Journal of Visualized Experiments

Zebrafish corneal wound healing: from abrasion to wound closure imaging analysis

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63605R2
Full Title:	Zebrafish corneal wound healing: from abrasion to wound closure imaging analysis
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biology
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TITLE:

Zebrafish Corneal Wound Healing: From Abrasion to Wound Closure Imaging Analysis

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KEYWORDS:

Cornea, epithelium, abrasion, ocular burr, wound closure, SEM, zebrafish.

SUMMARY:

This protocol focuses on damaging the ocular surface of zebrafish through abrasion to assess the subsequent wound closure at the cellular level. This approach exploits an ocular burr to partly remove the corneal epithelium and uses scanning electron microscopy to track changes in cell morphology during wound closure.

ABSTRACT:

As the transparent surface of the eye, the cornea is instrumental for clear sight. Due to its location, its tissue is prone to environmental insults. Indeed, the injuries most frequently encountered clinically are those to the cornea. While corneal wound healing has been extensively studied in small mammals (i.e., mice, rats, and rabbits), corneal physiology studies have neglected other species, including zebrafish, despite zebrafish being a classic research model.

This report describes a method of performing a corneal abrasion on zebrafish. The wound is performed *in vivo* on anesthetized fish using an ocular burr. This method allows for a reproducible epithelial wound, leaving the rest of the eye intact. After abrasion, wound closure is monitored over the course of 3 h, after which the wound is reepithelialized. By using scanning electron microscopy, followed by image processing, the epithelial cell shape, and apical protrusions can be investigated to study the various steps during corneal epithelial wound closure.

The characteristics of the zebrafish model permit study of the epithelial tissue physiology and the collective behavior of the epithelial cells when the tissue is challenged. Furthermore, the use of a model deprived of the influence of the tear film has produced new answers regarding corneal response to stress. Finally, this model also allows the delineation of the cellular and molecular events involved in any epithelial tissue subjected to a physical wound. This method can be applied to the evaluation of drug effectiveness in preclinical testing.

INTRODUCTION:

As most of the epithelia are in contact with the external environment, they are prone to physical injury, making them well suited for the study of wound healing processes. Among the well-studied tissues, the cornea is an extremely useful model in the investigation of the cellular and molecular aspects of wound healing. As a transparent external surface, it provides physical protection to the eye and is the first element to focus the light onto the retina. While the structure and cell composition of the retina differ between species[1], these elements of the cornea are generally similar in all camera-type eyes, regardless of species.

The cornea is composed of three main layers[2]. The first and outermost layer is the epithelium, which is constantly renewed to ensure its transparency. The second layer is the stroma, which contains scattered cells, called keratocytes, within a thick layer of strictly organized collagen fibers. The third and innermost layer is the endothelium, which allows nutrient and liquid diffusion from the anterior chamber to the outer layers. The epithelial and stromal cells interact via growth factors and cytokines[3]. This interaction is highlighted by the rapid apoptosis and subsequent proliferation of keratocytes after epithelial injury[4,5]. In case of a deeper wound, such as a puncture, keratocytes take an active part in the healing process[6].

Being in contact with the external environment, corneal physical injuries are common. Many of them are caused by small foreign objects[7], such as sand or dust. The reflex of eye rubbing can lead to extensive epithelial abrasions and corneal remodelling[8]. According to wound size and depth, these physical injuries are painful and take several days to heal[9]. The optimal wound healing characteristics of a model facilitate the understanding of the cellular and molecular aspects of wound closure. Furthermore, such models have also proved useful for testing new molecules with the potential to accelerate corneal healing, as previously demonstrated[10,11].

The protocol described here aims to use zebrafish as a relevant model to study corneal physical injury. This model is highly convenient for pharmacological screening studies as it allows molecules to be added directly to the tank water and, therefore, to come into contact with a healing cornea. The details provided here will help scientists perform their studies on the zebrafish model. The *in vivo* injury is performed with a dulled ocular burr. The impact on epithelial cells adjoining or at a distance from it can be analyzed by specifically removing the central corneal epithelium. In recent years, numerous reports focused on such a method on rodent cornea[12-17]; however, to date, only a single report has applied this method to zebrafish[18].

