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Isolation of intact eyeball to obtain integral ocular surface tissue for histological examination and immunohistochemistry

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June 26, 2019
Dear Dr. Alisha DSouza,

Enclosed please find a revised manuscript for JoVE60086: “Isolation of intact eyeball to obtain integral ocular surface tissue for histological examination and immunohistochemistry” that we previously sent to JoVE.

We have addressed all editorial and reviewer’s comments and believe that the revised manuscript meet all requirements for publication in JoVE. We sent a rebuttal letter separately in addressing the editorial and reviewers’ comments.

We are looking forward to a positive outcome.

Sincerely yours,
Chunqiao Liu

TITLE:

Isolation of Intact Eyeball to Obtain Integral Ocular Surface Tissue for Histological Examination and Immunohistochemistry

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KEYWORDS:

Eyeball, skull, orbital bones, ocular surface, conjunctiva, cornea, immunohistochemistry

SUMMARY:

This protocol describes a method for the isolation of the mouse eyeball with eyelid, ocular surface, anterior and posterior segments in relatively intact position.

ABSTRACT:

Ocular surface (OS) consists of an epithelial sheet with three connected parts: palpebral conjunctiva, bulbar conjunctiva and corneal epithelium. Disruption of OS would lead to keratitis, conjunctivitis or both (keratoconjunctivitis). In experimental animal models with certain genetic modifications or artificial operations, it is useful to examine all parts of the OS epithelial sheet to evaluate relative pathogenetic changes of each part in parallel. However, dissection of OS tissue as a whole without distortion or damage has been challenging, primarily due to the softness and thinness of the OS affixed to physically separate yet movable eyelids and eyeball. Additionally, the deep eye socket formed by the hard skull/orbital bones is fully occupied by the eyeball leaving limited space for operating dissections. As a result, direct dissection of the eyeball with associated OS tissues from the facial side would often lead to tissue damages, especially the palpebral and bulbar conjunctiva. In this protocol, we described a method to remove the skull and orbital bones sequentially from a bisected mouse head, leaving eyelids, ocular surface, lens and retina altogether in one piece. The integrity of the OS sheet was well preserved and could be examined by histology or immunostaining in a single section.

INTRODUCTION:

The ocular surface consists of a continuous sheet of regionalized epithelium including palpebral conjunctiva, bulbar conjunctiva and cornea[1]. Many glandular structures are associated with the ocular surface epithelium and together generate a layer of tear film protecting the cornea surface from drying and environmental invasions[2]. Disruption of OS would lead to keratitis, conjunctivitis

or both (keratoconjunctivitis). Both genetic factors and environmental irritants or their interactions contribute to pathological alterations of the OS[3,4]. Accordingly, a variety of genetically-engineered and physically or chemically-induced animal models have been used for studying disease processes of the human OS.

The structure and function of the mouse OS is similar to that of humans in many ways. The tear film components secreted by the ocular glands are also similar between mice and humans. A wealth of studies has been conducted using mouse models for elucidating mechanisms of human OS diseases[5-7]. In many occasions, it is critical to analyze global instead of local molecular changes of the OS to gain comprehensive information under the same experimental treatment. Therefore, sample preparation with good integrity is needed to ensure each part of the OS to be analyzed simultaneously.

The mouse OS tightly associates with the eyeball that was embedded in the eye socket/orbit (a bony cup made of several different skull bones) and connects to it through thin connective tissues. There exist tremendous challenges for dissecting the whole ocular surface without damaging the palpebral or bulbar conjunctiva. These challenges descend from: (i) the OS is soft and thin and affixed to physically separate yet movable eyelids and eyeball, therefore vulnerable for distortion and damage; and (ii) the limited space between the orbital bones and eyeball restrict the dissection operations. The challenges are much greater for the adult mouse. In the embryonic mouse, the orbital bone ossification is not complete and surrounding tissues are relative loose[8]. The head can be removed and bisected, and then directly subjected to paraformaldehyde (PFA) fixation and embedding[9]. By contrast, the postnatal and adult mouse orbital bones are fully ossified with thick surrounding tissues, making the penetration of fixatives less efficient. Furthermore, the orbital/skull bones are hard and brittle, easily broken when sectioning them in the soft embedding compounds such as paraffin. The broken pieces of bones will unanimously tear the nearby tissues resulting in inferior tissue morphology.

Many published studies often showed partial ocular surface, which may be sufficient for their particular research purposes[10,11]. A gross examination of literatures found only few studies showing the whole intact ocular surface being demonstrated without detailed description of dissection protocols[12,13]. In this protocol, we detailed a dissection method to obtain integral postnatal ocular surface, in which orbital and skull bones were orderly removed, leaving untouched ocular surface together with the eyeball and eyelids, minimizing the physical damages. We further examined the OS histology and performed immunohistochemistry using the tissue sections prepared with this protocol.

PROTOCOL:

All procedures involving the use of mice were approved by the Animal Care and Use Committee, Zhongshan Ophthalmic Center, and adhered to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

1. Dissection of eyeball with intact ocular surface and eyelids

1.1. Dissect the head.

1.1.1. Euthanize postnatal day 10 (P10) and P28 mice (see the **Table of Materials** for mouse strains) by cervical dislocation, cut the head off the neck by a pair of sharp scissors.

1.1.2. Bisect head with a pair of straight scissors along the sagittal midline beginning at the interparietal bone rostrally to the nasal (**Figure 1A,B**).

NOTE: The skull bone hardens with mouse aging, be careful to keep the scissors cutting along the midline as much as possible.

1.1.3. Place each half of the bisected head in a clean Petri dish, cut off the jaw and tongue first using sharp scissors. Free the optic nerve by cutting it off the brain prior to the optic chiasm location, and remove all brain tissues including olfactory bulb attached to the skull wall.

