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A custom Multiphoton Microscopy Platform for Live Imaging of Mouse Cornea and Conjunctiva

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Dear editor Nandita Singh,

We are submitting our manuscript entitled “Live Imaging of Mouse Cornea and Conjunctiva by a Multiphoton Microscopic Platform” for consideration of publication as an original research article in *Journal of Visualized Experiments*.

Corneal study using conventional tissue sectioning/staining and *ex vivo* cell culture is often unable to represent the *in vivo* physiological and pathological changes completely. An intravital imaging system capable of monitoring of cellular and extracellular changes of cornea and conjunctiva will be able to provide complementary information. With the advantages of non-invasiveness, minimal photodamage and deep imaging depths, multiphoton microscope has gained popularity in investigating cell dynamics *in vivo*. Although we (1-5) and other groups (6-7) have demonstrated multiphoton microscope as an effective tool for visualizing corneal structures, *in vivo* continuous monitoring across a longer period of time for studying cell dynamics in animal models remains challenging due to the lack of a suitable imaging platform. In this work, we describe the detailed technical protocol step-by-step for *in vivo* imaging of ocular surface using our imaging platform in dual fluorescent transgenic mice. By using transgenic mice with fluorescent protein labeling of distinct cellular compartments and cell populations, we showed that this platform enables visualization of distinct cells and structures from corneal epithelium to the corneal endothelium. This transgenic mouse line enabled us to investigate the cell dynamics of capillaries in the future, which is key to pathological corneal neovascularization. In addition, this *in vivo* imaging platform can also be used to explore immune responses in corneal pathologies by fluorescent labeling of different immune cells. In brief, we demonstrated a multiphoton microscopic stereotaxic platform for longitudinal 4-dimensional *in vivo* imaging of ocular surface with subcellular resolution.

We think this imaging platform will enable the application of multiphoton microscope to the 4-dimensional dissection of cellular dynamics and extracellular matrix structures in physiological and pathological changes and this work is of broad interest to the readers of *Journal of Visualized Experiments*.

As corresponding authors, we confirm that

1. The data in the manuscript is original and that the paper is not under consideration elsewhere.
2. None of its content has been published.
3. All authors have read and approved the version of the manuscript, its content, and its submission to *Journal of Visualized Experiments*.

Sincerely,

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TITLE:

A Custom Multiphoton Microscopy Platform for Live Imaging of Mouse Cornea and Conjunctiva

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KEYWORDS:

cornea, conjunctiva, ocular surface, multiphoton microscopy, MPM, intravital imaging, transgenic mouse

SUMMARY:

Presented here is a multiphoton microscopic platform for live mouse ocular surface imaging. Fluorescent transgenic mouse enables the visualization of cell nuclei, cell membranes, nerve fibers and capillaries within the ocular surface. Non-linear second harmonic generation signals derived from collagenous structures provide label-free imaging for stromal architectures.

ABSTRACT:

Conventional histological analysis and cell culture systems are insufficient to simulate in vivo physiological and pathological dynamics completely. Multiphoton microscopy (MPM) has become one of the most popular imaging modalities for biomedical study at cellular levels in vivo, advantages include high resolution, deep tissue penetration and minimal phototoxicity. We have designed an MPM imaging platform with a customized mouse eye holder and a stereotaxic stage for imaging ocular surface in vivo. Dual fluorescent protein reporter mouse enables visualization

of cell nuclei, cell membranes, nerve fibers, and capillaries within the ocular surface. In addition to multiphoton fluorescence signals, acquiring second harmonic generation (SHG) simultaneously allows for the characterization of collagenous stromal architecture. This platform can be employed for intravital imaging with accurate positioning across the entire ocular surface, including cornea and conjunctiva.

INTRODUCTION:

The ocular surface structures, including the cornea and conjunctiva, protect other deeper ocular tissues from external disturbances. The cornea, the transparent front part of the eye, functions both as a refractive lens for directing light into the eye and as a protective barrier. Corneal epithelium is the outermost layer of the cornea and consists of distinct layers of superficial cells, wing cells and basal cells. Corneal stroma is composed of sophisticatedly packed collagenous lamellae embedded with keratocytes. Corneal endothelium, a single layer of flat hexagonal cells, has an important role in maintaining the transparency of cornea by keeping corneal stroma in a relatively dehydrated state through its pumping functions[1]. Limbus forms the border between the cornea and the conjunctiva, and is the reservoir of corneal epithelial stem cells[2]. The highly vascularized conjunctiva helps to lubricate the eyes by producing mucus and tears[3].

Cell dynamics of the corneal surface structures are conventionally studied by either histological analysis or in vitro cell culture, which might not adequately simulate the in vivo cell dynamics. A non-invasive live imaging approach can, therefore, bridge such the gap. Due to its advantages, which include high resolution, minimal photodamage and deeper imaging depth, MPM has become a powerful modality in diverse areas of biological research[4-8]. For corneal imaging, MPM provides cellular information from intrinsic autofluorescence derived from the intracellular NAD(P)H. Second harmonic generation (SHG) signals derived from the non-centrosymmetric type I collagen fibers under femtosecond laser scanning provides collagenous stromal structures without additional staining procedures[9]. Previously, we and other groups have exploited MPM for imaging of animal and human corneas[9-15].