Because of its simplicity, the physical wound is useful in delineating the role of epithelial cells in wound closure. Another well-established model of corneal injury is the chemical burn, especially the alkali burn[19-21]. However, such an approach indirectly damages the entire eye surface, including the peripheral cornea and corneal stroma[19]. Indeed, alkali burns potentially induce corneal ulcers, perforations, epithelial opacification, and swift neovascularization[22], and the uncontrollable outcome of alkali burns disqualifies that approach for general wound healing studies. Numerous other methods are also used to investigate corneal wound healing according to the particular focus of the study in question (e.g., complete epithelial debridement[23], the combination of chemical and mechanical injury for partial-thickness

wound[24], excimer laser ablation for wounds extending to the stroma[25]). The use of an ocular burr restricts the focal point to the epithelial response to the wound and provides a highly reproducible wound.

As with each method of wound infliction, the use of an ocular burr has advantages and disadvantages. The main disadvantage is that the response being mostly epithelial, it does not perfectly reflect the abrasions seen in the clinical setting. However, this method has numerous advantages, including the ease with which it can be set up and performed, its precision, its reproducibility, and the fact that it is noninvasive, making it a method well tolerated by animals.

PROTOCOL:

All experiments were approved by the national animal experiment board.

1. Preparations

1.1. Prepare the tricaine stock solution used for anesthesia[26] in advance (0.4% stock solution used in this protocol). Use gloves and keep the materials in a fume hood whenever possible.

1.1.1. For 50 mL of a 0.4% solution, weigh 200 mg of tricaine powder into a 50 mL tube. Dissolve the powder in approximately 45 mL of double-distilled water.

1.1.2. Adjust the pH of the tricaine stock solution to 7 with 1 M Tris (pH 8.8, ~1.25 mL). Add the Tris solution to the tricaine stock in aliquots, mix the stock thoroughly after each aliquot, and check the pH after each addition of Tris.

1.2. Before the experiment, prepare a 0.02% working solution of tricaine.

1.2.1. Thaw 2 mL of 0.4% stock solution and add to 40 mL of system water (final concentration 0.02%). Place the solution in a small container.

1.3. Before the experiment, prepare the fixing solution (2.5% glutaraldehyde in 0.1 M sodium phosphate (Na_3PO_4) solution at pH 7.4). Use gloves and keep the materials in a fume hood.

1.3.1. For 10 mL of the fixing solution, pipette 5 mL of 0.2 M Na_3PO_4 into a tube. Add 0.5 mL of 50% glutaraldehyde, and add double-distilled water to obtain the final volume of 10 mL. Protect the solution from light, and keep it on ice or in the fridge prior to use.

NOTE: If samples must be collected for several hours after wounding, prepare the fixing solution just prior to use.

1.4. Prepare the equipment for wounding (**Figure 1**).

1.4.1. Fill the recovery tanks or smaller containers with system water.

1.4.2. Have the ophthalmic burr ready. Check that the burr tip is clean. If needed, remove cell debris with a moist cotton swab.

1.4.3. Make an incision to the side of a soft sponge, and moisten the sponge with system water. Place the sponge on the base/stage of a dissecting microscope. Ensure enough working space for using the burr and enough illumination from the sides and/or above to see the eye surface properly.

2. Anesthesia

2.1. Transfer a fish from the tank to the 0.02% tricaine solution with a net as gently as possible.

2.2. Monitor the anesthesia, checking for lack of response to light mechanical stimulus.

NOTE: For consistent anesthesia, a 2 min exposure to tricaine is used prior to abrasion with adult wild-type AB fish. With fish of other genetic background, a different duration may be needed.

3. Abrasion

3.1. Gently place the anesthetized fish with a spoon into the incision on the sponge, head protruding from the sponge surface.

3.2. Turn on the burr, and focus the microscope view onto the eye surface.

3.3. Carefully approach the eye surface with the burr tip. When touching the eye surface, start moving the burr tip on the eye surface with circular motion. Avoid sudden movement, as it might lead to the eye tilting in the socket and the burr tip to slip.

3.4. When the abrasion is done, carefully place the fish in fresh system water for recovery.

3.5. Clean the burr right after use with a moist cotton swab.

4. Collecting samples

4.1. At the desired time point, pick the fish up with a net and place it in 0.02% tricaine solution. Keep the animal in the solution until the opercular movement has ceased completely, and the fish does not react to touching.

4.2. Place the fish on a Petri dish with a spoon, and hold it with tweezers. Decapitate the fish with dissecting scissors. Avoid making any scratches on the eye surface when handling the sample.

4.3. Put the tissue into a sample tube containing 0.1 M Na_3PO_4.

4.3.1. Rinse the tissue by replacing the 0.1 M Na_3PO_4 with clean buffer so that no blood remains in the solution.

5. Sample processing for electron microscopy

5.1. Fix the tissue in 2.5% glutaraldehyde/0.1 M Na_3PO_4 (pH 7.4) for ~24 h at +4 °C. Keep the sample on a rotating/shaking sample holder to ensure proper fixation.