1.1.4. Now the remaining tissues shall have all skull/orbital bones associated with the eyeball (**Figure 1C,D**). Wash the remaining part with PBS to clean blood and hair debris.

1.1.5. Prefix tissues with 4% paraformaldehyde (PFA, in phosphate-buffered saline [PBS], pH 7.4) in a 50 mL conical tube at room temperature (RT) with rotation. The approximate fixation time is as follows: ~10 min for P0 to P7; ~20 min for P8 to P28; and ~30 min for P29 and older.

NOTE: The prefixation offers tissue rigidity making the ensuing dissection easier. Additionally, prefixation avoids leaving fresh tissue unfixed too long before dissection is complete.

CAUTION: PFA is hazardous and must be handled with care.

1.2. Remove skull/orbital bones.

1.2.1. After prefixation, quickly wash the dissected head in PBS three times to further eliminate the broken hairs and fixatives in order to proceed with further dissection.

CAUTION: Exposure to fixatives during dissection can be injurious to health.

1.2.2. Place the tissue in 10 cm Petri dish, cut the skull into three parts along the planes indicated in **Figure 1D,E**. The eyeball in the socket is hidden in the middle part of the skull under ethmoid, frontal and maxillary bones (**Figure 1D,E**).

1.2.3. Use two pairs of No. 4 straight forceps to peel off the ethmoid bone to fully expose the frontal and maxillary bones (**Figure 1F**).

1.2.4. Geographically divide the skull surface (including maxillary and the frontal bones) into 4 areas (**Figure 1F**). Insert the tip of curved forceps horizontally into each area to remove the maxillary and frontal bones sequentially (**Figure 1F**).

NOTE: The maxillary bones cannot all be removed at one time. Patiently dissect them piece by piece. The frontal bone is directly connected to the eyeball through soft connective tissues. Take caution when removing it from the eyeball to prevent stretching the eyeball and damaging the conjunctiva.

1.2.5. After removal of partial maxillary bone and all frontal bone, the underlying lacrimal and jugal bones would be exposed (**Figure 1G**). Remove the two bones and associated subcutaneous muscles and fats surrounding the eyeball, trim the eyeball with attached eyelids and skin into a small square-shape block to reduce tissue volume and facilitate orienting the tissue when embedding (**Figure 1H**).

1.2.6. Place the eyeball and associated tissues back into 4% PFA and continue to fix overnight at 4 °C. The fixed tissue can be preserved for at least one month at 4 °C in PBS with addition of 0.02% NaN_3. Alternatively, proceed to histological analysis immediately.

NOTE: If the tissue needs to be stored for longer periods, it can readily be stored in 70% ethanol.

2. Histological analysis

2.1. Paraffin section and hematoxylin and eosin (H&E) staining

2.1.1. Follow standard protocol described elsewhere[9] to perform paraffin embedding and sectioning.

2.2. Immunohistochemistry (IHC)

2.2.1. Dewax the paraffin sections with xylene and rehydrate the sections through alcohol series (100%, 95%, 80%) into distilled water (dH_2O).

2.2.2. Perform antigen retrieval by microwave treatment of the tissue slides in 0.01 M citric acid buffer (3 g of trisodium citrate, 0.4 g citric acid per 1 L double distilled H_2O) in a glass slide jar with low power (120 W). Energy should be intermittently delivered for total 5–10 min with each interval lasting about 2 min.

NOTE: High-power microwave or consistent heating would lead to detachment of tissue sections from the slide.

2.2.3. Pick out the slides from glass jar, carefully wipe off residual liquid surrounding each section using facial tissues and place them onto the slide rack in a histology box. Draw a square around each section with a waterproof histological pen. Place 100 µL of blocking buffer (0.1% triton X-100 and 10% donkey serum in 1x PBS) onto each square, and incubate for 30 min at RT.

2.2.4. Carefully remove the blocking solution with vacuum, add the primary antibody (see the **Table of Materials**) with desired dilution in blocking buffer, and continue to incubate the slides at 4 °C for 24 h or longer.

NOTE: The concentration for each primary antibody used for IHC varies, and needs to be tested out in pilot experiments.

2.2.5. Wash tissue slides with PBST (0.1% Triton in PBS) three times, for 10 min each. Repeat step 2.2.4 using the secondary antibody (see **Table of Materials**) together with 4',6-diamidino-2-phenylindole (DAPI) replacing the blocking buffer. Continue to incubate for at least 4 h or longer at room temperature (RT).

2.2.6. Remove the secondary antibody, wash tissue sections with PBST solution three times, for 10 min each, then wash with clean PBS for another 5 min.

2.2.7. Wipe off the residual PBS surrounding tissue sections, mount coverslips on sections with mounting medium. Perform fluorescent microscopy to obtain images (see the **Table of Materials**).

REPRESENTATIVE RESULTS:

The major skull bones viewed from different perspectives were illustrated in **Figure 1A–E**, with colors denoting different bones. We used four-week old animal for demonstration of the dissection processes. Following dissection steps 1.1.1–1.1.3 and a short prefixation (step 1.1.4), the eyeball with associated facial bones are demonstrated in **Figure 1E**. Further trimming to remove anterior (nasal and premaxillary) and posterior (e.g., parietal) as well as ethmoid bones generated tissue block with mainly maxillary and frontal bones atop the eyeball (**Figure 1F**). Sequential removal of these bones according to designated areas in **Figure 1F** exposed underlying lacrimal and jugal bones as well as Harderin gland (**Figure 1G**). The Harderin gland attached to the eyeball served as a landmark (**Figure 1G**), and should be kept in place at all times. Isolation of the eyeball was completed by removal of all bones and surrounding fats and muscle tissues (**Figure 1H**).

