol of a wounded cornea resulted in the total absence of staining[7] suggesting that the immunostaining is specific. Frozen sections (not shown) were similar to paraffin sections in this regard. However, because the slight but variable background staining in the epithelium, our lab has only quantified the stromal staining, which appears to have no background histological issues[7].

If wounding human corneas, obtaining corneas not used for transplant instead of full globes may be more cost effective[40]. In this case, the cornea will be wounded without the aid of the pressure afforded by the globe. Additional wounding strategies may include corneal burns, which have been extensively utilized *in vivo* to produce a scar[42].

In summary, the advantages of this system for a 3D tissue wound healing assay is its reproducibility and cost savings with only standard equipment needs, making it an excellent resource for observing and quantifying the effects of agents on tissue healing.

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**Disclosures:**
The authors declare that they have no competing financial interests.

**References:**
1 Stepp, M. A. *et al.* Wounding the cornea to learn how it heals. *Experimental Eye Research* . **121C** 178-193, doi: 10.1016/j.exer.2014.02.007, (2014).

2 Karamichos, D., Hutcheon, A. E. & Zieske, J. D. Transforming growth factor-beta3 regulates assembly of a non-fibrotic matrix in a 3D corneal model. *Journal of Tissue Engineering and Regenerative Medicine.* **5** (8), e228-238, doi:10.1002/term.429, (2011).

3 Ronkko, S., Vellonen, K. S., Jarvinen, K., Toropainen, E. & Urtti, A. Human corneal cell culture models for drug toxicity studies. *Drug Delivery and Translational Research.* **6** (6), 660-675, doi:10.1007/s13346-016-0330-y, (2016).

4 Bernstein, A. M., Twining, S. S., Warejcka, D. J., Tall, E. & Masur, S. K. Urokinase receptor cleavage: a crucial step in fibroblast-to-myofibroblast differentiation. *Molecular Biology of the Cell.* **18** (7), 2716-2727 (2007).

5 Zhu, Y. T. *et al.* Knockdown of both p120 catenin and Kaiso promotes expansion of human corneal endothelial monolayers *via* RhoA-ROCK-noncanonical BMP-NFkappaB pathway. *Investigative Ophthalmology & Visual Science.* **55** (3), 1509-1518, doi:10.1167/iov*s.*13-13633, (2014).

6 Shu, D. Y. & Lovicu, F. J. Myofibroblast transdifferentiation: The dark force in ocular wound healing and fibrosis. *Progress in Retinal and Eye Research.* **60** 44-65, doi:10.1016/j.preteyeres.2017.08.001, (2017).

7 Gillespie, S. R., Tedesco, L. J., Wang, L. & Bernstein, A. M. The deubiquitylase USP10 regulates integrin beta1 and beta5 and fibrotic wound healing. *Journal of Cell Science.* **130** (20), 3481-3495, doi:10.1242/jcs.204628, (2017).

8 Yang, Y. *et al.* TRPV1 potentiates TGFbeta-induction of corneal myofibroblast development through an oxidative stress-mediated p38-SMAD2 signaling loop. *PLoS One.* **8** (10), e77300, doi:10.1371/journal.pone.0077300, (2013).

9 Sta Iglesia, D. D. & Stepp, M. A. Disruption of the basement membrane after corneal debridement. *Investigative Ophthalmology & Visual Science.* **41** (5), 1045-1053 (2000).

10 Ljubimov, A. V. & Saghizadeh, M. Progress in corneal wound healing. *Progress in Retinal and Eye Research.* **49** 17-45, doi:10.1016/j.preteyeres.2015.07.002, (2015).

11 Echevarria, T. J. & Di Girolamo, N. Tissue-regenerating, vision-restoring corneal epithelial stem cells. *Stem Cell Reviews and Reports.* **7** (2), 256-268, doi:10.1007/s12015-010-9199-1, (2011).

12 Wilson, S. E., Mohan, R. R., Hong, J. W., Lee, J. S. & Choi, R. The wound healing response after laser *in situ* keratomileusis and photorefractive keratectomy: elusive control of biological variability and effect on custom laser vision correction. *Archives of Ophthalmology.* **119** (6), 889-896, doi:emo10005 , (2001).

13 Zieske, J. D., Guimaraes, S. R. & Hutcheon, A. E. Kinetics of keratocyte proliferation in response to epithelial debridement. *Experimental Eye Research .* **72** (1), 33-39 (2001).

14 Lassance, L., Marino, G. K., Medeiros, C. S., Thangavadivel, S. & Wilson, S. E. Fibrocyte migration, differentiation and apoptosis during the corneal wound healing response to injury. *Experimental Eye Research .* **170** 177-187, doi:10.1016/j.exer.2018.02.018, (2018).

15 Jester, J. V. & Ho-Chang, J. Modulation of cultured corneal keratocyte phenotype by growth factors/cytokines control *in vitro* contractility and extracellular matrix contraction. *Experimental Eye Research .* **77** (5), 581-592 (2003).

16 Gallego-Munoz, P. *et al.* Effects of TGFbeta1, PDGF-BB, and bFGF, on human corneal fibroblasts proliferation and differentiation during stromal repair. *Cytokine.* **96** 94-101, doi:10.1016/j.cyto.2017.03.011, (2017).

17 Hinz, B. & Gabbiani, G. Fibrosis: recent advances in myofibroblast biology and new therapeutic perspectives. *F1000 Biology Reports.* **2** 78, doi:10.3410/B2-78, (2010).
18 Lagares, D. *et al.* Targeted apoptosis of myofibroblasts with the BH3 mimetic ABT-263 reverses established fibrosis. *Science Translational Medicine.* **9** (420), doi:10.1126/scitranslmed.aal3765, (2017).
19 Hinz, B. Formation and function of the myofibroblast during tissue repair. *Journal of Investigative Dermatology.* **127** (3), 526-537 (2007).
20 Karamichos, D., Guo, X. Q., Hutcheon, A. E. & Zieske, J. D. Human corneal fibrosis: an *in vitro* model. *Investigative Ophthalmology & Visual Science.* **51** (3), 1382-1388, doi:io*vs.*09-3860 10.1167/io*vs.*09-3860, (2010).
21 Henderson, N. C. *et al.* Targeting of alphav integrin identifies a core molecular pathway that regulates fibrosis in several organs. *Nature Medicine.* **19** (12), 1617-1624, doi:nm.3282 10.1038/nm.3282, (2013).
22 Muro, A. F. *et al.* An essential role for fibronectin extra type III domain A in pulmonary fibrosis. *American Journal of Respiratory and Critical Care Medicine.* **177** (6), 638-645, doi:200708-1291OC 10.1164/rccm.200708-1291OC, (2008).
23 Shinde, A. V. *et al.* The alpha4beta1 integrin and the EDA domain of fibronectin regulate a profibrotic phenotype in dermal fibroblasts. *Matrix Biology.* **41** 26-35, doi: 10.1016/j.matbio.2014.11.004, (2014).
24 White, E. S. & Muro, A. F. Fibronectin splice variants: understanding their multiple roles in health and disease using engineered mouse models. *IUBMB Life.* **63** (7), 538-546, doi:10.1002/iub.493, (2011).
25 Walraven, M. & Hinz, B. Therapeutic approaches to control tissue repair and fibrosis: Extracellular matrix as a game changer. *Matrix Biology.* doi:10.1016/j.matbio.2018.02.020, (2018).
26 Rosenbloom, J., Ren, S. & Macarak, E. New frontiers in fibrotic disease therapies: The focus of the Joan and Joel Rosenbloom Center for Fibrotic Diseases at Thomas Jefferson University. *Matrix Biology.* **51** 14-25, doi:10.1016/j.matbio.2016.01.011, (2016).
27 Liu, J. *et al.* Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. *Cell.* **147** (1), 223-234, doi:10.1016/j.cell.2011.08.037, (2011).
28 Ritchey, E. R., Code, K., Zelinka, C. P., Scott, M. A. & Fischer, A. J. The chicken cornea as a model of wound healing and neuronal re-innervation. *Molecular Vision.* **17** 2440-2454 (2011).
29 DelMonte, D. W. & Kim, T. Anatomy and physiology of the cornea. *Journal of Cataract & Refractive Surgery.* **37** (3), 588-598, doi:10.1016/j.jcrs.2010.12.037, (2011).
30 Wilson, S. E. *et al.* Epithelial injury induces keratocyte apoptosis: hypothesized role for the interleukin-1 system in the modulation of corneal tissue organization and wound healing. *Experimental Eye Research .* **62** (4), 325-327 (1996).
31 Miron-Mendoza, M., Graham, E., Kivanany, P., Quiring, J. & Petroll, W. M. The Role of Thrombin and Cell Contractility in Regulating Clustering and Collective Migration of Corneal Fibroblasts in Different ECM Environments. *Investigative Ophthalmology & Visual Science.* **56** (3), 2079-2090, doi:10.1167/io*vs.*15-16388, (2015).
32 Saghizadeh, M. *et al.* Adenovirus-driven overexpression of proteinases in organ-cultured normal human corneas leads to diabetic-like changes. *Brain Research Bulletin.* **81** (2-3), 262-272, doi:10.1016/j.brainresbull.2009.10.007, (2010).