5.2. Remove the fixing solution and rinse the sample several times with 0.1 M Na_3PO_4.

5.3. Dissect the sample at this point.

5.3.1. Place the sample onto a drop of 0.1 M Na_3PO_4 on a dissecting plate. If both eyes from the same fish must be imaged, cut the head sample into two with fine dissecting scissors.

5.3.2. Alternatively, collect the eyes only by carefully placing the tips of fine tweezers into the eye socket from the side of the eye, taking extra care not to scratch the eye surface. Then, pull the eye out from the socket.

5.3.3. Transfer the dissected sample into a tube containing 0.1 M Na_3PO_4. Ensure there is no extra tissue in the sample tube, as it may adhere to the top of the eye during sample processing.

5.4. Store the sample in 0.1 M Na_3PO_4 (maximum one week) at +4 °C.

5.5. Process the samples for electron microscopy imaging.

5.5.1. Postfix the samples in 2% osmium tetroxide in 0.1 M Na_3PO4 buffer for 1 h at room temperature (RT).

5.5.2. Wash the samples 3 times for 5 minutes each wash in 0.1 M Na_3PO_4 at RT.

5.5.3. Dehydrate the samples successively in 30%, 50%, and 70% ethanol for 1 h in each solution at RT.

5.5.4. Immerse the samples in 96% ethanol for 2–3 h at RT.

5.5.5. Next, incubate the samples two times in 100% ethanol, first for 1 h and then in fresh 100% ethanol overnight at +4 °C.

5.5.6. Subject the samples to 30 cycles in an automated critical point dryer.

5.6. Embed and platinum-coat the samples.

5.6.1. Place an adhesive tab onto a mount. If the sample must be marked on top of the mount, leave a piece of tab cover paper on the tab and write the sample ID on the paper.

5.6.2. Place the mount with the tab on the base of a dissecting microscope.

5.6.3. Gently place the tissue sample on the mount with fine tweezers, cornea facing up.

5.6.4. Coat the specimen with platinum using the appropriate device. After coating, store the samples at room temperature until imaging.

6. Imaging (**Figure 2**)

6.1. Operate the devices as advised in the user's manual and by imaging experts.

6.2. Acquire images of the desired magnification, and use 2,000–2,500x images for analysis.

6.3. Adjust the brightness and contrast so that there are no overexposed areas in the image, and cell borders and microridges are seen as clearly as possible.

NOTE: The position and angle of the tissue affect the brightness and contrast settings. They may need to be adjusted from sample to sample and between different regions of the tissue.

7. Measuring cell shape, size, and microridge pattern

7.1. Open the TIFF image in Fiji ImageJ 1.53[27]. Set the scale using the scale bar of the image: create a line equal in size to the scale bar with the **Line** tool. Select **Analyze | Set scale**, and type in the known distance. Open the **ROI manager** from the **Analyze | Tools** menu.

7.2. For cell size and roundness, select **Analyze | Set measurements | Shape descriptors**. Use the **Magnifying glass** tool to see the cells under magnification. Select cells with the **Polygon** tool, and add each selection to the ROI manager. Finally, measure the selected cells, and save the measurement.

7.3. Microridge analysis (**Figure 3** and **Figure 4**)

7.3.1. Ensure that the image is in 8-bit format from the **Image | Type** menu.

7.3.2. Select a cell with the **Polygon** tool, and clear the background from **Edit | Clear outside**.

7.3.3. Smoothen the image one to three times by selecting **Process | Smooth**, and adjust the brightness and contrast from **Image | Adjust | Brightness/Contrast** so that the microridges stand out as clearly as possible.

7.3.4. Convolve the image from **Process | Filters | Convolve**, turn into binary from **Process | Binary | Make binary**, and skeletonize the black-and-white image by selecting **Process | Binary | Skeletonize**.

7.3.5. Use the **Analyze skeleton** function in the **Analyze | Skeleton** menu to measure the microridge parameters and save the values.

NOTE: In SEM, individual images may differ in brightness and contrast. Thus, the steps in the analysis may need adjustments from image to image.