Transgenic mouse lines exhibiting fluorescent proteins in specific cell populations have been widely used for various studies in cell biology, including development, tissue homeostasis, tissue regeneration, and carcinogenesis. We used transgenic mouse strains labeled with fluorescent proteins for in vivo imaging of corneas[9,10], hair follicles[10] and epidermis[10] by MPM. The dual fluorescent mouse strain with cell membrane labeled with tdTomato and cell nucleus tagged with EGFP is bred from two mouse strains: R26R-GR (B6;129-Gt (ROSA)26Sor$^{tm1Ytchn/J}$, #021847) [16] and mT-mG (Gt(ROSA26) $^{ACTB\text{-}tdTomato\text{-}EGFP}$, #007676)[17]. R26R-GR transgenic mouse line contains a dual fluorescent protein reporter constructs, including an H2B-EGFP fusion gene and mCherry-GPI anchor signal fusion gene, inserted into the Gt (ROSA)26Sor locus. The mT-mG transgenic strain is a cell membrane-targeted tdTomato and EGFP fluorescent Cre-reporter mice. Prior to Cre recombination, cell membrane protein with tdTomato fluorescence expression is widely present in various cells. This transgenic mouse strain enables us to visualize nuclei-EGFP and membrane with tdTomato without Cre excitation. Two females (R26R-GR$^{+/+}$) and one male (mT-mG$^{+/+}$) transgenic mouse were bred together to produce sufficient mice for experiments. Their offspring with R26R-GR$^{+/-}$;mT-mG$^{+/-}$ genotype, a dual fluorescent mice strain, were used in this study.

Compared with one fluorescent reporter mouse line as previously described[9,10], this dual fluorescent reporter mouse strain provides us with a 50% reduced acquirement of imaging time.

In this work, we describe a detailed technical protocol for in vivo imaging of the ocular surface in a step-by-step manner using our imaging platform and dual fluorescent transgenic mice.

PROTOCOL:

All animal experiments were conducted in accordance with procedures approved by the Institutional Animal Care and Use Committee (IACUC) of the National Taiwan University and Chang Gung Memorial Hospital.

1. Multiphoton microscopy setup

1.1. Build a system based on an upright microscope with water immersion 20x 1.00 NA objective **(Figure 1A)**.

1.2. Use Ti: Sapphire laser (with tunable wavelength) as the excitation source. Set the laser output wavelength at 880 nm for EGFP and 940 nm for tdTomato **(Figure 1A)**.

1.3. Include two dichroic mirrors (495 nm and 580 nm) for the separation of SHG/EGFP and EGFP/tdTomato **(Figure 1A)**. Spectrally separate the SHG signals, EGFP and tdTomato by bandpass filters 434/17nm, 510/84nm and 585/40nm **(Figure 1A)**.

1.4. In order to optimize the image quality and to avoid photobleaching and tissue damage, set the laser power to be about 35 mW for imaging cornea and 50 mW for limbus. Measure the laser power before the laser passes the optical system. The exact laser power on samples is about 8-9 mW. The complete microscopic design is shown in **Figure 1A.**

NOTE: The upper limit of laser power is set to 70 mW to avoid photo-bleaching and tissue damage.

2. Animal preparation for live imaging

2.1. Use 8-12-week-old mice for the experiment. Intramuscularly inject 50-80 mg/kg of tiletamine HCl and zolazepam HCl for general anesthesia. Check for the lack of response to withdrawal reflex by pinching a toe. Sufficient anesthetization is important to allow stable breathing rate monitoring.

NOTE: Mice at the age of 8 weeks or above are recommended because their eyeballs are matured.

2.2. Place the mouse under anesthesia on a heated stage and insert the temperature monitoring probe into the anus.

CAUTION: The probe must be inserted fully into the anal cavity without exposure to the air, to avoid overheating of the heater and induction of heatstroke.

3. Eye holding for live imaging of ocular surface

3.1. For live imaging of the ocular surface, use the custom designed stereotaxic mouse holder consisting of two parts: a head holder to stabilize the head and an eye holder to retract the eyelids and expose the entire ocular surface **(Figure 1B-D)**.

3.2. Insert ear bars into the external auditory meatus and maintain the three-point fixation of the head holder **(Figure 1B,D)**.

3.3. Topically apply a solution of 0.4% oxybuprocaine hydrochloride in saline and leave it for 3 min to anesthetize the ocular surface.

3.4. Ensure the eyeball is protruded by proper manual eyelid retraction. Otherwise, ischemia and bleeding of the eyeball can occur.

3.5. Carefully place a loop of the polyethylene tube of eye holder along the eyelid margin to expose the ocular surface. Stabilize the eyeball with the eye holder composed of a No. 5 Dumont forceps with its tips covered with the loop of polyethylene tube **(Figure 1C,D)**.

3.6. Screw forceps using a knob in distal forceps of eye holder to keep the eyeball stable **(Figure 1D)**.

3.7. Apply an eye gel with the refractive index of 1.338 on the corneal surface as an immersion medium to maintain the moisture of the ocular surface every hour. In addition, regular application of the eye gel every hour avoid clouding in cornea during imaging.

3.8. Rotate the eyeball with the holder that locked on the stepper-motorized stage for imaging across the entire corneal surface from the central cornea to the peripheral region **(Figure 1C,D)**.

CAUTION: Both excess and insufficient amounts of eye gel can impact the quality of images during imaging. Therefore, supplementing eye gel every hour to keep the surface moist regularly is important for imaging.

4. Z-serial image acquisition

NOTE: Set the first and last slide in every stack to reduce the dropping motion artifacts.

4.1. Before taking the images, image the targeting field with a mercury light source.

4.2. Click the symbol of the microscope software to turn on the software.

4.3. Select proper PMT gain and digital gain to visualize the cellular structure in the ocular surface.

4.4. Set the first slide and the last slide to acquire a stack.

4.5. Enter numerical values for image resolution and z-step, e.g., 512 x 512 and 1 µm as z-step.

4.6. Click on the **Start** button to collect z-serial images.

4.7. Acquire live images twice in the same area, first at 880 nm excitation for SHG/EGFP signals collection and second at 940 nm excitation for EGFP/tdTomato signals collection.

NOTE: The combination of two stacks provides 3 channels images. The image resolution and scan format size were 512 x 512 pixels and 157 µm x157 µm, respectively.