After paraffin embedding, the eyeball was sectioned and H&E-stained. Representative images are shown in **Figure 2**. The relative intact positions of eyelids, corneal and conjunctival surface were visualized in one section (**Figure 2A**), parts of which were magnified in **Figure 2B,C**. The lens morphology is hard to maintain (**Figure 2A**) because of its rigidity and fragility. We applied this dissection method to other postnatal ages and showed an example of H&E histology of P10 eyeball section (**Figure 3**). In general, the younger the mouse is, the better the eyeball morphology is preserved. Immunostained paraffin sections from serial postnatal ages are shown in **Figure 4**, with keratin 12 (K12) (**Figure 4A–F**) and keratin 14 (K14) (**Figure 4G–L**) staining the cornea and the whole OS epithelium, respectively.

FIGURE LEGENDS:

Figure 1: Illustration of the eyeball dissection in 4-week old adult mouse. (A–D) The schematic diagram of the skull composition viewed from top (A), bottom (B), lateral (C) and middle plane (D), respectively. Dashed lines in (A) and (B) indicate bisected planes. (**E**) Bisected-half skull at 4-week old age that exactly matches (D). (**F**) Medial-plane view of dissected tissue between the two transverse planes in (D) and (E). The eyeball (dashed circle) was mainly embedded underneath the frontal (Fron) and maxillary (Max). Colored dashed lines geographically divide the bisected plane into 4 areas, in which bones were roughly removed as per the order or numbers. Note that the ethmoid (Eth) bone has been removed. (**G**) After removal of frontal bone and partial maxillary, Harderin gland (Har) and jugal (Jug) and lacrimal (La) bones were exposed. Circle dashed lines indicate the eyeball position. (**H**) Isolated eyeball viewed from inside after dissection completed. Arrow points to optic nerve (On). E = Eye, Na = Nasal, Pre = Premaxilla, Max = Maxillary, La = Lacrimal, Fron = Frontal, Ali = Alisphenoid, Jug = Jugal, Pal = Palatine, Ptery = Pterygoid, Squ = Squamosal, Par = Parietal, Eth = Ethmoid.

Figure 2: H&E histology of the ocular surface tissue at postnatal 4-week old. (**A**) Eyelid, conjunctiva and cornea are visualized in one tissue section. Boxed areas of "b" and "c" were magnified in (B) and (C), respectively. (**B**) The cornea epithelium, stroma and endothelium. (**C**) The conjunctiva with goblet cells. ce = cornea epithelium, st = stroma, ed = endothelium, gc = goblet cell.

Figure 3: H&E histology of the ocular surface tissue at postnatal day 10. (**A**) Intact eyeball at P10. Boxed areas of "b" and "c" were magnified in (B) and (C), respectively. (**B**) The cornea. (**C**) The conjunctiva. Ce = cornea epithelium, st = stroma, ed = endothelium, cj = conjunctiva.

Figure 4: Immunostaining of the ocular surface from P5 to P15. Square-boxed area in each panel was magnified in their right. (**A–F**) Keratin 12 was specifically expressed in the cornea epithelium (arrows). (**G-L**) The keratin 14 expressed in the ocular surface. ac = anterior chamber, pc = palpebral conjunctiva. This figure was modified from a previous publication Guo et al.[14].

DISCUSSION:

One critical reminder for preparation of the intact eyeball is that all orbital bones must be removed completely, especially the juga and lacrimal bones, which are small and located near the bottom of eye socket. Any left-over bones may complicate the ensuing histology. In case a tiny piece of bone was not completely removed from dissection by accident, it may be picked out from the embedding paraffin block using a pair of sharp tweezers. The hole left behind should be filled with melted paraffin after this operation.

Additional caution should be exercised when handling the lens. The broken pieces of lens are usually scattered through the whole section. This will considerably affect microscopy examination and image analysis. Attempts to remove lens would damage either the ocular surface (if the operation was conducted from facial side) or the retina (if the operation was from the optic disc side of the retina). An alternative way to reduce the negative impact of the presence of lens tissue without dissection is to change sectioning directions. For instance, to save

the ocular surface morphology, sectioning of the paraffin block should be performed from anterior of the eyeball to the posterior, otherwise, from the opposite direction.

A useful note to make for the PFA-fixed paraffin section is that antigen retrieval is usually necessary for immunostaining such as using K12 and K14 antibodies. However, there are many exceptions that antigen retrieval would generate non-specific signals increasing the background staining. Thus, one should be cautious when using antigen retrieval technology, and should test antibodies in pilot experiments if possible, beforehand.

In general, there are two methods for isolation of the eyeball from the skull/orbital bones: (i) directly dissect the eyeball from facial side; and (ii) remove the inside skull/orbital bones to free the eyeball. The challenge for the first method is that eyeball sinks deep into the orbital socket with a narrow space in between for dissection. Furthermore, the movable eyeball would stretch the conjunctiva epithelium at any given point when the dissection tools touch it, creating unintentional damages. By contrast, the second method begins with dissection of skull/orbital bones, which are farther from the eyeball and associated ocular surface tissue, thus reducing the risk of damaging the conjunctiva. Moreover, there are no spatial constrictions for performing dissections. Even though the first method can work if great cautions were taken, the second one is definitely easier and better.