33 Saghizadeh, M., Kramerov, A. A., Yu, F. S., Castro, M. G. & Ljubimov, A. V. Normalization of wound healing and diabetic markers in organ cultured human diabetic corneas by adenoviral delivery of c-Met gene. *Investigative Ophthalmology & Visual Science.* **51** (4), 1970-1980, doi:10.1167/io*vs.*09-4569, (2010).
34 Kramerov, A. A., Saghizadeh, M. & Ljubimov, A. V. Adenoviral Gene Therapy for Diabetic Keratopathy: Effects on Wound Healing and Stem Cell Marker Expression in Human Organ-cultured Corneas and Limbal Epithelial Cells. *Journal of Visualized Experiments.* (110), e54058, doi:10.3791/54058, (2016).
35 Cho, S. Y., Kim, M. S., Oh, S. J. & Chung, S. K. Comparison of synthetic glues and 10-0 nylon in rabbit lamellar keratoplasty. *Cornea.* **32** (9), 1265-1268, doi:10.1097/ICO.0b013e31829a3760, (2013).
36 Sharma, A., Mehan, M. M., Sinha, S., Cowden, J. W. & Mohan, R. R. Trichostatin a inhibits corneal haze *in vitro* and *in vivo*. *Investigative Ophthalmology & Visual Science.* **50** (6), 2695-2701, doi:io*vs.*08-2919 10.1167/io*vs.*08-2919, (2009).
37 Marino, G. K., Santhiago, M. R., Torricelli, A. A., Santhanam, A. & Wilson, S. E. Corneal Molecular and Cellular Biology for the Refractive Surgeon: The Critical Role of the Epithelial Basement Membrane. *Journal of Refractive Surgery.* **32** (2), 118-125, doi:10.3928/1081597X-20160105-02, (2016).
38 Marino, G. K., Santhiago, M. R., Santhanam, A., Torricelli, A. A. M. & Wilson, S. E. Regeneration of Defective Epithelial Basement Membrane and Restoration of Corneal Transparency After Photorefractive Keratectomy. *Journal of Refractive Surgery.* **33** (5), 337-346, doi:10.3928/1081597X-20170126-02, (2017).
39 Marino, G. K. *et al.* Epithelial basement membrane injury and regeneration modulates corneal fibrosis after pseudomonas corneal ulcers in rabbits. *Experimental Eye Research .* **161** 101-105, doi:10.1016/j.exer.2017.05.003, (2017).
40 Janin-Manificat, H. *et al.* Development of *ex vivo* organ culture models to mimic human corneal scarring. *Molecular Vision.* **18** 2896-2908 (2012).
41 Mohan, R. R. *et al.* Apoptosis, necrosis, proliferation, and myofibroblast generation in the stroma following LASIK and PRK. *Experimental Eye Research .* **76** (1), 71-87 (2003).
42 Anumanthan, G. *et al.* KCa3.1 ion channel: A novel therapeutic target for corneal fibrosis. *PLoS One.* **13** (3), e0192145, doi:10.1371/journal.pone.0192145, (2018).
43 Chandler, H. L., Colitz, C. M., Lu, P., Saville, W. J. & Kusewitt, D. F. The role of the slug transcription factor in cell migration during corneal re-epithelialization in the dog. *Experimental Eye Research .* **84** (3), 400-411, doi:10.1016/j.exer.2006.10.010, (2007).