5. Image processing and 3D reconstruction

5.1. Load the z-serial images into Fiji software[18].

5.2. Select the plugin **Median 3D filter** in Fiji to reduce background noises.

5.3. Select the Package **Unsharp Mask filter** in Fiji to sharpen the images.

5.4. Click "**Auto**" in brightness/contrast to automatically optimize the quality of images.

5.5. Save the images as image sequences to be able to export the z-serial images.

5.6. Load z-serial images into commercial software (e.g., Avizo lite) for 3D reconstruction using volume rendering.

5.7. In all MPM images, present EGFP, tdTomato, and SHG signals in pseudo-green, red, and cyan color respectively.

5.8. Capture 3D structure pictures by the snapshot.

REPRESENTATIVE RESULTS:

Using this live imaging platform, the mouse ocular surface can be visualized at cellular levels. To visualize individual single cells in the ocular surface, we employed the dual fluorescent transgenic mice with EGFP expressed in the nucleus and tdTomato expressed in the cell membrane. The collagen-rich corneal stroma was highlighted by SHG signals.

In corneal epithelium, superficial cells, wing cells and basal cells (**Figure 2**) were visualized. In the dual fluorescent transgenic mice, we were able to map single cells from the basal layer to the superficial layers in both corneal and limbal epithelium (**Figure 2**). The hexagonal superficial cells were observed (white arrowhead in **Figure 2**). Both nuclear size and internuclear spacing from

the basal layer toward the outer layers increased in corneal epithelium (**Figure 2**). The cytoplasmic signals of tdTomato fluorescence indicated that the membrane protein-rich intracellular vesicular system, including the Golgi apparatus, endoplasmic reticulum, were scattered in the wing cells (**Figure 2**).

Within the collagenous stroma, the stellate-shaped keratocytes were outlined by the membrane-targeting tdTomato fluorescence in dual fluorescent transgenic mice (yellow arrowhead in **Figure 3** and **Figure 4**). The keratocytes embedded in the collagen stroma were more loosely spaced than the endothelial cells. In addition, thin branching nerves in corneal stroma were also visualized by membrane-targeting tdTomato signals (white arrowhead in **Figure 3**). Monolayer of corneal endothelial cells showed a relatively homogenous hexagonal shape connected into a honeycombed pattern (white arrowhead in **Figure 4**). The limbal epithelium consisted of 1–2 layers of epithelial cells **(Figure 5).** The dual fluorescent reporter transgenic mouse strain also enabled us to image the capillaries within conjunctiva **(Figure 6A).** 3D architecture of capillaries was reconstructed by outlining vascular endothelium **(Figure 6B,6C)**.

FIGURE AND TABLE LEGENDS:

Figure 1: The setup of MPM and rotating mouse holder. (**A**) The setup of the MPM. (**B**) The design of the mouse holder. The mouse holder consists of a rotating head holder and an eye holder. (**C**) The design of the mouse eye holder. The eye holder retracts eyelids by a plastic loop to expose the cornea and conjunctiva. The head holder and eye holder are screwed together (B) on the stage to allow precise and controllable rotation and imaging of ocular surface. (**D**) Photograph of live mouse for corneal imaging with eye holder.

Figure 2: Live imaging of corneal epithelium in dual fluorescent transgenic mice. Corneal epithelium was imaged layer-by-layer, to include superficial cells, wing cells, and basal cells. Superficial cells are marked with a white arrowhead. Scale bar = 50 μm. (Z = depth from the top surface of epithelium (μm)).

Figure 3: Live imaging of the corneal stroma. Within the corneal stroma, the keratocytes (yellow arrowhead) and the nerve fibers (white arrowhead) were embedded in the collagenous stroma (in pseudo-blue color). Scale bar = 50 μm.

Figure 4: Live imaging of corneal endothelium in central cornea. Monolayer of hexagonal corneal endothelial cells (white arrowhead). Scale bar = 50 μm.

Figure 5: Live imaging of limbal epithelium. Live images of the limbal epithelium in dual fluorescent transgenic mice showed vacuolated nuclei. Scale bar = 50 μm.

Figure 6: Live imaging and the three-dimensional reconstruction of vascular network in conjunctiva. (**A**) Capillaries in conjunctival stroma were visualized. Scale bar = 50 μm. **(B)** 3D reconstruction of capillary networks performed using a commercial software. Scale bar = 50 μm. (**C,D**) Magnified 3D views of the image shown in panel B. Fluorescent vascular endothelial cells

outlined the capillaries (white and yellow arrowheads). Scale bar for panel C = 6.26 µm and for panel D = 10 µm.

DISCUSSION:

This custom-built MPM imaging platform with a control software was used for intravital imaging of mouse epithelial organs, including skin[10], hair follicle[10] and ocular surface[9,10] (**Figure 1A**). The custom-built system was used for its flexibility in changing the optical components for various experiments, since the beginning of our project. This imaging methodology is versatile for commercial MPM products. This protocol describes a detailed method for intravital imaging of the mouse ocular surface by MPM imaging platform. Using a stereotaxic mouse holder (**Figure 1B**), we were able to visualize different regions across the entire ocular surface. The accurate positioning capability makes it possible to monitor temporal cell dynamic changes in a specified region.

Although several studies of murine corneal MPM imaging have been reported in vivo[19-22], there are several technical limitations which one needs to overcome to achieve continuous imaging over a long period e.g., holding the eyeball in a stable position, decreasing motion artifacts during imaging and acquiring the images for a long period without photobleaching. Compared to the plastic eyecup designed for corneal imaging in vivo with relatively limited access to the eye surface[20], our eye holder not only keeps the eyeball fully exposed but also makes the entire ocular surface accessible to the objective lens (**Figure 1C**).