In summary, we have described a method for isolation of mouse eyeball with intact eyelid and associated ocular surface from the postnatal mice. The described protocol is suitable for isolation of the eyeball with intact eyelid and associated ocular tissues in postnatal mice. The protocol is especially useful when integrity of the OS or entire ocular tissues is required. We have used this method to prepare tissue samples for examination of keratin markers in OS under normal and pathological conditions. We conclude that the ocular tissues from such preparations are particularly helpful for looking at differential regulations of a protein in different parts of the OS on a single section.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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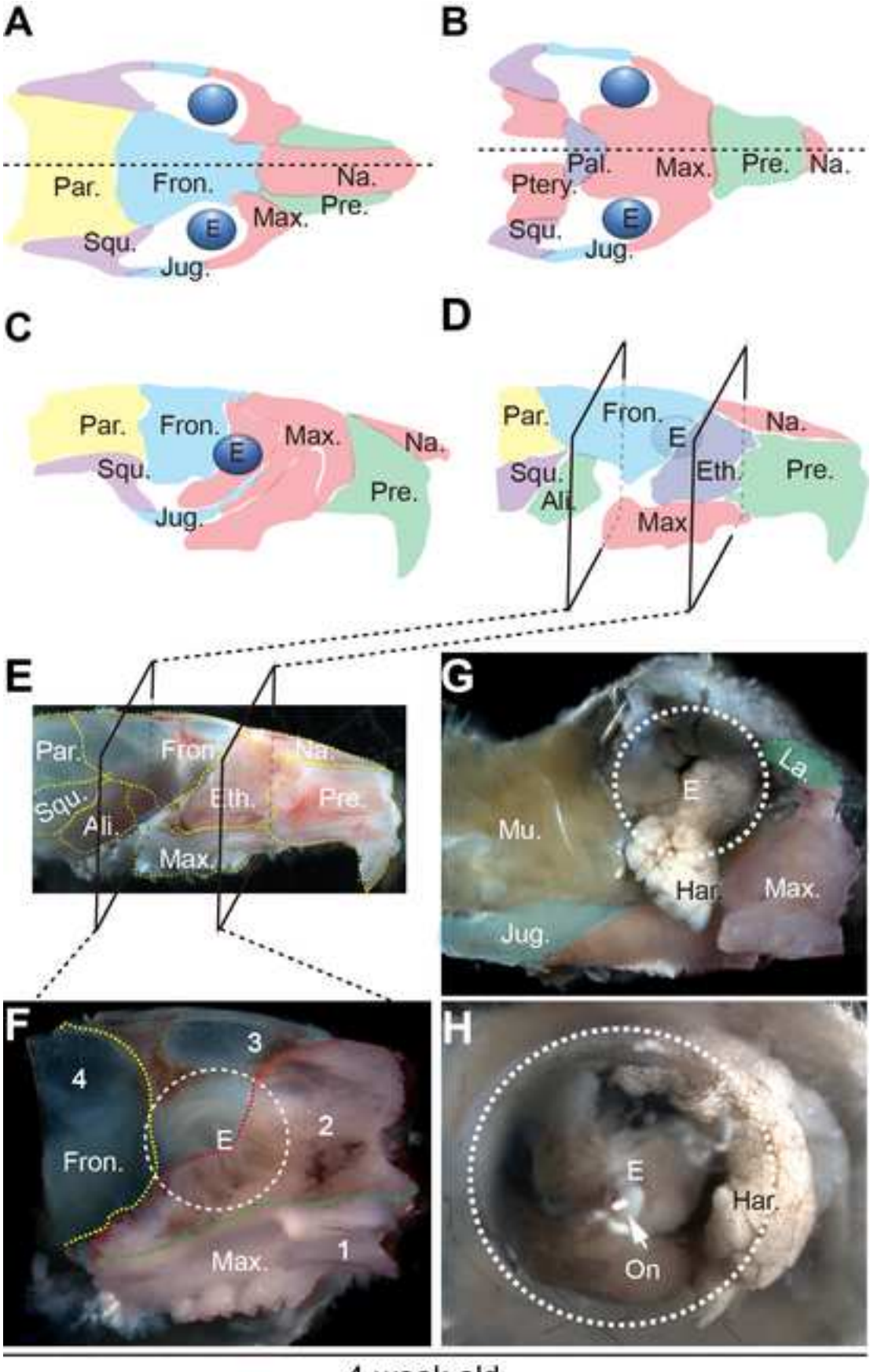