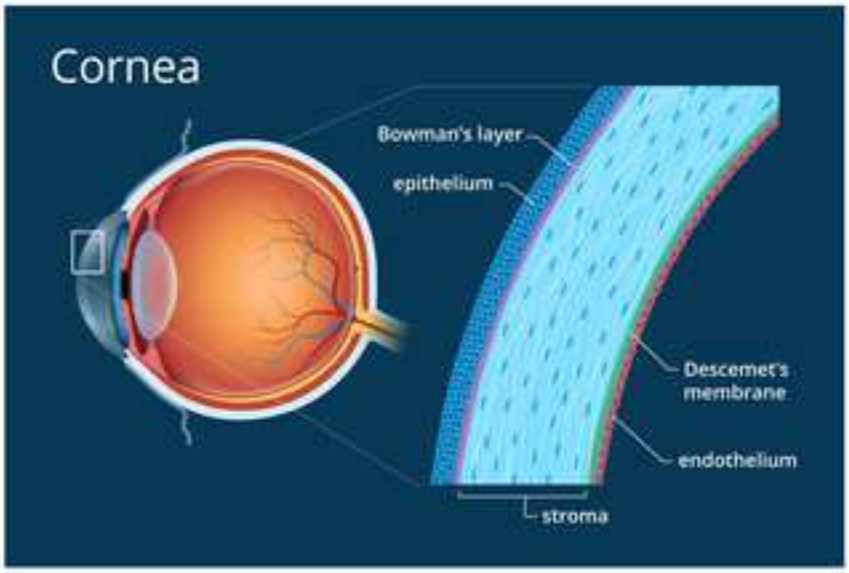


Figure 1

Figure 2



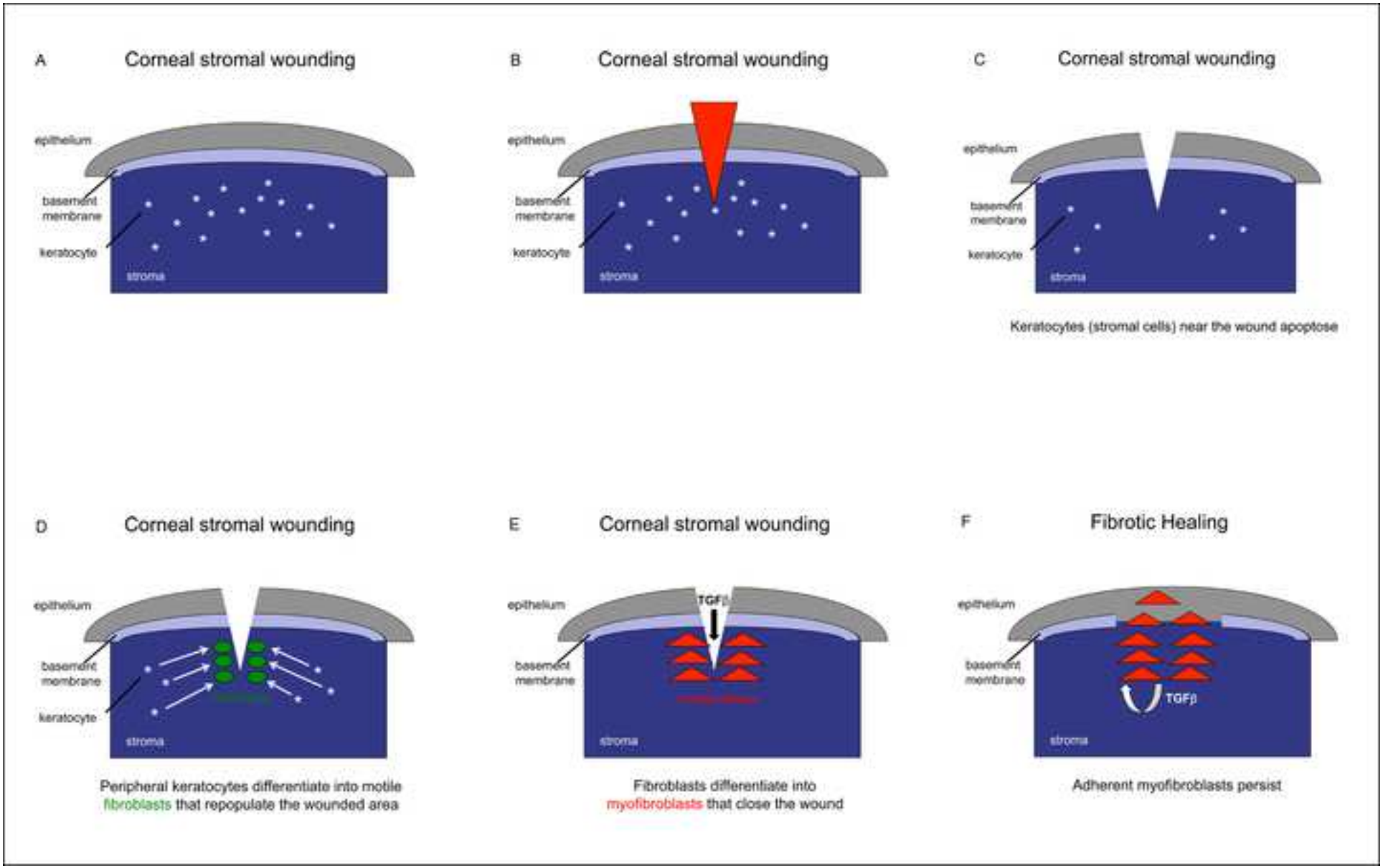


Figure 3

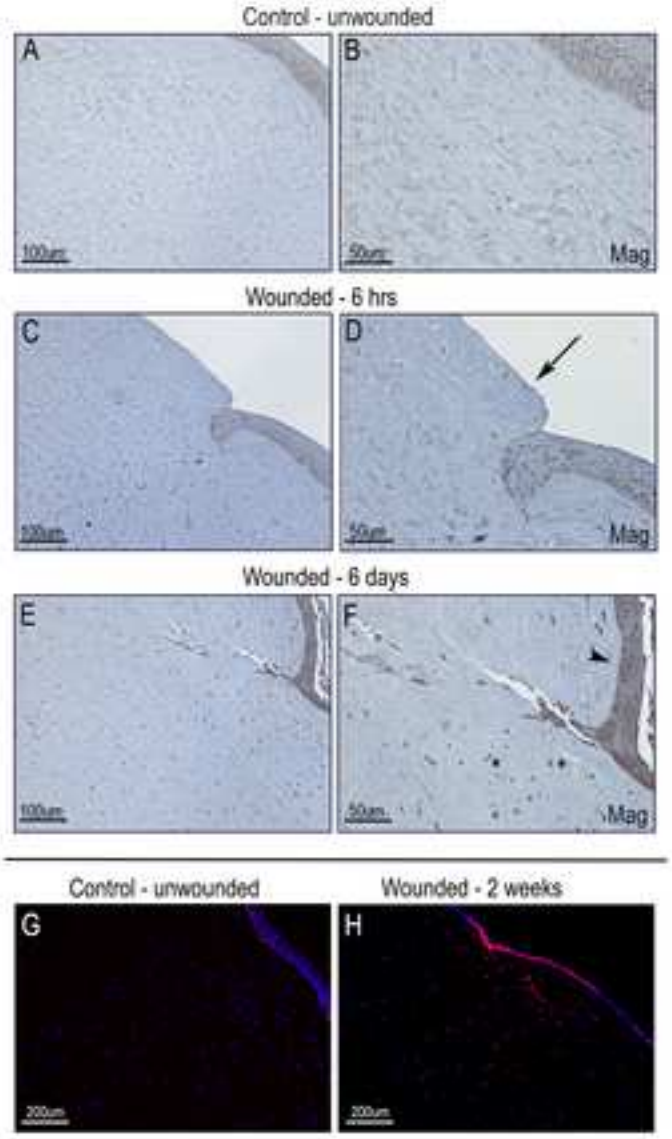


Figure 4

Figure 5 Click here to access/download;Figure;Figure 5-final(3).tif

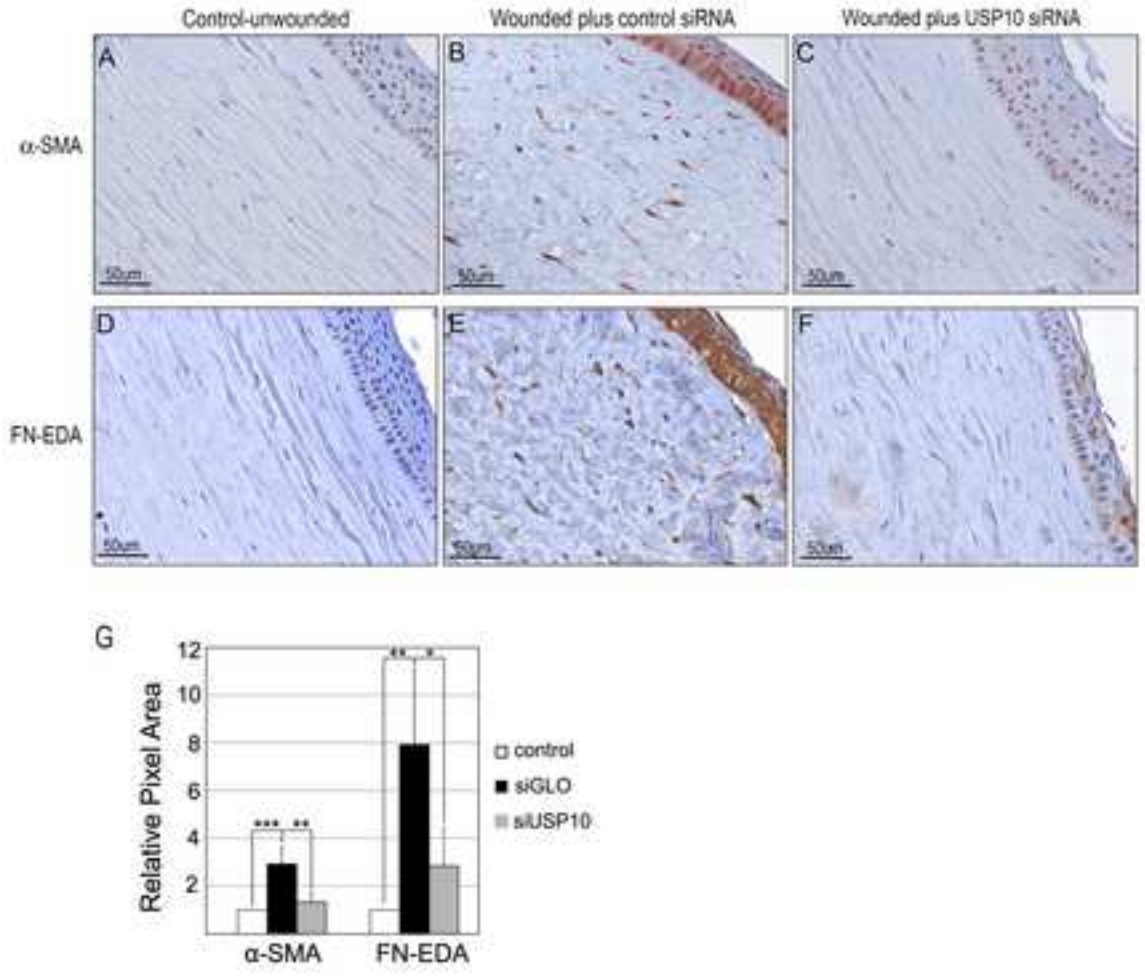


Figure 5

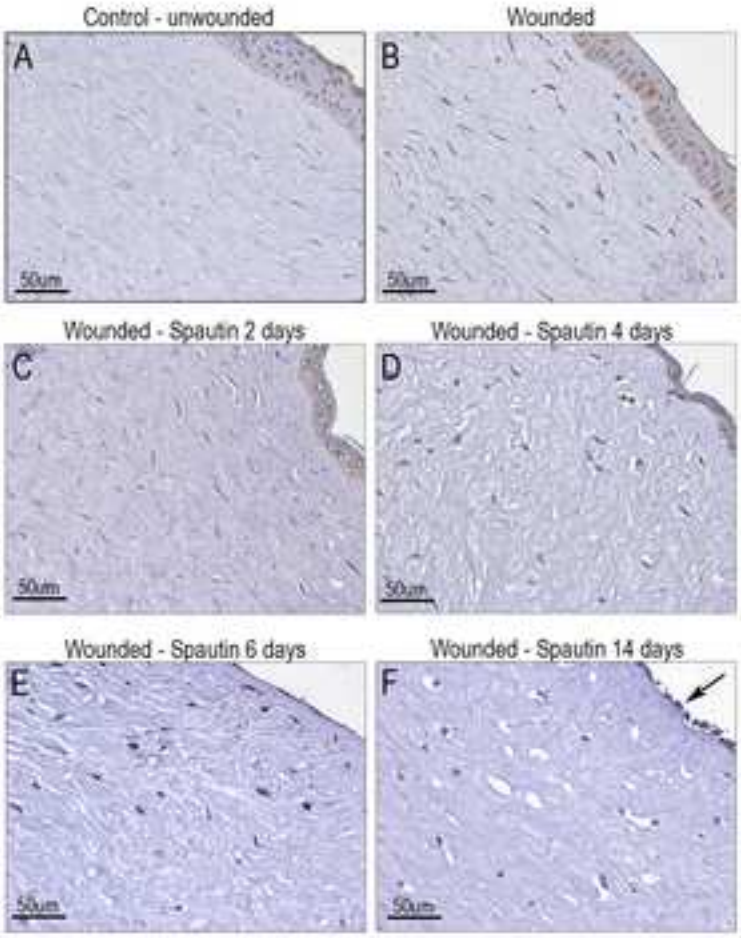


Figure 6

| Name of Reagent/ Equipment | Company | Catalog Number |
|---|---|---|
| PBS | Gibco | 10-010-023 |
| Pen Strep | MP Biomedicals | 91670049 |
| Bovine Collagen Solution | Advance Biomatrix | 5005 |
| Pig eyes with lids attached | Pel-freeze, Arkansas | N/A |
| 6.0 mm trephine | Katena | K28014 |
| Surgical Blade | Personna | 0.009 |
| Small scissor | Fisher | 895110 |
| Forceps | Fisher | 08953-F |
| Kim Wipes | Kimberly-Clark™ 34120 | 06-666 |
| 60 mm cell culture dishes | Falcon | 08-772B |
| | | |
| **Supplemented Serum- Free media (SSFM)** | **Add all of the following components to DMEM/F-12: ITS, RPMI, Gl** | |
| DMEM/F-12 | Gibco | 11330 |
| ITS Liquid Media Supplement | Sigma | I3146 |
| RPMI 1640 Vitamins Solution | Sigma | R7256 |
| Glutathione | Sigma | G6013 |
| 1% L-glutamine solution | Gibco | 25030-081 |
| MEM Non-essential amino acids solution | Gibco | 11140 |
| MEM Sodium pyruvate solution | Gibco | 11360 |
| ABAM | Sigma | A7292 |
| Gentamicin | Sigma | 30-005-CR |
| Vitamin C | Wako | 070-0483 |
| | | |
| 10% Iodine | Fisher Chemical | SI86-1 |
| Tissue Path Cassettes | Fisher | 22-272416 |
| Normal Goat Serum (NGS) | Jackson Immuno Research | 005-000-121 |
| Mounting Media | Thermo Scientific | TA-030-FM |
| Safe Clear | Fisher | 314-629 |
| Ethyl Alcohol | Ultra Pure | 200CSGP |
| Sodium citrate | Fisher | BP327 |