Although vital dyes or probes are routinely used as fluorescent reporters to label cells[20-22], the invasiveness of needle injection may disrupt the natural cell dynamics of organs during the physiological homeostatic state or tissue regeneration process. R26R-GR transgenic mice strain expresses stable and bright green fluorescent protein in the cell nucleus and has been used for the visualization of the cell dynamics of muscle progenitors in ghost fibers[23]. mT-mG reporter transgenic mouse line was used to analyze the differentiation of epidermal cells in homeostasis[24]. Compared with a single reporter fluorescent transgenic mouse line, this dual fluorescent transgenic mouse strain reduced the time of image acquisition to half by simultaneously providing the cell structure information of both cell nuclei and cell membranes. By rotating the mouse holder, flat nuclei of both endothelial and blood cells were visualized within the vascular lumen using dual fluorescent transgenic mouse strain **(Figure 6)**. Therefore, this transgenic mouse line can be used to investigate the cell dynamics of capillaries in the future, which is the key to the pathological corneal neovascularization. In addition, this in vivo imaging platform can also be used to explore immune responses in corneal pathologies by fluorescent labeling of different immune cells, such as neutrophils[25], langerhans cells[26], regulatory T cells (Tregs)[27] and mast cells[28].

In conclusion, we demonstrated a powerful intravital MPM imaging platform for mouse ocular surface study. The combination of live MPM stereotaxic stage and special fluorescent transgenic mice enables real-time visualization of distinct cellular and extracellular structures in the ocular surface, including cornea, limbus and conjunctiva. This intravital imaging platform can help to explore the cell dynamics of the ocular surface and, with further development, can be potentially

used for ophthalmic drug screening in the future.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

