STAR Protocols

Protocol

A whole-mount immunohistochemistry protocol for detection of mouse corneal nerves

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Highlights

This protocol will assist in maintaining appropriate morphology of corneal nerves

Neurons are sensitive to fixation methods and durations

1.3% PFA is the ideal fixative for fluorescent imaging of corneal nerves

This protocol can be used to guide fluorescent imaging at other tissues

A cornea is innervated by sensory nerves, which branch into thick trunks, subbasal plexuses, and sensory endings. Appropriate assessment of nerve structure in a tissue provides a more complete understanding of the role of nerves in health and disease. Here, we present a whole-mount immunohistochemistry protocol that facilitates evaluation of nerve architecture throughout the mouse cornea. The fixation step in this protocol allows for reliable detection of nerve structures within the cornea and likely other tissues.

Protocol

A whole-mount immunohistochemistry protocol for detection of mouse corneal nerves

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SUMMARY

A cornea is innervated by sensory nerves, which branch into thick trunks, sub-basal plexuses, and sensory endings. Appropriate assessment of nerve structure in a tissue provides a more complete understanding of the role of nerves in health and disease. Here, we present a whole-mount immunohistochemistry protocol that facilitates evaluation of nerve architecture throughout the mouse cornea. The fixation step in this protocol allows for reliable detection of nerve structures within the cornea and likely other tissues.

For complete details on the use and execution of this protocol, please refer to Yun et al, (2020).

BEFORE YOU BEGIN

Prepare buffers

⏱ Timing: 15 min

1. Prepare permeabilization buffer (1% Triton-X100 in 1× PBS) and keep the buffer at 4°C.
2. Prepare blocking buffer (0.3% Triton-X-100/0.1% Tween-20 in 1× PBS) and keep the buffer at 4°C.
3. Prepare washing buffer (0.1% Tween-20 in 1× PBS) and keep the buffer at 4°C.

Note: Normally, we do not make these buffers more than three days in advance. Older buffers likely work; however, it has not been tested.

Prepare paraformaldehyde solution

⏱ Timing: 5 min

4. Prepare fresh 1.3% Paraformaldehyde (PFA) in 1× PBS from 16% methanol-free paraformaldehyde solution.
5. Prepare cold 1× PBS that is 4°C.

Note: PFA is susceptible to degradation by light and oxygen, so it must be made fresh on the day of sacrifice.
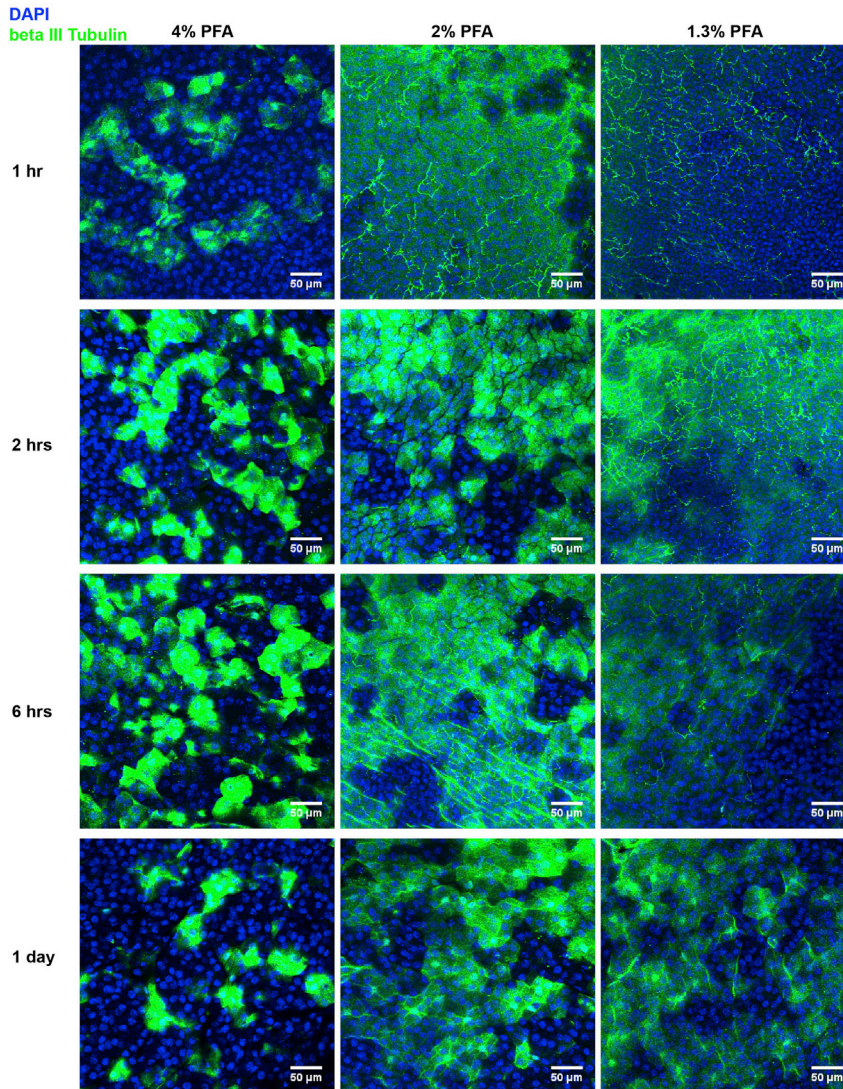
Fix mouse eyes