| Hematoxylin | EMD Millipore | M10742500 |
| Bluing agent | Ricca Chemical Company | 220-106 |

| | | |
|---|---|---|
| 1% Triton X-100 | Fisher | 9002-93-1 |
| 0.1% Tween 20 | Fisher | BP337 |
| 3% Hydrogen Peroxide | Fisher | H324 |
| DAB Kit | Vector Laboratories | SK-4100 |
| Agar | Fisher | BP1423-500 |
| Parafilm | Bermis | 13-374-12 |
| Moist Chamber | | |
| Lipofectamine 2000 | | |
| Qiagen RNAprotect Cell Reagent | Qiagen | 76104 |
| Ambion PureLink RNA Mini Kit | Thermo Scientific | 12183018A |
| Anti-Fibronectin-EDA Antibody | Sigma | F6140 |
| Anti-alpha smooth muscle actin Antibody | Sigma | A2547 or C6198 (cy3 conjugated) |
| Permafluor | Thermo Scientific | TA-030-FM |
| DAPI | Invitrogen | P36931 |
| Gt anti -MS IgG (H+L) Secondary Antibody, HRP | Invitrogen | 62-6520 |
| Gt anti -MS IgM (H+L) Secondary Antibody, HRP | Thermo Scientific | PA1-85999 |
| Gt anti -MS IgG (H+L) Secondary Antibody, Cy3 | Jackson Immuno Research | 115-165-146 |
| Zeiss Axioplan2 | Zeiss | |
| SPOT-2 | Diagnostic Instruments, Sterling Heights, Michigan | |

**Comments/Description**

**utathione, L-Glutamine, MEM Non essential amino acids, MEM Sodium Pyruvate, ABAM, Gentamicin, Vitamin C.**

100X
100X
Use at 1 µg/mL. Freeze aliquots; do not reuse after thawing.
100X
100X
1 M Stocks (1000X) and freeze in single use aliquits. Use from freezer each time media is made.
100X
200X
2-0-aD Glucopyranosyl-Ascorbic Acid. 1 mM stocks (1000x)

We use 3% NGS

200 Proof, diluted at 100%, 70%, 50%)
10mM, pH 6.4

Diluted in PBS
Diluted in PBS

Agar solution: prepare 1% agar and 1 mg/mL bovine collagen in DMEM-F12 up to 20 mL

Use any chamber, cover it with wet Wipe Tissue and then put a layer of Parafilm over it.