Figure 1

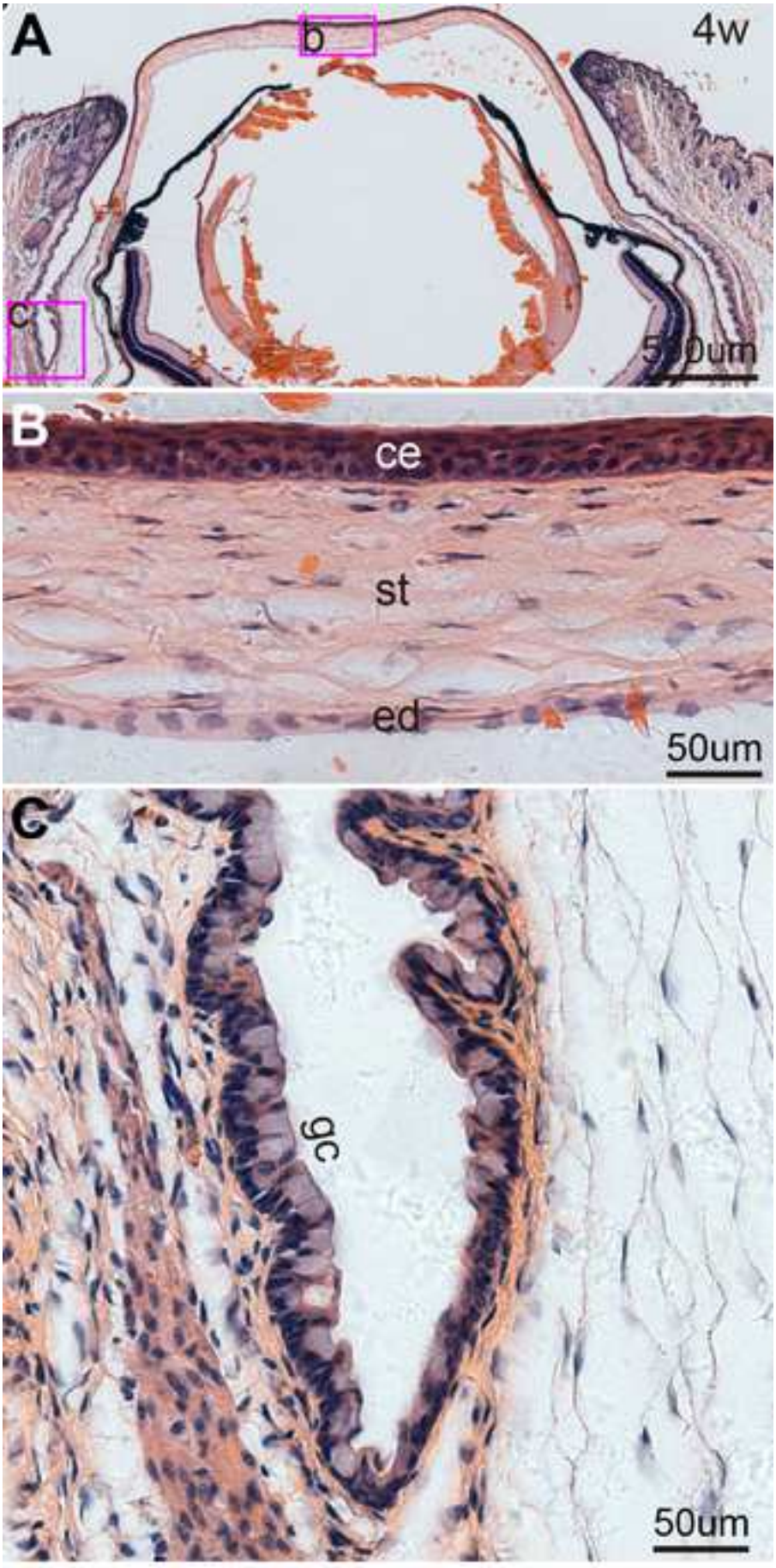

Figure 2

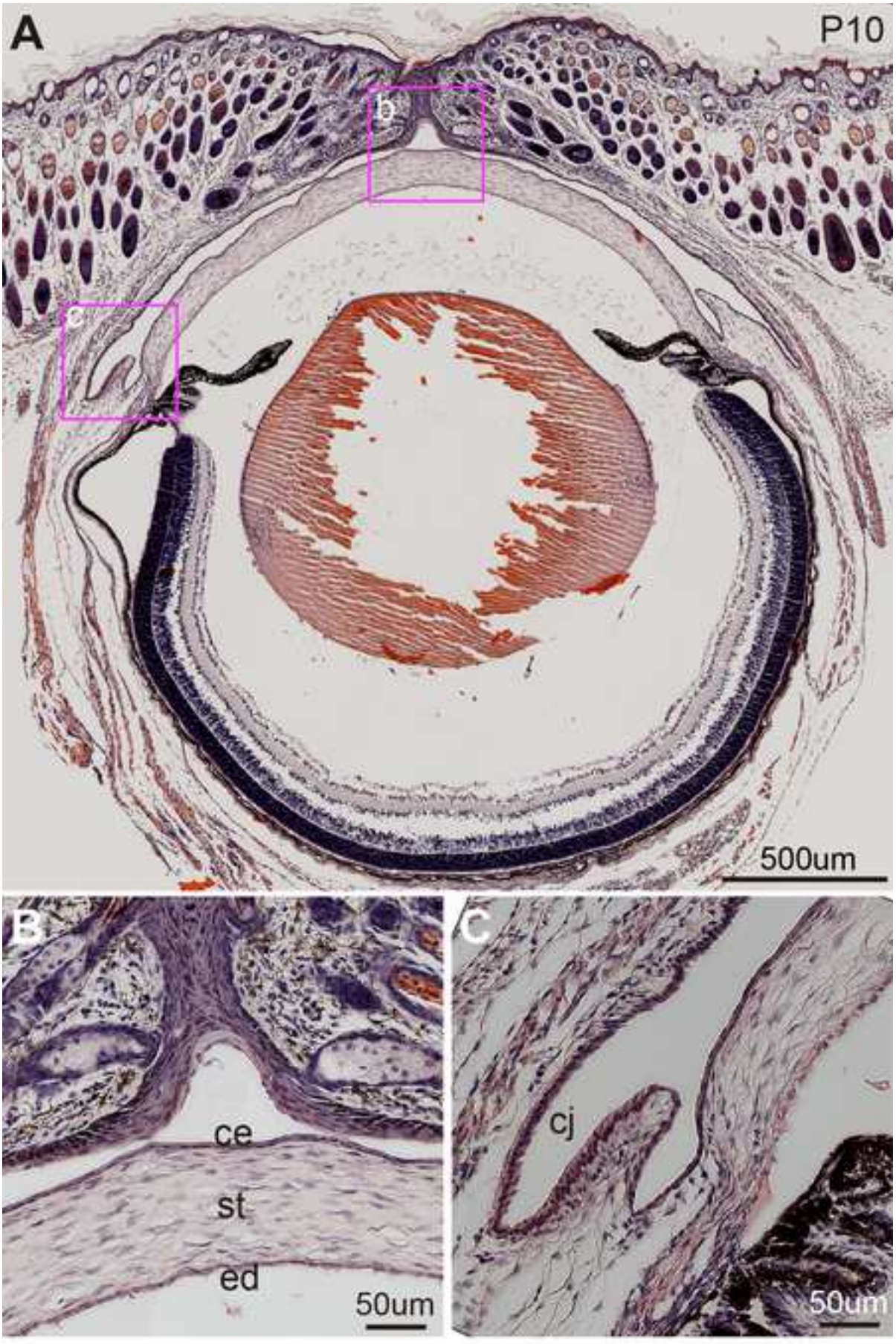

Figure 3

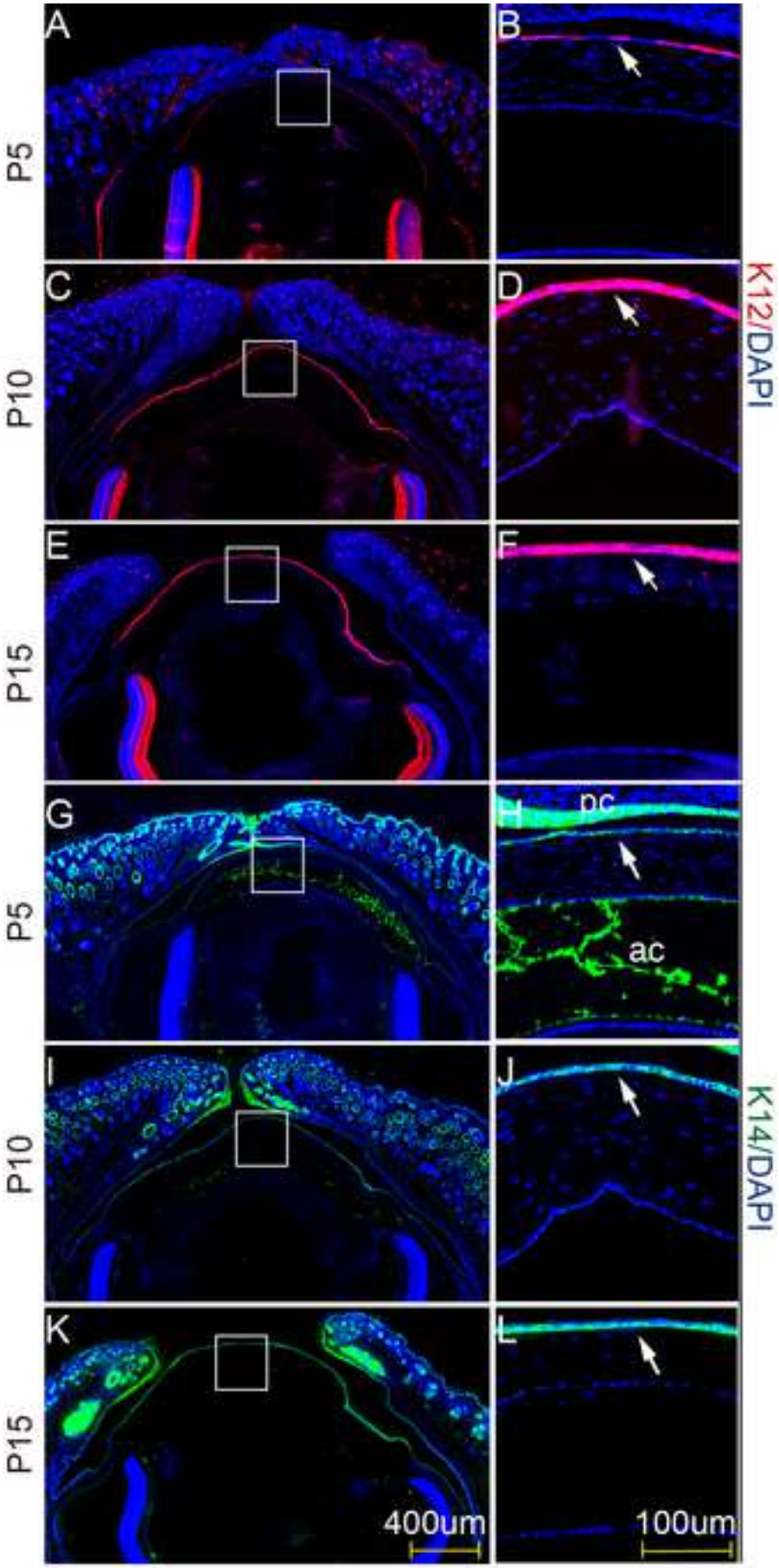

Figure 4

Name of Material/ Equipment	Company	Catalog Number
1× Phosphate buffered saline (PBS)	Transgen Biotech	FG701-01
50ml centrifuge tube	Corning	430829
Adhesive microscope slides	Various	
Alexa Fluor 488 Phalloidin	Invitrogen/Life Technologies	A12379
Alexa Fluor 568 Phalloidin	Invitrogen/Life Technologies	A12380
Anti-K12 antibody	Abcam	ab124975
Anti-K14 antibody	Abcam	ab7800
Citric acid	Various	
Cover slide	Various	
Curved forceps	World Precision Instruments	14127
Dissecting microscope.	Olmpus	SZ61
Ethyl alcohol	Various	
Fluorescent Microscope	Zeiss	AxioImager.Z2
Fluoromount-G Mounting media	SouthernBiotech	0100-01
Micro dissecting scissors-straight blade	World Precision Instruments	503242
Microwave ovens	Galanz	P70D20TL-D4
Mouse strains	C57/BL6 and Sv129 mixed	
No.4 straight forceps	World Precision Instruments	501978-6
Normal Goat Serum	Various	
Paraformaldehyde (PFA)	Various	
Tissue culture dish	Various	
Trisodium citrate	Various	
Triton X-100	Sigma-Aldrich	SLBW6818

Comments/Description

Suggested concentration 1:500 - 1,000
Suggested concentration 1:500 - 1,000
Suggested concentration 1:1,000
Suggested concentration 1:800

Prepare a 4% solution in 1× PBS and filter with 0.45µm filter membrane

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Comments are italicized and underlined.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have carefully examined spelling and grammatical errors.

• Protocol Detail:

1) 1.1.1: What is the mouse age exactly?

The postnatal mouse ages are given in section 1.1.1 (line 93)

2) 2.1: Cite references.

A reference is now cited for the relevant statement (line 149)

• Protocol Numbering: *Please add a one-line space between each protocol step.*

Added (line 95, 100, 106, 113, 118, 122, 125, 129, 134, 140).

• Protocol Highlight: *Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.*

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3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Highlighted (line 90~144).

• Discussion: *JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.*

We ensured that the discussion covered relevant details in above categories.

Previous line 208 "we described a method for isolation of mouse eyeball with intact eyelid and associated ocular surface from the postnatal ages" was moved to line 264 of this revision.