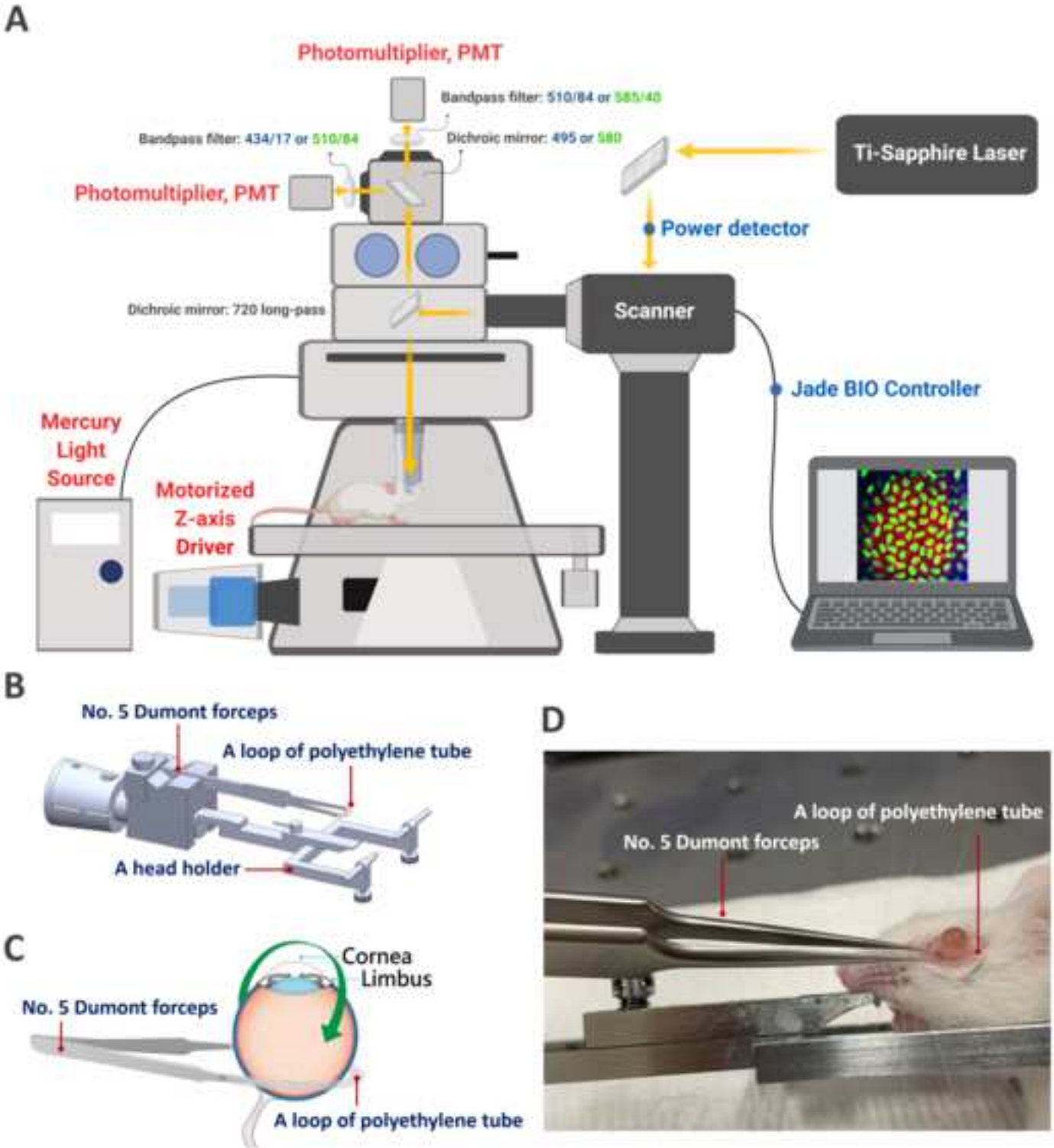

Figure 2

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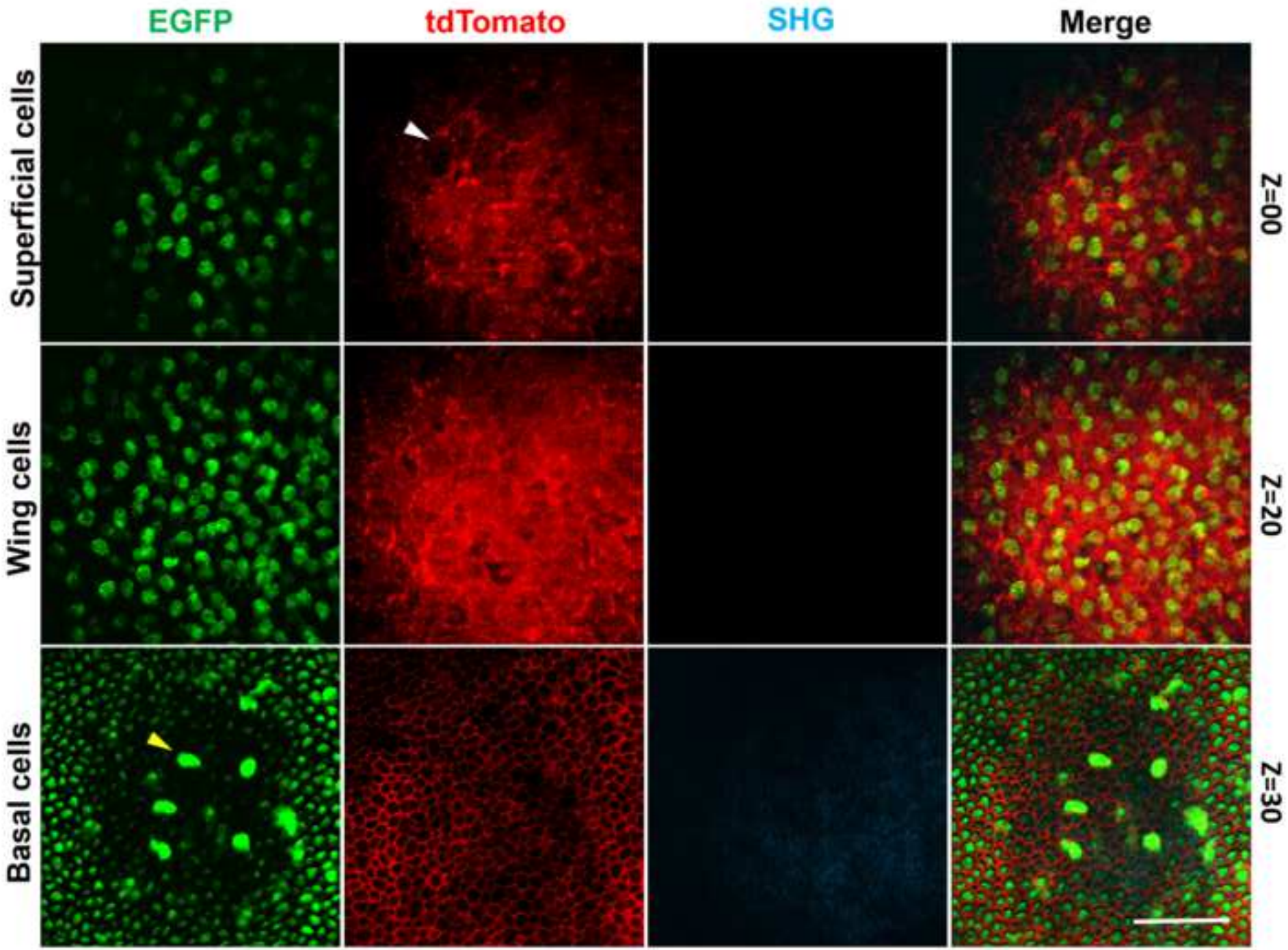