REPRESENTATIVE RESULTS

This study describes a method using an ophthalmic burr in zebrafish corneal wound healing experiments. The method is modified from previous studies on mice, where the burr was shown to remove the epithelial cell layers efficiently[13]. The challenges in zebrafish corneal wounding include the relatively small size of the eye, and in the case of time-consuming experiments, the need to maintain a constant water flow through the gills (as described by Xu and colleagues[28]). The main benefits of this method are its simplicity and speed. A standard dissecting microscope is used for controlled use of the burr (**Figure 1**). As the procedure is of short duration (approximately 3 min from the start of anesthesia), the fish recover well from the handling, and no extra equipment is needed for the maintenance of anesthesia and oxygen delivery.

There are several ways of visualizing the corneal wound. This protocol uses scanning electron microscopy (SEM, **Figure 2**), which has a long history of use in corneal studies[29,30]. Although this approach does not allow an assessment of the lower layers of the epithelium, it provides an easy method of estimating the wound healing speed and comparing the corneal surfaces of different regions of the eye. At 3 h post wound, while the wound area is closed (**Figure 2**), the site where the wound borders are joined remains visible (**Figure 2**).

The superficial cells on zebrafish cornea contain pronounced microridges[31]. Recently, a study reported these structures as lost in elongated cells adjacent to wounds on zebrafish skin[32]. However, the presented results show that on abraded corneal epithelium, microridges can be observed in some elongated cells next to the wound site (**Figure 4B**). In some peripheral regions, the microridges are lost from the center of the cell (**Figure 4C,D**). For a more detailed analysis, apical cell area and roundness are quantified, in addition to microridge amount and average length in ImageJ[27] (**Figure 3** and **Figure 4E—H**).

The microridge analysis is done using the **Skeleton** function (modified from van Loon and colleagues[33]). A comparison between the two peripheral regions (**Figure 4A** (regions C and D), **Figure 4C**, and **Figure 4D**) reveals that the cells in **Figure 4D** are more elongated (indicating cell rearrangements as a reaction to wounding) and have shorter average microridges than cells in **Figure 4C**. This result suggests that the change in cell shape correlates with the microridge modification and emphasizes the heterogeneity within the corneal epithelium in wound response.

Measuring the apical cell area and roundness on SEM images is a simple and reproducible way to obtain quantitative data on cell appearance in different regions of the cornea. Though

limited to 2D, this approach helps acquire an overall understanding of the dynamics and speed of cell rearrangements during wound closure. The SEM images are utilized for analyzing the microridge patterns on the apical cell surface. The image processing described here gives an approximation of the changes in the microridge parameters, which would be tedious to measure by hand.

FIGURE AND TABLE LEGENDS:

Figure 1: The setup for corneal abrasion. (**A**) A dissecting microscope is necessary for the controlled abrasion on the small zebrafish eye. (**B**) The sponge helps to stabilize the anesthetized fish during the procedure. (**C**) The fish is anesthetized on a Petri dish, and the anesthetized animal is transferred to the sponge with a small spoon. An ocular burr with a 0.5 mm tip is used to abrade the cornea.

Figure 2: Visualization of the wounded cornea with scanning electron microscopy. The overview of the abraded cornea collected at 0, 1, 2, or 3 h post wound (HPW). The dashed outline indicates the wound border. Scale bars = 500 µm.

Figure 3: An example of the image modification prior to microridge measurement. Although not an exact replica of the original cell surface, the final skeletonized pattern captures the differences between the cell center and periphery. Scale bar = 10 µm.

Figure 4: A comparison of apical cell area, roundness, and microridge values between two peripheral regions after corneal abrasion. (**A**) An overview of the wounded eye. The boxes indicate the location of the higher magnification images (in **B**, **C**, and **D**). (**C, D**) Cells selected for shape descriptor analysis are marked with a green outline. (**E, F**) Apical cell area (**E**) and roundness (**F**) values of selected cells. (**G, H**) Microridge total length (**G**) and average length (**H**) of the same cells. Groups were statistically compared with a two-tailed *t*-test (*$p \leq 0.05$, **$p \leq 0.01$) Scale bars = 500 µm in **A**, 50 µm in **B**, **C**, and **D**.

DISCUSSION:

Corneal physical injuries are the most common cause of ophthalmology patient visits to the hospital. Therefore, it is important to establish relevant models for the study of different aspects of corneal pathophysiology. So far, the mouse is the most commonly used model for the study of corneal wound healing. However, adding eyedrops on murine wounded eyes to validate the impact of specific drugs on corneal wound healing can be difficult. In this respect, the zebrafish model is particularly useful for the pharmacological screening of molecules impacting corneal wound healing. The method described here is very similar to that described for mouse[13].