Previous line 209~218 (Two ways…..and better) was moved to line 253~262 of this revision.

Previous line 219~237 (one critical….beforehand) was moved to line 234~252 of this revision.

• Figures: *1) Fig 2B, 3B: Add scale bar.*

Scale bars are now added to each panel.

• References: *Please spell out journal names.*

The journal names are now spelled out (line 285~321)..

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We eliminated commercial names in the manuscript and referred them to the "table of materials" (line 93, 167, 173, 180)

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Trademarks were removed from the "table of materials".

• Table of Materials: *Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as animal strain.*

Mouse strains were added to the "table of materials".

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Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

Liu and Guo present a short descriptive paper on a preferred method of removing an intact postnatal mouse eyeball while retaining its ocular surface tissue, including the palpebral and bulbar conjunctiva of the eyelid. While this has not been published in this form previously, there are clearly many alternate ways to achieve this, one of which is presented here as a preferred method by these authors.

We thank the reviewer for this comment. We agree that there are other ways of dissection for this purpose, and this is a way we tried better indeed.

Overall the description of the process is well delivered with only minor grammatical errors and the occasional typographical error, but for most, it flows well and the narrative is clear. There are some minor issues that can readily be addressed and text emended.

We thank the reviewer for the positive comment. We corrected typos and grammatical errors as suggested.

While it is relatively easy to remove (enucleate) an intact eyeball from a postnatal mouse, this does not usually come with the intact eyelid as is required here. This reported method that removes all the surrounding bones to allow easier extraction of the eye is described and the resultant tissue is processed for histology and immunolabeling.

We are thankful to the reviewer for understanding the purport of this protocol.

Major Concerns:

Authors state that lens morphology is hard to keep. While this is very true, there are indeed methods to retain its structural integrity for histology. The authors refer to the lens as rigid and fragile, yet later compare it to bony tissues, that are far from fragile. The lens tissue is no doubt the most problematic of the eye tissues to section for histology, and this comes from its very dense protein composition. Because of this, it allows relatively poor penetrance of fixatives, PBS washes and especially histological reagents such as

xylene and molten wax. While this aspect is not the major thrust of the paper, I will only spend a short period here informing the authors on how best to avoid this histology artefact. When sectioning a postnatal eye, simply dampen the block face with a damp tissue and this will allow the next series of lens sections to come out intact. Once the lens starts to fragment again, reapply damp tissue and repeat as above.
The damp tissue is thought to partially hydrate the lens fiber mass and allow it to section cleanly. Poor preservation of this tissue unfortunately requires this. This step is not as necessary for embryonic or fetal eyes that are better preserved, and sometimes even neonatal eyes are forgiving.

We agree that the comparison of lens to bony tissues may not be appropriate, thus omitted the relevant statement (previous line 225: "which behaves like bony tissue).

We very much appreciate the reviewer for time and patience sharing with us valuable experience for preparation of the lens section. This will be very helpful for our researches.

While the histology validates the presence of the intact tissue, this nominated dissection procedure does not necessarily offer any improvement to the histological staining or immunolabeling procedures.

We agree that the focus of this manuscript is the dissection method, and the histological and immnohistochemical analyses were used to prove its general application.

Line 221. While any left-over bones may complicate the histology leading to a score mark on sections, it would not ruin the immunostaining in any way. This should be corrected or qualified.

We corrected the sentence as: "Any left-over bones may complicate the ensued histology" (line 236).

As a suggestion, have the authors ever considered making a square incision of the facial skin surrounding the eye and then simply by using curved forceps enucleate the whole eye from its socket, with the intact eyelid attached?

We thank the reviewer for the helpful suggestion. We were able to manage dissections in the suggested way. However, tremendous efforts were required to avoid stretches and damages of OS caused by frequently touching the OS and/or eyeballs by the dissection tools.

The first paragraph of the Representative Results is closely repetitive of the Experimental Steps included on Page 4. The results can be re-written in a more concise manner.

We rewrote the first paragraph of the Results section to make it more concise(line 183-193).

Minor Concerns:

When removing all brain tissues, does this impact on the optic nerve, hence eye? Was it necessary to remove all brain tissues or was this simply for aesthetics?

We thank the reviewer pointing out this issue. We managed not to damage the optic nerve by cutting the nerve off the brain prior it reaches optic chiasm. We added description in step 1.1.3 in this revision (line 102-103: “Free the optic nerve by cutting it off the brain prior to the optic chiasm location”). Removal of brain tissue exposes the underneath facial bones, which makes dissection easier.

Also, was it necessary to prefix tissues (despite this only being for a relatively short period). The benefit of this is lost on the reader and it should be explained, if indeed even necessary.

Prefixation offers tissue rigidity making dissection easier. Additionally, prefixation avoids leaving fresh tissue unfixed too long before dissection is complete. We noted in step 1.1.4 (line 110~112).

One would think that any PBS washes to eliminate loose hairs would be done prior to any fixation steps.

We thank the reviewer for the comment. Another important purpose of PBS wash is to eliminate the fixatives in order to proceed further dissection (exposure to fixatives harms health during dissection). We noted this in step 1.2.1(line 116~117).

If the labels/sections in Figure 1E would exactly match those in Figure 1D, this would be more useful.

We replaced Figure 1D & E panels to make them exactly match.

It is not clear what Figures 1G and 1H add. 1G is mostly shown in 1F, and 1H appears to be the close up of any mouse eye.

Figure 1G now becomes Figure 1H offering an inside view of eyeball with associated tissues after dissection completed. We leave out the original Figure 1H agreeing that it does not add more information.

In lines 121-122, the authors should clarify exactly what they mean by trimming the eyeball with attached eyelids and skin, onto squares? Do they mean into squares (or even cubes) etc. Again the purpose of this is lost somewhere in the translation.