1:200 Diluted in  3% normal goat serum
1:200 Diluted in 3% normal goat serum

1:100 diluted in 3% normal goat serum (for $\alpha$-SMA, DAB staining)
1:100 diluted in 3% normal goat serum (for FN-EDA, DAB staining)
1:200 Diluted in  3% normal goat serum (for $\alpha$-SMA, Fluorescence staining)
Microscope
CCD camera

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**CORRESPONDING AUTHOR:**

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Article Title: Ex vivo corneal organ culture model for wound healing studies

Signature: [signature] Date: 6/1/18

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All of these are accomplished. I have written in red if I needed to respond.

**Editorial comments:**

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”
3. Please rephrase the Long Abstract to more clearly state the goal of the protocol.
4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.
5. Please define all abbreviations before use.
6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
7. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.
8. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Gibco, MP Biomedicals, Advance Biomatrix, Pel-freeze, Katena, Personna, Fisher, Falcon, Kim Wipe, Vector Laboratories, etc.
9. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. There are no animal studies in this manuscript. This does not apply.
10. Please revise the protocol to be a numbered list: step 1 followed by 1.1, followed by 1.1.1, etc. Please refrain from using bullets, dashes, or indentations.
11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
12. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
13. Lines 112-128, 188-203: Please move the solutions, materials and equipment information to the Materials Table.
14. Lines 130-141: Please write the text in the imperative tense.
15. Line 147: Please describe how to remove excess tissue from the eye. What tool is used?
16. 1.1-1.3: What is used to hold the globe, etc.?
17. Lines 154-183, 207-211: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step and that they are described in the imperative tense in complete sentences. Please move the discussion about the protocol to the Discussion.
18. 1.15: Please add more details to your protocol step. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
19. Please include single-line spaces between all paragraphs, headings, steps, etc.
20. After you have made all of the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The protocol is not long. The entire protocol needs to be demonstrated.
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22. Figures 4-6: Please include a space between the numbers and their units and use the micro symbol µ instead of u (i.e., 100 µm and 50 µm).
23. Please shorten the figure legends. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
24. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique.
25. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
26. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Thank you to the reviewers for their comments.

**Reviewers' comments:**

Reviewer #1:

Manuscript Summary:
The paper deals with the description of an important model of wound healing using corneal organ cultures. The use of organ cultures allows studying live human tissue, which is a unique opportunity for an experimental system. The difference of the used model from the majority of other studies is related to stromal wounds that recapitulate the emergence of myofibroblasts and scarring, which are seen clinically. The authors present an organ culture model system for assessing scarring versus regenerative healing using rabbit, pig, and human eyes. Corneas still in the globe are wounded with a trephine removing central epithelium and anterior part of the stroma. After wounding, corneas are cut out, mounted on a collagen/agar base, and cultured for up to one month. This model can be used to assay improvement in healing, the effects of various anti-scarring agents and for toxicology studies. The authors also present their data on testing the effects of small molecule inhibitors and lipid-mediated siRNA transfection for gene knockdown. The paper is well written and this reviewer has only minor comments on the manuscript.

Major Concerns:
None

Minor Concerns:
1. Please describe in more detail the markers used for the general reader including alpha-SMA, fibronectin ED-A and Spautin-1 (and why it is used).

Spautin targets USP10 non-specifically. We have added this information now to the results with the description of the siRNA, see below. We have added a description of a-SMA and FN-EDA to the results section of Figure 5.

2. It is unclear what is "experimental siRNA". Please just call it by the target, e.g., siRNA to fibronectin.
It has been added to the text for Figure 5. It had been omitted because the explanation of the target may be beyond the scope of the paper but hopefully it fits into the construct of the paper.

3. It is unclear why the epithelium stains for alpha-SMA. Is this a paraffin embedding problem? In fresh-frozen sections this does not happen.

True. We have tried to eliminate this but have not been successful. However, others have seen this as well in organ culture and when we induce “regenerative healing” with our USP10 siRNA, the staining is significantly reduced suggesting it is EMT. These concepts are published and now better explained and cited in this paper. Our studies have focused only on quantifying myofibroblast and fibrotic development in the stroma. We have made clear that our quantification is only of the stroma using this technique because of any background in the epithelium. I have augmented the prose on this entire issue in the Discussion.

4. JOVE has recently published one paper on human corneal organ cultures used for epithelial wound healing studies. The authors might like to cite it (Kramerov et al. Adenoviral gene therapy for diabetic keratopathy: effects on wound healing and stem cell marker expression in human organ-cultured corneas and limbal epithelial cells. J Vis Exp, 2016;110:e54058.

Inserted.

Reviewer #2:

Manuscript Summary:
The authors present an organ culture, ex vivo model for creating, assessing, and investigating corneal wound healing.

Major Concerns:
-Why do the authors highlight the need for wounding prior to corneal removal/cut from the globe?
Please see the answer to the next question.

-Cant the model be used by scientists when only the corneal rim is available? and not the whole globe?
Yes. It may be a bit more difficult to wound as the wounding is performed in the globe because the pressure of the globe plus the hand pressure on the globe aids in creating a smooth, taut surface for wounding. However, we have added this possibility to the Discussion and cited a paper that used human corneas as the starting point of the experiment.

Minor Concerns:
-Short abstract: The authors state "organized 3D multi cellular environment". It is just the cornea. Please correct.
The cornea is a multi-cellular environment. Epithelial cells, keratocytes, endothelial cells, limbal cells, resident immune cells, etc…and the cornea is also highly innervated.

-It is not clear why the model is limited to trephine wounds? Unless there are problems with using other wounding protocols (burn, penetrating, and so on), the authors should highlight the flexibility of their model.

Thank you for the suggestion. Although we have not done this ourselves, we have added the possibility to the Discussion as an alternative wounding strategy.

Reviewer #3:

General comments:
Authors describe a corneal wound healing model using porcine corneas stored in a Petri dish that aims to study "stromal scar" generation. Authors do not study "stromal scar formation" (extracellular matrix deposition, transparency,…), but only a surrogate that is myofibroblast formation in the stroma.

We have removed the word scar in many places and replaced it with myofibroblast development and fibrotic marker development..

They also used siRNA in this model but this part of the protocol is not fully described. The protocol is rather well described but certain details should be provided to enable others to reproduce the results. Several sentences and concepts described in the introduction and the discussion are oversimplified or incorrect.

Specific comments:
Introduction
- Line 70
o Epithelial wound healing primarily involves several factors not limited to "activation of limbal stem cells", such as migration of epithelial cells from the leading edge
o Location of epithelial stem cells, especially in animal corneas is a debated subject (Majo et al. Nature 2008)
o You should discuss these point as wound healing model are not that simple.
- Line 78 to 90:
o please provide reference for each assertion
- Line 81:
This paper is not intended in any way to discuss the activation of limbal stem cells. We have added this prose.