Two specific points of difference, however, must be kept in mind. First, the use of an ophthalmic burr requires practice to ensure wound reproducibility, particularly with respect to the pressure exerted on the eye, which is critical for proper abrasion. In addition, the abrasive tip should be changed when the epithelium is no longer removed efficiently. Second, while the structure and morphology of the zebrafish cornea are similar to other corneas[31], this animal possesses regenerative capacities that are unparalleled in mammalian organisms[34-36]. While wound closure in mouse lasts for 48–72 h[11,14,37], a timeline of 3 h is reported for zebrafish. Due to structural and molecular similarities, the cellular behavior

induced by a corneal physical wound is probably similar in most vertebrates. However, the swift response in zebrafish is probably guided by an advanced regenerative mechanism that is specific to that animal.

The described protocol uses SEM to track wound closure. Numerous other studies have used fluorescence microscopy instead to track this process[15,17,38]. However, the use of SEM facilitates the analysis of cell shape modification following epithelial abrasion. The downside of that technology is that the stratification steps cannot be tracked, as SEM permits only the imaging of the most external layer. To study the epithelium in 3D during full corneal healing, fluorescent models, such as Zebrabow[39], or immunolabeling should be used.

The use of zebrafish as a corneal wound healing model enhances the scope of investigation as it allows the application of numerous molecular tools available, such as genetically modified fish lines, morpholinos, and chemical screening, to significantly expand the possible range of corneal wound healing studies. Furthermore, the size of the zebrafish eyes allows the development of new imaging strategies for studying epithelial cell dynamics in greater detail than can be done with murine eyes.

The main aim of this report is not only to adapt the physical corneal wounding approach to the zebrafish model but also to demonstrate that the use of new models allows new questions to be asked and answered and points to new ways of investigating fundamental biological phenomena. These advantages will ultimately be beneficial to patients.

ACKNOWLEDGMENTS:

The authors thank Pertti Panula for the access to the Zebrafish unit and Henri Koivula for the guidance and help with the zebrafish experiments. This research was supported by the Academy of Finland, the Jane and Aatos Erkko Foundation, the Finnish Cultural Foundation, and the ATIP-Avenir Program. Imaging was performed at the Electron Microscopy unit and the Light Microscopy Unit, Institute of Biotechnology, supported by HiLIFE and Biocenter Finland.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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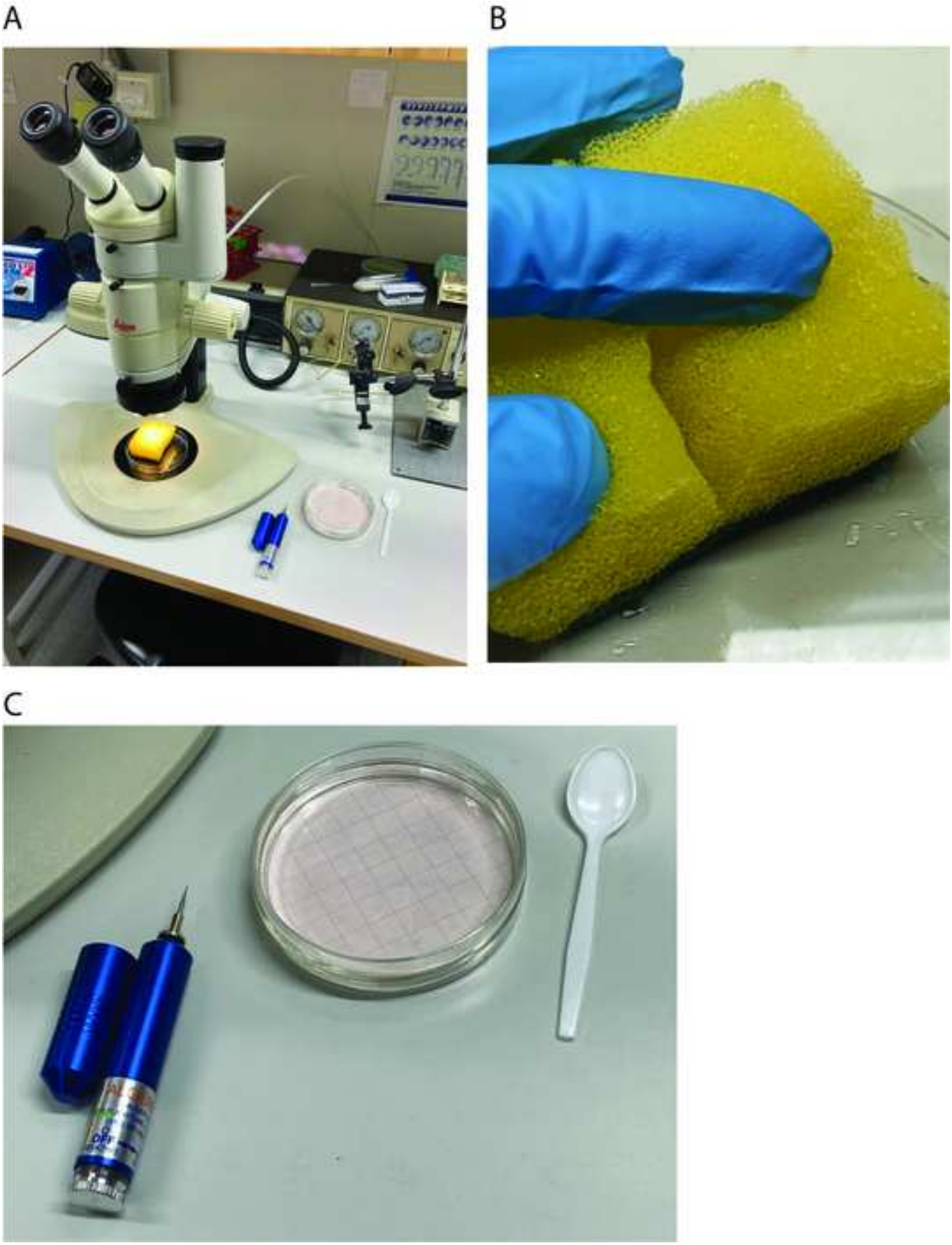