Figure 3

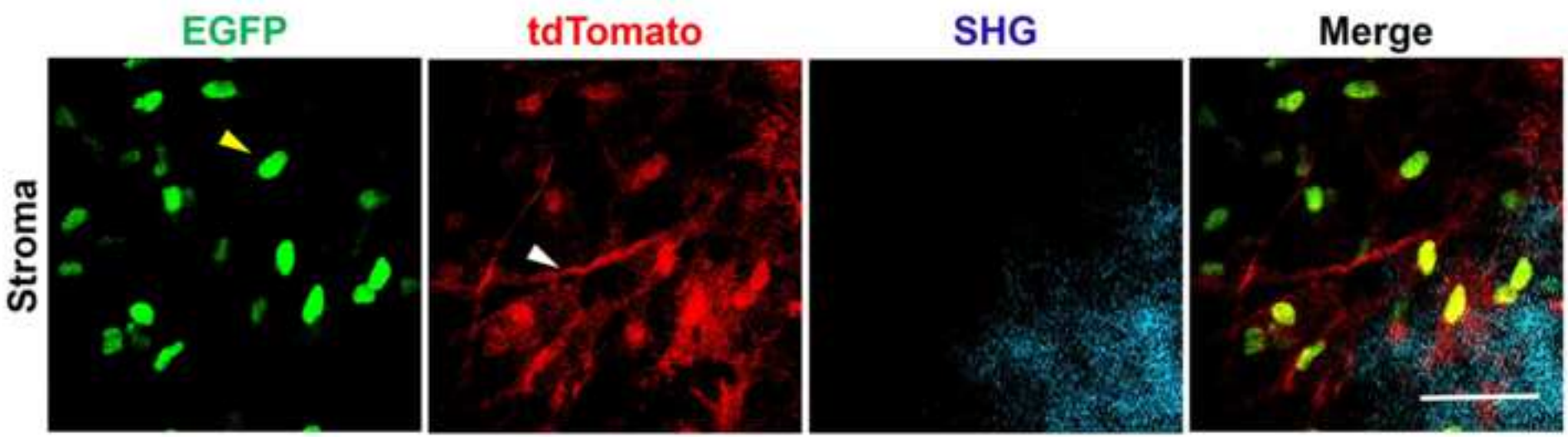

Figure 4

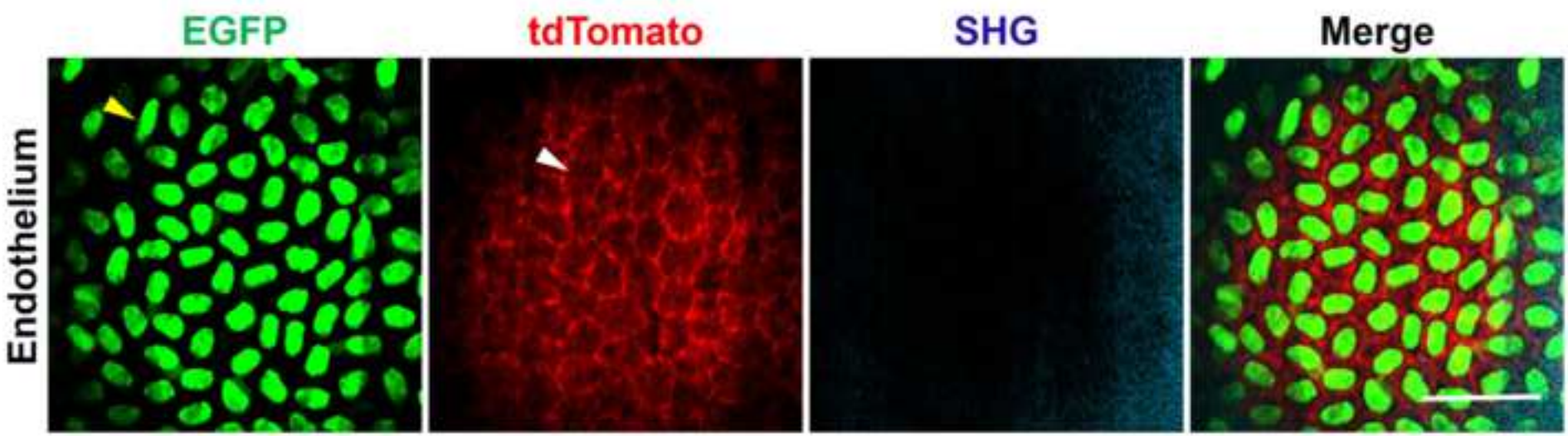

Figure 5

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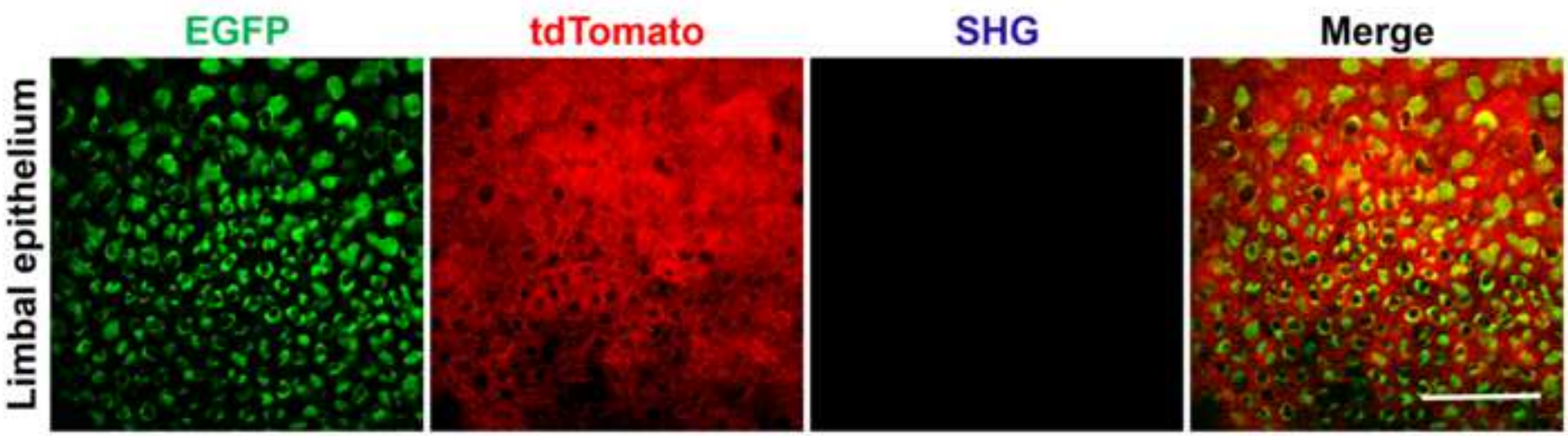
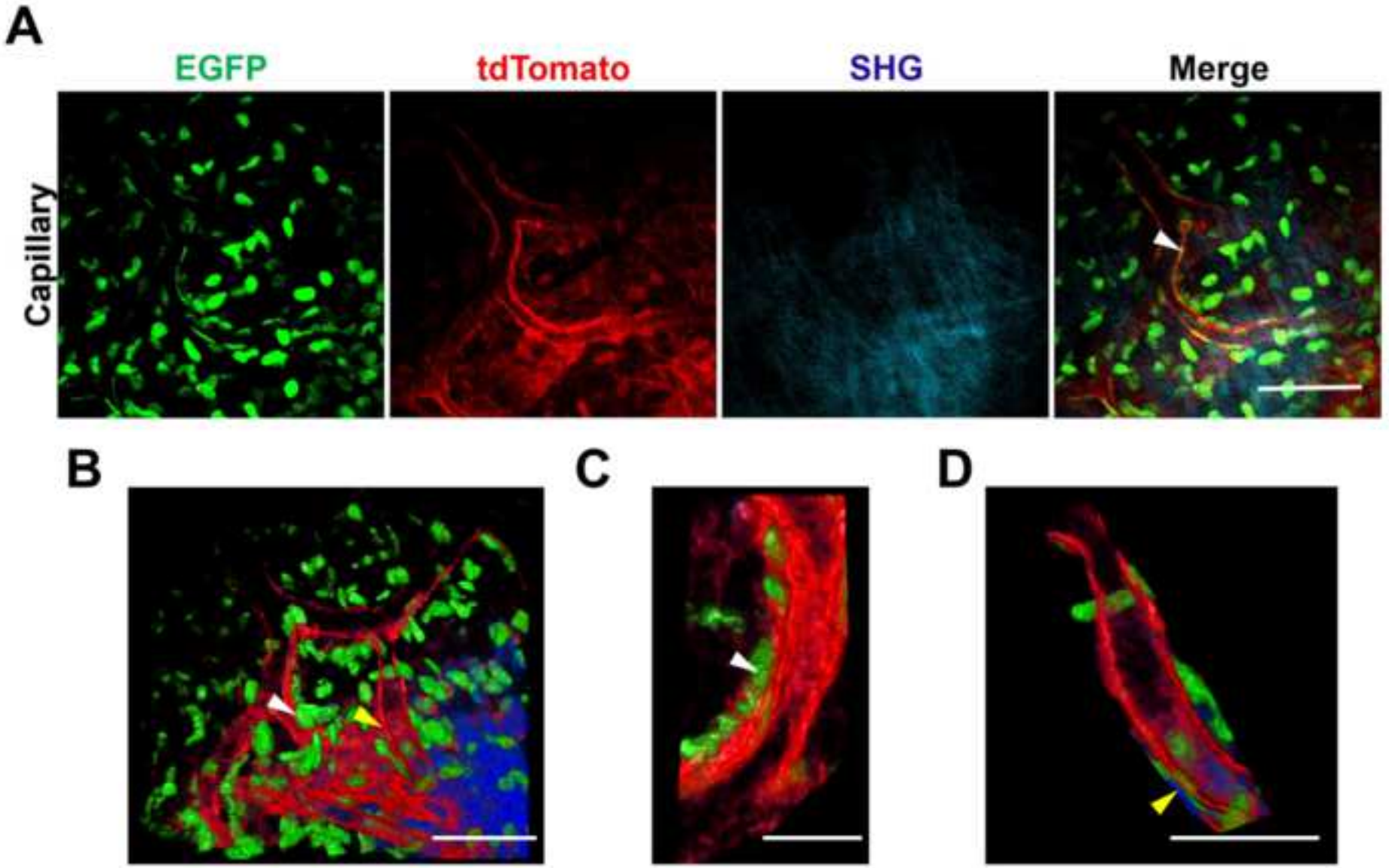