Corneal epithelial wounding that does not breach the epithelial basement membrane normally closes within 24-72 h [9]. Soon after wounding, the cells at the edge of the epithelium start spreading and migrating into the epithelial free surface, to reestablish epithelial barrier function. This activity is sequentially followed by activation of corneal basal cell proliferation first and, in a later stage, of precursor cells located at the outer limbal zone to achieve recovery of epithelial cell mass [10,11].

o "inducing integrin mediated myofibroblast differentiation"
o Not only Integrin mediated, several factors could be involved, TGFß, CD147, etc…
The word integrin was removed. We had already named other factors.
o Please rephrase
o Provide reference
- Line 98 to 102:
I have added a reference to our paper.

o "The corneas are cultured in serum-free media plus vitamin C. Neither the addition of serum nor growth factors are needed to induce scar formation."
o Discuss the addition of Vitamin C
We have added the reason for adding stabilized vitamin C and referenced this point.

Methods
- Line 170
o "Before use, the agar should come to a warm (not hot) temperature."

o Temperature is critical, please specify the range of temperature used.
- Line 176
We have now listed “(approximately 25 ºC)”

o Use International nomenclature for volumes. "mL"
- Line 178
done
o "Wet the corneal surface every day to maintain moisture."
o Specify how many times
“once” is added
o Specify incubation environment
5% CO2 and 37°C is added.

o Specify if you placed a lid
- Line 181-182:
“cover with the lid” was added.
o "For gene knockdown, the wound is treated with experimental or control siRNA that is complexed to Lipofectamine 2000 (Invitrogen) by the standard protocol."
o As it is a protocol, please specify what is the "standard" protocol" and how you proceeded for "wound treatment"
- Line 227 & 234:
We have removed the phrase by the standard protocol and added the method. We are not allowed to state the name of the company in the text.

o Specify the antibody and the dilution that were used
added 1:250
- Did you estimated the depth of the trephination?
This wound is not a “perfectly controlled wound” like PRK as reviewer 4 suggests and is stated in the Discussion. Although after performing hundreds of these wounds, we seem to produce the approximate same depth every time. It is about the top 1/3 of the cornea if one follow’s our procedure. Wounding mechanically is not a perfect science. We view this as an advantage to the model as clinical wounds, (unless it is in a clinician’s office) are irregular in nature. Just as burning a cornea with NaOH or other agents is done routinely in vivo, it can’t be completely controlled. This method is reproducible and has been routinely taught to junior staff, who mastered it on the first try.

Figure 1.
Line 302-303 "An intact membrane separating the epithelium from the stroma"
- Specify "Basement membrane" as it is not clear if you refer to the Bowman layer or to the Basement membrane

I was purposely vague because I am generalizing to all mammals. I have added an intact Bowman’s membrane or basement membrane separating the epithelium from the stroma is a necessary to prevent scarring in all mammals.

Figure 2.
On your schematic representation, in F you represent myofibroblast in the epithelium and a restoration of the Bowman layer. Can you correct or provide a reference for this?

We have redone this to continue a “break” in the membrane.

After epithelial closure, the TGFß signal is reduced. As you emphasized TGFß comes from tears, epithelial cells and an autocrine loop of myofibroblast. Do you have a reference to emphasize the persistence of such autocrine loop after epithelial closure and basement membrane restoration? After

epithelial closure, the drop in TGFß level in the stroma induces apoptosis of myofibroblast but indeed not all of them. This point should be discussed in the discussion.

We have added in the Discussion with references that restoration of the basement membrane reduces fibrotic outcomes.

- Can you provide the result of your staining after 4 weeks?

It is the same, but I have taken out the words up to 4 weeks as we are not showing it here.

- Did you assess the deposition of extracellular matrix? The goal would be to assess if the aSMA cells are functionally active

Figure 5D, E is fibronectin-EDA. This is a splice variant of fibronectin, also called cellular fibronection. It is secreted from cells under fibrotic conditions, whereas “normal” plasma fibronectin circulates through the body. Thus, FN-EDA serves as one marker of fibrotic ECM expression. As reviewer 1 suggests as well, we have added text about FN-EDA and other markers that are routinely used.

- Can you provide a reference assessing the migration of fibroblast in the depopulated stroma after wound induction?

Inserted:

After corneal stromal wounding, the stroma is populated with cells of multiple origins including differentiated resident stromal cells as well as bone marrow-derived fibrocytes [3-5].

- A strong staining is observed in the epithelium, can you provide the negative control of each staining?
We have published this control (*Gillespie, et al Journal of Cell Science, 2017*). I have more thoroughly addressed this issue in the Discussion as stated above.

- Line 260 you say it could be related to EMT ("There is also an increase in epithelial reactivity that may suggest EMT transition."). Have you made other staining to confirm that hypothesis? Can you provide references from other team to emphasize this observation? Can you provide images in the unwounded area to compare the staining? Also provide the negative control images as it could be some background staining. Please see the new prose in the Discussion, which addresses these concerns.

Line 270
- The corneal surface is wetted every day and media is changed every two days.
- Contradicts line 177?
Added:
5.3 Wet the corneal surface once every day by adding 1 drop of SSFM from the conditioned media in the dish to maintain moisture. (Take the dish out of the incubator, put under the hood, remove the lid, wet surface with media from dish using a sterile pipette, cover again and put it back at the incubator).

Please provide specification for the SiRNA and the control used.
Done.
Figure 4 and 5
You said you quantified the immunostaining. Can you provide the results?
The results are published with extensive quantification (*Gillespie, et al Journal of Cell Science, 2017*). Fig 4-6 are examples of what can be done with this method. We have added quantification of Figure 5 (Fig 5G) with permission, amended and reproduced from the *JCS* article.

Figure 4-5-6
Can you also explain your findings and provide pictures in the center of the wound at distance of the wound edge? Was the aSMA stained cells distributed evenly?

We have added two images to Figure 4 at 10X (Fig 4G,H) demonstrating the margins of the wound, and the gradient of a-SMA activation. These are fluorescent images so we have added this method of staining to the protocol.

Line 277:
- "fibronectin-EDA"
- Provide antibody reference, dilution in the method section
We have taken this out of the Figure legend and listed the antibodies with dilutions in the Methods

Line 280:
- "In addition, performing qRT-PCR can assure gene knockdown in the tissue"
- Can you provide these results?

We started doing this for in vivo studies (beyond the scope of this paper). We have done it for other targets in organ culture studies. Although I don't have the data for the USP10 study, I think it is worth mentioning as it is absolutely standard and anyone that can isolate RNA, can do this.

Did you observe a modification in stromal organization and matrix deposition?
Yes, we state this in the Results and we have shown the staining for FN-EDA.

Line 282-286:
- For RNA isolation, specify what part of the tissue you use or if you get rid of the sclera, endothelium, epithelium

There is no sclera. We have inserted:

**Methods:**

1. **Preparing the Tissue:**

1.1) After a two week incubation if using some of the tissue for qRT-PCR analysis, before fixing, cut the cornea in half through the wound.
1.2) Put this half or only ¼ (either is enough tissue) into stabilizing RNA protect reagent.
1.3) Using a standard isolation kit, isolate RNA and perform qRT-PCR. Note: Alternatively, the wounded part only, can be isolated and tested for gene expression.
1.4) For the other half of the cornea, place into Tissue Path Cassettes and submerge in fixative (10% formalin) for 2-4 days at Room Temperature (RT).