⏱ Timing: 1–2 h

Figure 1. The concentation of PFA affects the nerve morphology and structure within the cornea
Eyes of non-infected C57BL/6 mice that were 2–3 months old were enucleated and fixed with 1.3%, 2% and 4% PFA for 1 h, 2 h, 6 h and 1 day, respectively. The eyes were then washed with 1× PBS and the corneas were dissected, flat mounted and labeled with anti βIII Tubulin antibody (green) and 4′,6-diamidino-2-phenylindole (DAPI, blue). Images were acquired by confocal microscope and the brightness levels in the figures were adjusted for display. The cornea fixed with 1.3% PFA for 1 h demonstrated clear sensory endings in the epithelium level which can be identified by the morphology of DAPI staining in the nuclei. Higher PFA concentration and longer fixation led to high background and patchy staining and making it unable to illustrate the sensory nerve terminus.

6. Sacrifice a single mouse by CO_2 exsanguination and cervical dislocation without perfusion. Mice (male and female) in this protocol were less than 12 months old; however, this protocol should work for any age of mouse.

   △ CRITICAL: Make sure to thoroughly wash off any blood and attached tissues.
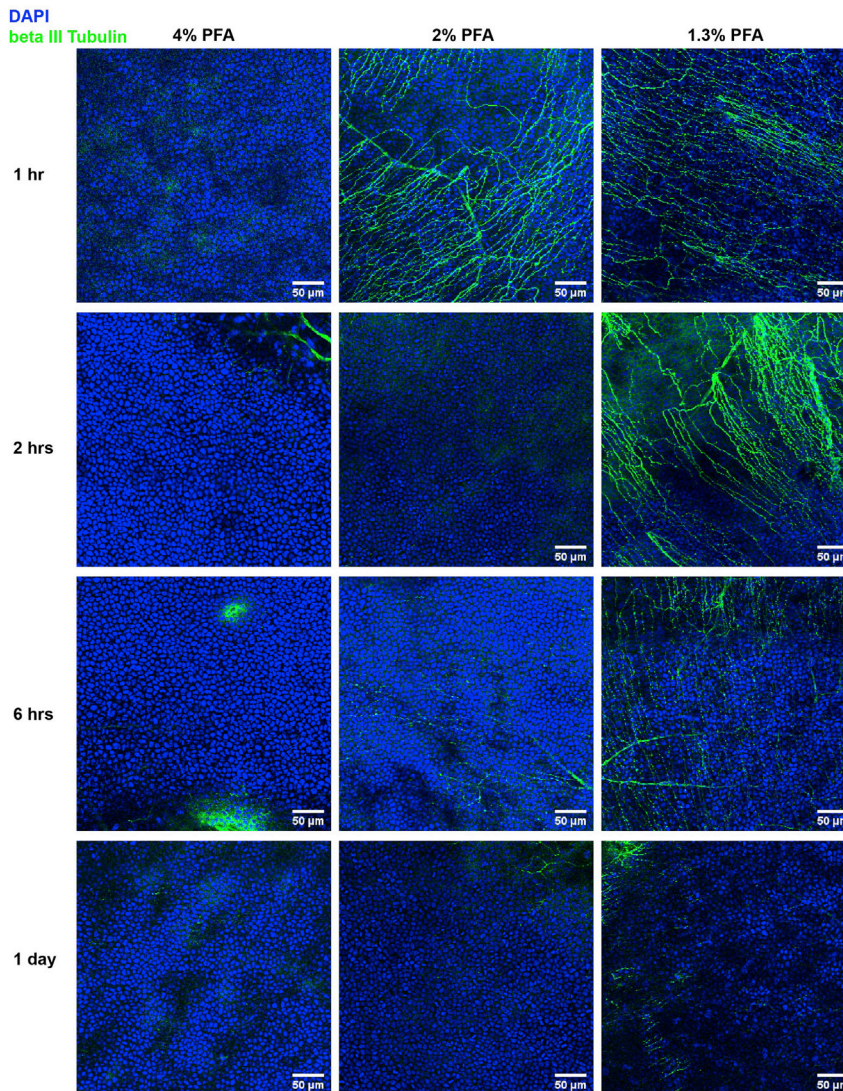
7. Enucleate eyes.