Figure 2

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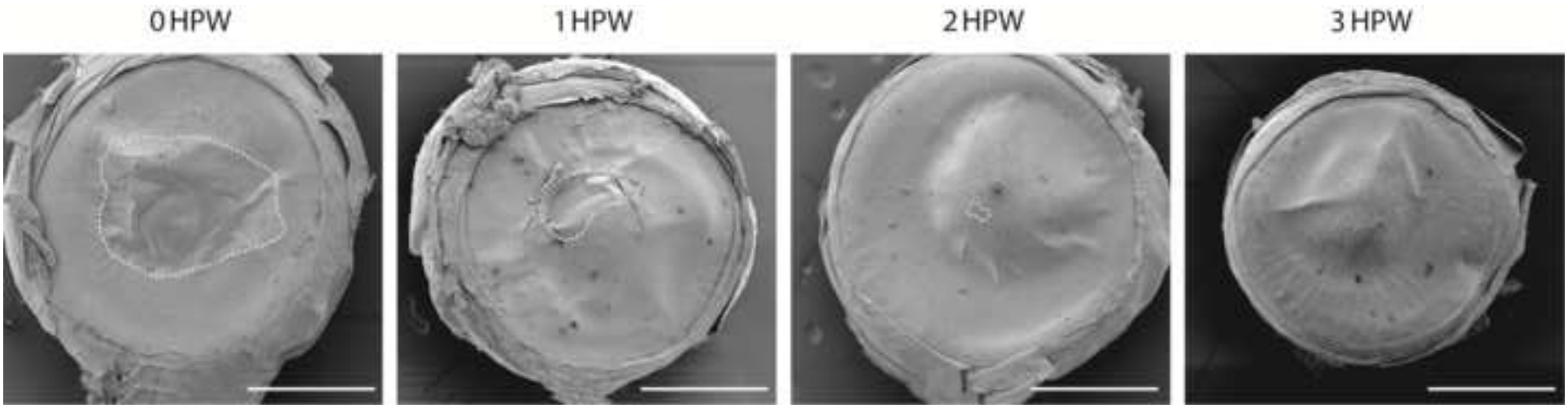