Discussion
Line 366:
You don't provide results in rabbit corneas, consequently you can't say the results are equivalent.
I didn't say that the results are equivalent. I am saying that others have performed the trephine wound in rabbits in vivo and so you can perform organ culture with rabbit corneas and determine IF they are equivalent.

Line 378-379:
- "In terms of translation to in vivo studies, this same procedure can be accomplished in rabbits and

thus organ culture results can be directly compared to in vivo results"
- You can't directly compare two completely different experiments (in vivo vs ex vivo).
- Wound healing involves tears, immune system, neurotrophic factors, etc…
- You don't provide in vivo results to sustain this assertion
- In mouse model as much as 70% of myofibroblast comes from bone marrow-derived precursor cells, what is not reproduced in an ex-vivo setting. Discuss that limitation
- Reformulate your sentence.

I removed the word “directly”. No model system is perfect. The elegant work of Dr. Wilson shows that in mice a large percentage of the myofibroblasts derive from bone marrow. However, these are mice and thus if held to this standard we must also note that we do not know if this is true in humans. Furthermore, as Dr. Wilson writes “No differences in function between myofibroblasts derived from keratocytes and myofibroblasts derived from bone marrow-derived cells have been discovered.” *(Gustavo et al.J Refract Surg. 2016;32(2):118-125.)*

We already say that : “Since the organ culture corneas lack a functional limbal vasculature, tears, and aqueous humor, each investigator much assess if this will be a useful model for their studies. Resident activation of immune cells has been demonstrated, but the exact parallel to *in vivo* studies is not yet clear [6].”

We are demonstrating a 3D cellular model system that has been extremely consistent for producing myofibroblasts after wounding. Given the number of papers using TGFb to stimulate myofibroblast development in cell culture, a system that itself is flawed given the concentrations used (as an example), it seems quite reasonable to publish an assay in which wounded tissue without growth factor and serum addition produces myofibroblast-rich tissue and one in which experimental drugs can be easily applied to test if myofibroblast development and persistence can be regulated.

Line 388-391:
- "However, if assaying for fibrotic endpoints, or the effects on reepithelialization, bacterial infection, proliferation, migration, or apoptosis, the exact depth of the wound is not critical as long as the wound penetrating the anterior stroma is similar between corneas."
- In corneal wound in vivo model using PRK, differences in myofibroblast activation could be observed if the stromal bed is regular or irregular, or between 2 stromal wounds with a difference in the depth of the wound of only 50 to 100µm
- Most wound healing model trying to reproduce corneal scar study anterior stroma wounds. The density of keratocytes is uneven between the anterior and posterior part of the cornea
- Please discuss that point and correct your statement

To satisfy this request we have changed it to:

A limitation of this model is that use of a trephine to produce a wound is uneven and cannot be reproduced identically from cornea to cornea compared to PRK laser-induced wounds [7]. However, naturally occurring wounds are not all equivalent in depth and a large body of data suggest that any breach in the basement membrane generates myofibroblast development and haze in the stroma, whereas regeneration of the basement membrane leads to diminished scarring [8-10]. Our pig corneal organ culture model employs a severe wound in which the basement membrane is removed within the area of the trephine. Development of myofibroblasts and fibrotic markers in the corneal stroma have been consistently and reproducibility achieved using this model system.

Line 391:
- "In addition, we have found that with the ex vivo organ culture tissue, using paraffin embedding has been more successful than frozen sections."
- Please explain

We have chosen to remove this.

End of comments.

Reviewer #4:

Manuscript Summary:
This manuscript describes the process of wounding a cornea and maintaining it as an ex vivo organ culture model for the testing of interventions for modulating corneal wound healing, etc.

Major Concerns:
The description of this protocol at first glance seems reasonable, but gong through it in detail gives rise to many questions about the repeatability of this process. I have no doubt that the authors have had success developing and maintaining this organ culture system, however, if a reader were to attempt to replicate this procedure, there would be much trial and error to develop their own protocol. There is not enough detail in this manuscript for someone else to be able to replicate it.

We have now made it as clear as possible. It is very easy to do and to replicate.

Abstract does not have nearly enough background to introduce the model or the goals or the outcomes that are to be measured.
We have rewritten the abstract.

If human corneas are used that are not of sufficient quality to be used in in vivo transplantation for human patients, how can they reliably demonstrate mechanistically the responses to wounding and healing that a normal cornea would have - this needs to be addressed as a limitation. There are considerable differences between different species (cell layers, layer thickness, lack of or robust nature of Bowman's layer, corneal diameter and curvature, overall thickness) that needs to be discussed and the differences in protocol (determining depth of wound, how much agarose necessary, etc.) need to be outlined specifically.

I removed human cornea. Typically the human corneas that one receives for research have no mechanical issues. They have infiltrates or the reason for donor death is not clear. In terms of the differences between species, if the basement (and/or Bowman's membrane) is breached and doesn't heal, persistent myofibroblasts will be present. The species doesn't matter. I have many references to this in all of the reviews and we have inserted (as reviewer 3 suggested) references to show that when the basement membrane heals, scarring is diminished.

The use of vitamin C is not discussed, just a passing mention, and this does not recapitulate the normal wounded state and degree of resultant fibrosis. This is induced fibrosis that is not natural.

The reason for the addition of Vitamin C is discussed above and added to the text. We are not using serum or growth factors to induce myofibroblast development. We are comfortable with adding vitamin c in supplemented serum-free media and using this as a model system. It does not compromise the results as others have shown. This is now better cited in the text.

Protocol text: Agar solution - what temperature, how long?
Inserted.

Give a specific description and name to your surgical instruments (not just a catalogue number).

There is only one real surgical instrument, a trephine. This is named. There is nothing else to name.

How is the chopping board prepared? Is it sterilized and how? What material is it made of? How big? Need exacts volumes or weights of ingredients for supplemented serum-free media.

We have added ethanol sterilized chopping board. It doesn't matter how big or how small. An eye isn't that big. As per the journal's instructions, we have put the SSFM recipe in the Methods.

Methods description: Need much more detail about preparation of globe? What tissues are removed? How much? How long are they dipped in things? What do you mean by hold globe with Kim-Wipe and create pressure. Description is lacking here. Description of wounding is imprecise - "about 5 times"? Need to have a better method of determining depth? A precise trephine that only goes to a certain depth perhaps? How are you determining depth beyond "eyeballing it"? The goal should be to keep wound depth precise and repeatable between globes. Also, a razor blade is very crude for removing the wound button? Consider a Martinez dissector or something that will more precisely separate the lamellae. Where are you making short incision in the globe/cornea to remove the cornea from the globe? I assume you mean sclera posterior to the limbus, but it does not read this way? Also, how far posterior to the limbus?

We have stated exactly what we do. I am hoping that it is clear now. As I have said, the depth is not exactly precise as PRK (of course) but it is very easy and it works every time. Anyone I have taught can do it on the first or second try. Use of this technique has also been repeatedly published.

Need exact temperature of agar and volume necessary (will vary between species).
"Until full" . Temp is now stated.

Incubation - how much media is necessary during a change? How did you determine that changing once every other day is sufficient? What are the outcomes, factors you are assessing here? What are you moistening the cornea with - how much, how often? Once daily dose not mimic the in vivo state and adds the tremendous stress of exposure to the organ which does not recapitulate normal wound healing state. Are you leaving the cornea to sit in any experimental drugs you are applying topically? This also does not mimic what would happen with pulse therapy in the living animal. Need volumes that would need to be ordered to perform this protocol and how long those amounts would likely last.

I have clearly stated how to do this technique.