We rephrase the sentence as “trim the eyeball with attached eyelids and skin into a small square-shape block to reduce tissue volume and facilitate orienting tissue for later embedding” (line 137~139). We hope this will make it all clear.

While fixed tissues can be retained in PBS at 4˚C for up to a month, if tissue is required to be stored for longer periods, it can readily be stored in 70% ethanol.

This is a very important piece of information, we added in the text (line 143~144).

King Wipes (maybe referring to 'Kim Wipes'), are trade-marked, and should be listed as such. Regardless, reference to facial tissues (and not necessarily any specific brand) can be alternatively mentioned here.

We thank the reviewer for the suggestion, we replace “kim wipes” with “facial tissues” (line 163).

As a suggestion, have the authors ever considered making a square incision of the facial skin surrounding the eye and then simply by using curved forceps enucleate the whole eye from its socket, with the intact eyelid attached?

We already addressed the same question above.

On several occasions the authors refer to the OS sheet being well 'reserved', when I think they are meaning 'preserved".

Thanks for pointing out the wording, we corrected(line 37, 142, 200).

The make-up of the PBST solution is repeated on lines 147 and 150/1.

We omitted the redundant description in step 2.2.6

Authors need to ensure that all Materials and Equipment mentioned in the final table are indeed also mentioned somewhere in the text, at least once.

We examined the text carefully to ensure the materials and equipment in table be mentioned.

Reviewer #2:

Manuscript Summary:
The manuscript is clear and concisely written except few corrections (Please see in the minor concern section). This Protocol can help the scientific community dealing with ocular histology. The dissection protocol seems promising, yielding untouched ocular surface together with the eyeball and eyelids, minimizing the physical damages that is mainly caused by other dissection techniques.

We thank the reviewer for endorsing the manuscript.

Major Concerns:
NIL

Minor Concerns:

1. At line 103- postnatal day (P) 0 to P7 is P0 to P7 ? Please correct.

Corrected (line 108).

2. At line 135, 136 & 137- The Low and High power microwave is not clear. Please give details in Watts of the Low and High power of the microwave. It can be found in the user

manual of the Microwave. This will help others to replicate the protocol easily as the microwave come with different watts (1000, 700, 500 etc) and it will be easier to standardize.

We standardized the microwave power (120 watts) in the text as suggested (line 157).

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Ocular surface pathogenesis associated with precocious eyelid opening and necrotic autologous tissue in mouse with disruption of *Prickle 1* gene

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ABSTRACT

Ocular surface disease is one major type of eye diseases. Different etiologies trigger distinct pathological responses of the ocular surface. We previously reported that genetically engineered mice with ablation of *Prickle 1* manifested precocious eyelid opening with ensuing cornea dysplasia. The current study aimed to characterize the molecular traits and the direct cause of ocular pathology associated with precocious eyelid opening in the *Prickle 1* mutant mouse. *Prickle 1* mutant mice exhibited a slew of ocular surface pathology including cell proliferation, cell fate transformation and inflammatory infiltration coinciding with the timing of the precocious eyelid opening. Forced eyelid opening in wild type mice did not induce cornea pathology comparable to that of the *Prickle 1* mutants. Necrotic tissue debris was found associated with the lesioned cornea. RNAseq analysis of the mutant cornea revealed an expression profile shared by a range of dermatological diseases involving immune responses and cancer. Taken together, the data suggest that the necrotic eyelid debris plays an important role in ocular pathogenesis of the *Prickle 1* mutant mouse, which may represent a type of non-infectious keratoconjunctivitis caused by damaged autologous tissues. Additionally, *Prickle* 1 mutant cornea pathogenesis may offer molecular insights into other types of epithelial pathogenesis.

1. Introduction

Ocular surface disorders can be caused by a variety of foreign stimuli including non-infectious atopic and immunopathic agents and immunogenic damaged tissues, and infectious microbial pathogens. The ocular surface pathology is often manifested as keratitis, conjunctivitis or both (keratoconjunctivitis), which may also be accompanied with hyperplasia, metaplasia, or squamous neoplasia with abnormal growth of the ocular surface epithelium (http://eyewiki.aao.org/Ocular_Surface_Squamous_neoplasia).

The eyelids in conjunction with the ocular surface play vital roles in maintenance of the normal eye function. The outer surface of the eyelid is keratinized skin tissue, a physical barrier against mechanical injuries and microbial invasions. The inner lining is conjunctival epithelium harboring goblet cells producing mucins of the inner layer of the tear film over the cornea. In between is the eyelid pocket comprising the Meibomian gland, continuously secreting lipid components forming the outer layer of the tear film, and the retractors and nerves responsible for eyelid blinking to refresh otherwise dry or irritants-contaminated tear film. Additionally, resident and bone marrow-derived immune cells in the eyelid serve as sentinels constantly performing surveillance of foreign pathogens and irritants (Foulsham et al., 2018; Suzuki et al., 2015; Ueta and Kinoshita, 2010, 2012).

In human embryonic development, the eyelid starts to form about 40–45 days of gestation and is completely closed about 15 days later. Eyelid does not reopen until seven months of fetal life (Pearson, 1980; Sevel, 1988). The closed eyelid during this period is thought to create an isolated environment from potentially toxic amniotic fluid to ensure proper development of the ocular surface (Sevel, 1988; Zieske, 2004). In rodents such as the mouse, eyelid remains closed postnatally for a couple of weeks. Despite this difference, the closed eyelid is considered to have similar protective function as in humans for the developing ocular surface (Zieske, 2004). A series of changes in ocular surface epithelial differentiation were correlated with the timing of postnatal eyelid reopening (Chung et al., 1992; Watanabe et al., 1993; Zieske, 2004), suggesting that ithe integrity of eyelid might be crucial for maturation of the ocular surface differentiation. Nevertheless, the essence of the postnatal on-time opening of the eyelid in mouse in regard

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