Figure 6



Table

Name of Material/Equipment	Company	Catalog Number
AVIZO Lite software	Thermo Fisher Scientific	
Bandpass filters	Semrock	FF01-434/17 FF01-500/24 FF01-585/40
Dichroic mirrors	Semrock	FF495-Di01-25x36 FF580-Di01-25x36
Galvano	Thorlabs	GVS002
Jade BIO control software	SouthPort Corporation	Jade BIO
Oxybuprocaine hydrochloride	Sigma	O0270000
PMT	Hamamatsu	H7422A-40
Polyesthylene Tube	BECTON DICKINSON	427401
Stereotaxic mouse holder	Step Technology Co.,Ltd	000111
Ti: Sapphire laser	Spectra-Physics	Mai-Tai DeepSee
Upright microscopy	Olympus	BX51WI
Vidisic Gel	Dr. Gerhard Mann Chem-pharm. Fabrik GmbH	D13581
Zoletil	Virbac	VR-2831

Comments/Description
Version: 2019.3.0

Dear Editor and Reviewers:

Thank you for your letter and the reviewer's comments concerning our manuscript entitled: A novel Multiphoton Microscopy Platform for Live Imaging of Mouse Cornea and Conjunctiva. Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our research. We have studied comments carefully and have made the correction which we hope to meet with approval. **The modification is marked in red.** The main corrections in the paper and the response to the editor's and reviewer's comments are as following:

Point-by-point responses

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.

The manuscript is carefully checked.

2. Please revise lines 172-174, 197-199, and 242-244 to avoid textual overlap with previously published work.

The sentence is corrected.

3. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

The personal pronouns are removed from the protocol text in the manuscript.

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. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names.

Examples of commercial sounding language in your manuscript are: Mai-Tai DeepSee, Tsunami oscillator, Zoletil, Jade BIO, etc.
The commercial language, including Mai-Tai DeepSee, Tsunami oscillator, Zoletil, Jade BIO, are removed from the protocol text in the manuscript.

5. Everything in the protocol (except for the introductory ethics statement) should be in a numbered step (in the imperative tense and with no more than 4 sentences), numbered header, or a "NOTE". Please move the introductory paragraphs of the protocol (e.g., 90-103, 107-109, 118-123, 194-201) to the Introduction, Results, or Discussion (as appropriate) or break into steps.
The protocol text is re-written.

6. Section 2: The Protocol should contain only action items that direct the reader to do something. Please move this section to the Introduction.
The information about transgenic mouse strain is moved to the Introduction section. (line 84)

7. Section 3, 4.6: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "NOTE".
This section is re-written.

8. 5.2-5.4, 6.1-6.6: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g., button clicks or menu selections for software actions, numerical values for settings, etc.).
The sentence is corrected. (line 183-216)

9. Please remove the embedded figure(s) from the manuscript.
The figures are removed from the manuscript.

10. Figures: Please reference each figure panel in the manuscript. For figures showing the experimental set-up (Figure 1), please reference them in the Protocol.
The figure panels are referenced in the Protocol.

11. References: Please do not abbreviate journal titles; use full journal name.

The full journal name is updated in the manuscript.

12. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please remove any ™/®/© symbols and sort the materials alphabetically by material name.