Figure 3

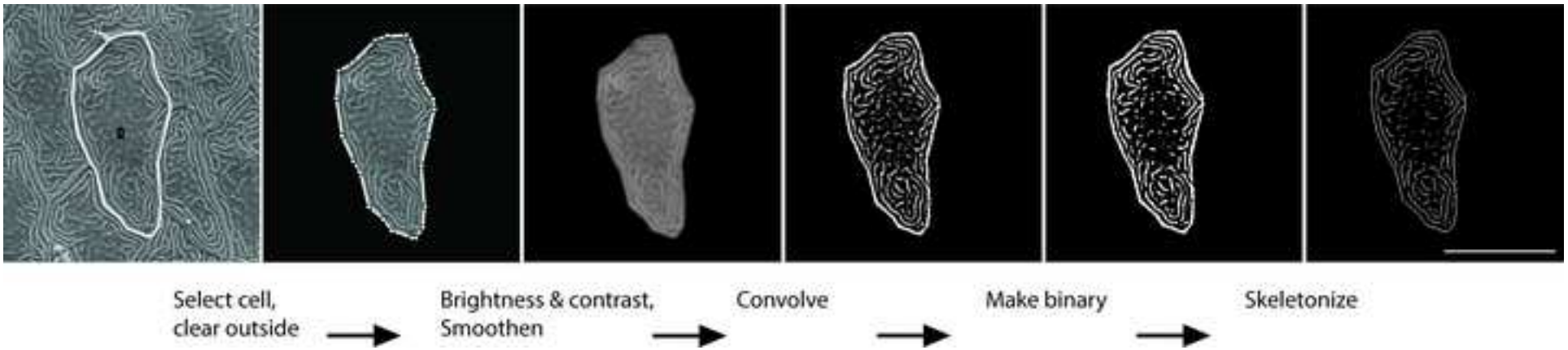
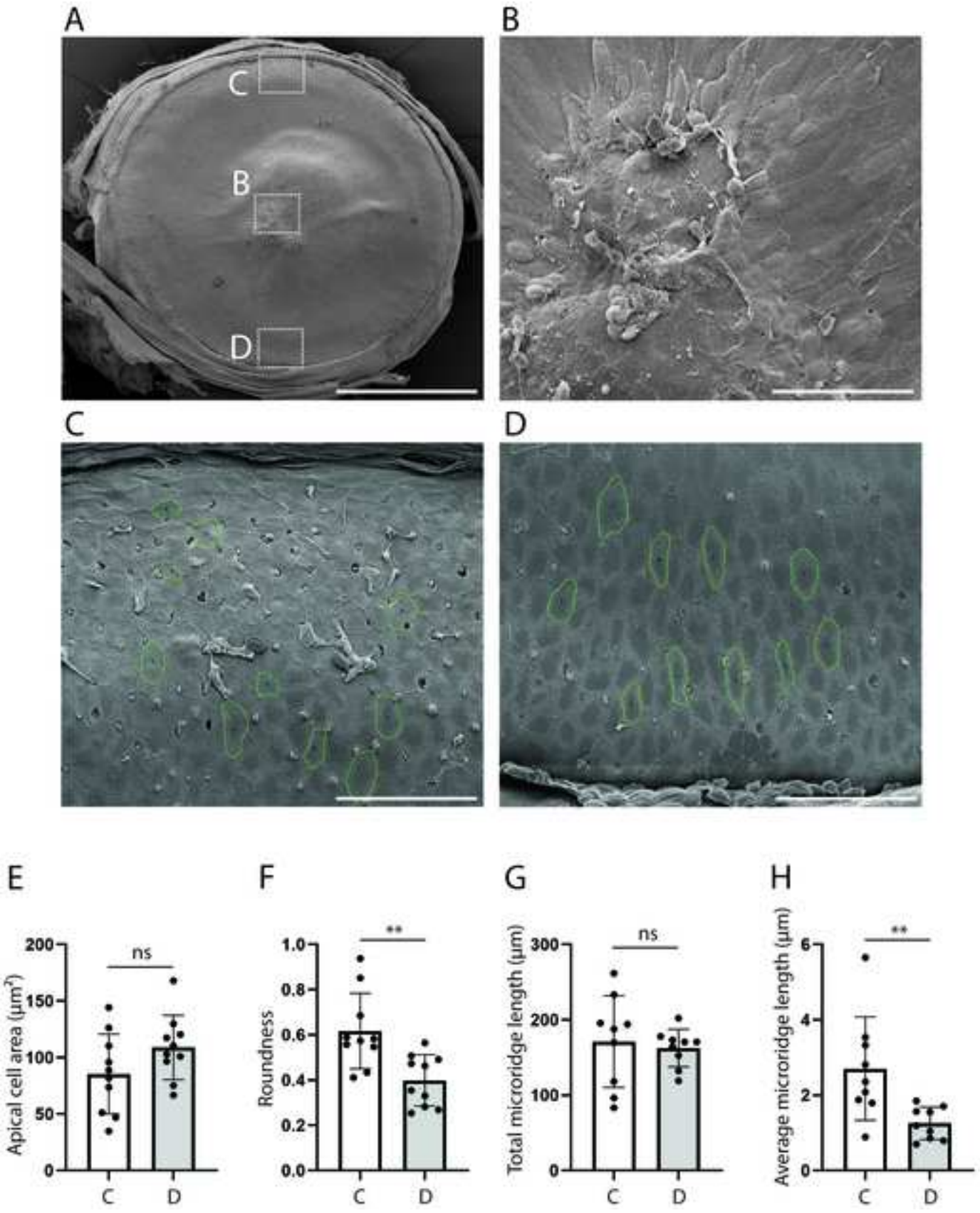