Histo prep: RT = room temperature (?). How are you maintaining corneal curvature in your processed corneas? Need more precise volumes of fixatives and reagents.

A core facility inserts the cornea into a paraffin block. This is not an issue. We explained the orientation.

Representative results: Background is lacking as are goals and outcomes assessments? The methods you describe do not evaluate time to re-epithelialization (rate of), so making an argument about using your methods for evaluating this ins imprecise and spurious.

I have been as clear as possible. Hopefully the changes will satisfy these requests.

Need much more detail about what you are assessing with Image J - are you assessing wound size, depth character, area, intensity of scar (and if so, how?). Be very specific if you expect someone to be able to repeat your protocol. If you are going to discuss this particular experiment as an example, it needs much more background regarding what you are looking to assess, what the agents you are using are expected to do and what the results were, how they were determined, assessed and

evaluated. Need more discussion and explanation of a "regeneratively healed wound" and the difference between a normal wound - how you are assessing this and how it is achieved.

We have added the steps of quantification to the protocol and added a graph of the quantification of Figure 5, now Fig 5G.

Minor Concerns:
Figure 1 does not illustrate what it is purported to in the text.

???

Figure 3 A and B are not noted in the body of the text.

Yes, they are there.

A few minor grammatical and punctuation errors. Figure legends - It is not true that primates and chickens are the only species that have a Bowman's layer.

I have asked 3 other leaders in the field. This is the answer that I consistently received. If you disagree please tell us what is the other species with the reference.

What tissues are you removing when you are preparing the globes - be specific. Discuss quantification of corneal staining with Image J in detail. This method as described is not useful for determining effects on wound re-epithelialization as described - there need to be a method for determining time to complete re-epithelialization. Need to discuss how you would assess this (fluorescein at different time points, sacrificing representative samples at different time points, confocal imagery? Also, if epithelium is to be assessed only after fixation and processing, there are major concerns for artifact formation. Discuss in detail your camera settings for photography (focal distance, aperture, etc.)? Is there any post-production necessary? How are the images analyzed?

Most of this has been addressed. Imaging is done with a microscope at any setting to image histological samples.

The references below are for this response only. The numbers don't coordinate with the numbers in the manuscript.


1 Ljubimov, A. V. & Saghizadeh, M. Progress in corneal wound healing. *Prog Retin Eye Res.* **49** 17-45, doi:10.1016/j.preteyeres.2015.07.002, (2015).

2 Echevarria, T. J. & Di Girolamo, N. Tissue-regenerating, vision-restoring corneal epithelial stem cells. *Stem Cell Rev.* **7** (2), 256-268, doi:10.1007/s12015-010-9199-1, (2011).

3 Wilson, S. E., Mohan, R. R., Hong, J. W., Lee, J. S. & Choi, R. The wound healing response after laser in situ keratomileusis and photorefractive keratectomy: elusive control of biological variability and effect on custom laser vision correction. *Arch Ophthalmol.* **119** (6), 889-896, doi:emo10005 [pii], (2001).

4 Zieske, J. D., Guimaraes, S. R. & Hutcheon, A. E. Kinetics of keratocyte proliferation in response to epithelial debridement. *Exp Eye Res.* **72** (1), 33-39 (2001).

5 Lassance, L., Marino, G. K., Medeiros, C. S., Thangavadivel, S. & Wilson, S. E. Fibrocyte migration, differentiation and apoptosis during the corneal wound healing response to injury. *Exp Eye Res.* **170** 177-187, doi:10.1016/j.exer.2018.02.018, (2018).

6 Stepp, M. A. *et al.* Wounding the cornea to learn how it heals. *Exp Eye Res.* **121C** 178-193, doi:S0014-4835(14)00044-X [pii]
10.1016/j.exer.2014.02.007, (2014).
7 Sharma, A., Mehan, M. M., Sinha, S., Cowden, J. W. & Mohan, R. R. Trichostatin a inhibits corneal haze in vitro and in vivo. *Invest Ophthalmol Vis Sci.* **50** (6), 2695-2701, doi:iovs.08-2919 [pii]
10.1167/iovs.08-2919, (2009).
8 Marino, G. K., Santhiago, M. R., Torricelli, A. A., Santhanam, A. & Wilson, S. E. Corneal Molecular and Cellular Biology for the Refractive Surgeon: The Critical Role of the Epithelial Basement Membrane. *J Refract Surg.* **32** (2), 118-125, doi:10.3928/1081597X-20160105-02, (2016).
9 Marino, G. K., Santhiago, M. R., Santhanam, A., Torricelli, A. A. M. & Wilson, S. E. Regeneration of Defective Epithelial Basement Membrane and Restoration of Corneal Transparency After Photorefractive Keratectomy. *J Refract Surg.* **33** (5), 337-346, doi:10.3928/1081597X-20170126-02, (2017).
10 Marino, G. K. *et al.* Epithelial basement membrane injury and regeneration modulates corneal fibrosis after pseudomonas corneal ulcers in rabbits. *Exp Eye Res.* **161** 101-105, doi:10.1016/j.exer.2017.05.003, (2017).

**From:** **Liz Segre** ls@allaboutvision.com
**Subject:** RE: website image
**Date:** March 22, 2018 at 12:13 PM
**To:** Audrey bernstea@upstate.edu

LS

Hi Audrey,

They agreed, as long as you can provide credit and a link to us. Would that be OK?

Here's the credit I would suggest:

Image provided with permission from AllAboutVision.com.

Could the link be to http://www.allaboutvision.com/resources/cornea.htm ? Or if not, to our home page at http://www.allaboutvision.com/ ?

Attached are four sizes of the image – not sure which one JOVE would want. (We serve the appropriate size depending on whether our site visitors are using a retina screen or not.)

Thanks,

Liz Segre
Editorial Director
AllAboutVision.com

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**From:** Audrey [mailto:bernstea@upstate.edu]
**Sent:** Thursday, March 22, 2018 8:13 AM
**To:** Liz Segre <LS@allaboutvision.com>
**Subject:** Re: website image

Great! Thank you!

> On Mar 22, 2018, at 11:11 AM, Liz Segre <LS@allaboutvision.com> wrote:
>
> Dear Audrey,
>
> Thanks for reaching out! I am checking with our publishers now and will get back to you.
>
> Liz
>
> ---
> Liz Segre

**From:** **permissions** permissions@biologists.com
**Subject:** RE: The deubiquitylase USP10 regulates integrin β1 and β5 and fibrotic wound healing - 2017 publication
**Date:** August 15, 2018 at 3:22 AM
**To:** Audrey Bernstein bernstea@upstate.edu, permissions permissions@biologists.com

Dear Audrey,

Thank you for your permissions enquiry.

As one of the original contributing authors to this article you do not need specific permission to reproduce any of the images. However, I normally suggest a courtesy email to your fellow authors to avoid any future conflict.

The acknowledgement should state "reproduced/adapted with permission" and give the source journal name - the acknowledgement should either provide full citation details or refer to the relevant citation in the article reference list - the full citation details should include authors, journal, year, volume, issue and page citation.

Where appearing online or in other electronic media, a link should be provided to the original article (e.g. via DOI).

Journal of Cell Science: http://www.biologists.com/journal-of-cell-science

We wish you the best of luck with your project.

Kind regards

Richard



**From:** Audrey Bernstein <bernstea@upstate.edu>
**Sent:** 13 August 2018 16:50
**To:** permissions <permissions@biologists.com>
**Subject:** The deubiquitylase USP10 regulates integrin β1 and β5 and fibrotic wound healing - 2017 publication