The Table of Materials is corrected.

Reviewers' comments:

Reviewer #1:

Major Concerns:

1) I am not convinced that the entire imaging system is "novel". There are several laboratories also have similar multiphoton imaging system for eye imaging. What is novel, however, is the eye holder. Therefore, I would recommend refraining from using the word "novel" from the title, or may be replace "novel" with "custom"?

The title changes into A custom Multiphoton Microscopy Platform for Live Imaging of Mouse Cornea and Conjunctiva (line 2)

2) The manuscript needed to be edited by some English proofreading services. There are many places in the manuscript that the meaning is unclear, grammatically incorrect, or may even lead to misunderstanding.

For instance,

a. Page 3, line 52: "Dual fluorescent transgenic mice enable us to visualize cell nuclei, cell membranes, nerve fibers and capillaries on the ocular surface." The expression "dual fluorescent transgenic mice" is strange, and maybe it should be reworded as "Transgenic mice with dual fluorescent labels"? "ON the ocular surface" - how can the cell nuclei etc. can be visualized ON the ocular surface? Should it be "within the ocular structure"?

The sentence is corrected. (line 51)

b. Page 3, line 59: "The ocular surface structures, including the cornea and conjunctiva, protect other deeper ocular tissues from external impacts." "Impacts" is too strong of a word; I guess "intrusions" or may be even "disturbances" is a better choice of words?

The sentence is corrected. (line 60)

c. Page 4, line 131: "To regularly monitor a stable breathing rate is an important step for adequate anesthetization." Is grammatically incorrect, thus the true meaning of the sentence is unclear. May be the sentence can be rewritten as "Sufficient anesthetization is important to allow stable breathing rate monitoring"?
The sentence is corrected. (line 131)

d. Page 5, line 146: "a designed stereotaxic mouse holder" should be "a custom designed stereotaxic mouse holder".
The sentence is corrected. (line 147)

e. There are many little things like these, and I think the authors should pay some time and attentions to revise these expressions to improve readability of the paper, and more importantly avoid confusions in understanding the meanings.
Thank you for your treasured opinions.

Reviewer #3:
Minor Concerns:
Figure 1 A: The orientation of the upper dichroic mirror (not filter!) is wrong. The lower dichroic mirror is not labelled.
The upper dichroic mirror in figure 1 is corrected.
The lower dichroic mirror in figure 1 is labeled.

Figure 3: I still think the SHG signal of the stroma is quite weak and inhomogeneous compared to other published data. See e.g. Fig 9 in Annals of Anatomy, 188, 5, p 395-409 (2006); www.sciencedirect.com/science/article/pii/S0940960206000513 or Fig. 9 in Latour, Gaël, et al. "In vivo structural imaging of the cornea by polarization-resolved second harmonic microscopy." Biomedical optics express 3.1 (2012): 1-15.
The live imaging platform allows visualizing of a murine ocular surface at the cellular-resolution for 11 hours. For long-term live imaging, SHG signals are relatively weak because the laser power is set at minimal to avoid photo-bleaching and tissue damage. We can also adjust the laser power to approach higher SHG signals; however, we chose the images using long-term conditions to avoid photo-bleaching or photo-damages.

Line 95-96: The laser description is still not clear. MaiTai DeepSee and Tsunami are both lasers. Do you use one of them or both or do you mean either one of them can be used?

We used Mai-Tai DeepSee as a laser source and the sentence is corrected. A wavelength-tunable ULTRAFAST Ti: Sapphire laser was used as the excitation source. (line 95)

Line 68: "helps to lubricate of the eyes" should read "helps to lubricate the eyes"

The sentence is corrected. (line 68)

Line 94 "upright microscopy" should read "upright microscope"

The sentence is corrected. (line 94)

Line 95,96 Do you mean you use two lasers (MaiTai DeepSee + Tsunmai)? MaiTai DeepSee with Tsunami oscillator 880 nm 940 nm. Which wavelength was used for SHG imaging?

We used ULTRAFAST Ti: Sapphire laser as the excitation source. In addition, the SHG signals were collected by 880nm.

Line 102 upper laser power limit of 70 mW: Why? How was that power level derived?

We explored the effects of various power laser, ranged from 40 mW to 120mW, on cornea and conjunctiva in vivo (our unpublished data). We found that the phenomenon of photo-bleaching occurs when the laser power was over 70mW. Therefore, we set the maximum of laser power is 70mW.

Line 103 "..microscopic design were shown.." should read "..microscope design is shown.."

The sentence is corrected. (line 104)

Line 200 "was listed below" should read "is listed below"

The sentence is corrected. (line 201)

Line 272 Please correct sentence Scale bars in Fig. C and Fig. D are..

The sentence is corrected. (line 272)

Line 282 "across" should read "over"

The sentence is corrected. (line 281)

Line 287 clarify reference "2120"

The sentence is corrected. (line 287)

Line 306 "enables" should read "enable"

The sentence is corrected. (line 306)

Reviewer #6:

Why is there no "discussion" section in revised manuscript? I think "discussion" section is needed and important.

The discussion is re-written. (line 269-306)

1) Figure 1A is largely improved, but the spec of MPM is lacked. Which scanner do you use, galvano or resonant? This thing has a profound effect on the observation conditions (scan speed(fps), scan size etc). In the same way, what kind of PMT set up do you use? Is your setup NDD? These information of MPM may be included in the "Table of materials".

1. Galvano, GVS002, Thorlabs
2. PMT, H7422A-40, Hamamatsu
3. Yes, it is non-descanned detectors (NDD)

The detailed information about scanner and PMTs is included in the Table of materials

2) As for the laser power, the authors provided the exact power on sample in "Point-by-point responses". Please add this to the manuscript, because this information is very important. The reason is that the loss of laser power depends on the setup.

The sentence is corrected. (line 101)

Sincerely,

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