Figure 4

Click here to access/download;Figure;Jove_fig4_quantification_2 PC.tif



Rebuttal letter

We are grateful for all the comments we received from the Editor and Reviewers which helped improving drastically the quality of the submitted manuscript. We have adapted the text to the remarks and editorial constrains.

In the new version of our manuscript, the text that was highlighted in yellow has been drastically revised. The text highlighted in blue should be included in the video. We hope that this new version of the manuscript will meet the standard to be accepted for publication in JoVE.

Please, find below the detailed answer to the various comments.

Best wishes,

The authors

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. Please define all abbreviations at first use.

The manuscript has been proofread by a language specialist

2. Please keep the short abstract word count between 10 and 50 words.

We have reduced the short abstract to 48 words

3. Line 90: What is (ref ZFIN)?

This was corrected

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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.

For example: Sigma etc

The manuscript has been revised accordingly

5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The manuscript has been revised accordingly.

6. Line 147 (step 5.4.1): please use English names for all materials.

The manuscript has been revised accordingly

7. Step 5.4: Please use full sentences while describing steps. Steps 5.4.3-5.4.5: the samples must be immersed in these solutions for these times and under these conditions?

The manuscript has been revised accordingly

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. 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. Please ensure the inclusion of specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Kaisa, in collecting samples, in embedding, in imaging, in measurements

9. Step 5.5: please provide more details OR cite a publication describing this process in detail.

The manuscript has been revised accordingly

10. Step 7.1: Please remember to convert all citations into the required superscript format (before punctuation).

The references have been checked

11. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

We have paid attention to follow these indications

12. As we are a methods journal, please ensure that the Discussion covers the following in detail with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

The discussion has been largely modified to include these aspects.

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

The references have been checked

Reviewers' comments:

Reviewer #1:

Major Concerns:

One major concern from this reviewer is as follow,

Since the adult zebrafish cornea contains all five major layers found in the human cornea, it is highly suggested that this study should also present TEM in different time points of wound closure from 1 to 3 hours post abrasion to show not only the re-epithelialization but also re-stratification at the cellular level in the wound healing process.

The two aims of the manuscript are: 1. Presenting a new animal model to study corneal wound healing; 2. Presenting a new visualization system that is underused when studying wound closure. SEM is a powerful approach to study cell dynamics in a wounded tissue. However, because of surface scanning, it is impossible to see what is happening below the surface layer. This is the reason why we have chosen to display only wound closure.

Ad the Reviewer pointed it out, the stratification would be visible through TEM. We chose to focus on cell surface because this type of analysis is not as common as studying the layer dynamics, via confocal microscopy for example. Furthermore, we feel that showing the kind of image analysis that can be used with apical microridges is more beneficial in this context than TEM.

Minor Concerns:

One minor point, on page2 line 55, a wrong statement regarding "stroma is mainly acellular" needs to be rephrased. Cornea stroma is not acellular but contains the most important neural crest-derived keratocytes responsible for corneal transparency. Moreover, during epithelial wound healing, keratocytes undergo dramatically transient cellular transformation into myofibroblast to facilitate the healing process.

Epithelial abrasion leads to a removal of the epithelial cell layers, with very little impact on the stroma, which is different to what happens with a wound impacting the stroma, such as a puncture wound. We modified the text to explain this nuance.

Reviewer #2:

Manuscript Summary:

The manuscript describes a protocol of wound induction in the upper cellular layer of the zebrafish eye with further investigation of the sample using SEM. The manuscript is well-written, and clearly describes the protocol, providing images and step-by-step procedures from the start till the sample preparation and investigation.

Major Concerns:

No concerns

Minor Concerns:

Authors may find a better justification of using zebrafish as a valid model for this kind of experiment other than the fact that zebrafish are currently not used; e.g., reduce the number of small animals for laboratory experiments for ethical considerations, etc.

We have followed this comments and added in the introduction the fact that zebrafish is a very convenient model to be used in pharmacological screening studies.