Figure 2. PFA concentration and fixation time effect the staining of the nerve plexus in the epithelial-stroma interface

In the exact frames illustrated in Figure 1, continuing to check the deeper z-stack layer for the staining of the nerve plexus located in the epithelial-stroma interface showed that 1.3% PFA with 1 h of fixation allowed the best revealing of the fine structure of nerve plexus. 2% PFA with 1 h of fixation or 1.3% PFA with 2 h of fixation reveals the majority of the nerve plexus. Higher PFA concentration and longer fixation led to partial to complete loss of the signal of the nerve plexus. (blue, DAPI; green, βIII Tubulin).

8. Briefly but thoroughly wash the enucleated mouse eyes with cold 1× PBS, and immediately immerse the eyes into 1.7 mL Eppendorf tubes with at least 200 μL 1.3% PFA in 1× PBS. Keep the tubes at 20°C–25°C for 1 h.

   △ CRITICAL: Fixing eyes immediately after enucleation prevents nerve degradation, which is vital when staining the corneas with antibodies for anti-sensory neurotrophic factors such as substance P (SP) and calcitonin gene-related peptide CGRP. When the nerves are severed, the distal axon fragmentation is triggered within seconds, but immediate fixation after harvesting preserves the cornea nerves from degradation (Stepp et al., 2017). See troubleshooting: problem 1.

⚠ CRITICAL: The concentration of PFA and the fixation time both affect the binding of anti-bodies. Increased concentration or fixation time cause patchy staining in the corneal epithelium and the epithelium-stroma interface, resulting in poor detection of sensory nerve endings (Figure 1) and nerve plexuses (Figure 2). See troubleshooting: problem 1 and 2.

9. Wash the eyes with 1× PBS 2 times/5 min.
10. Keep the eyes in 1× PBS at 4°C.

Note: After fixation, the eyes can be stored in 1× PBS at 4°C for >1 year, but relatively fresh corneas are required to detect sensory neurotrophic factors such as SP and CGRP. See troubleshooting: problem 1 and 2.

Note: We recommend diluting PFA from 16% methanol free paraformaldehyde solution, because methanol can permeabilize cell membranes and affect the morphology of cellular structures like the actin cytoskeleton.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor 488 goat anti-rabbit IgG(H+L) (dilution 1:500)	Abcam	ab150077 GR233725-3
Anti-Beta III Tubulin Rabbit Polyclonal (dilution 1:200)	Abcam	ab18207 GR220660-1
Chemicals, peptides, and recombinant proteins		
Paraformaldehyde, 16% w/v aqueous solution, methanol free	Thermo Fisher Scientific	43368
Goat serum	Cedarlane, Burlington, NC, USA	n/a
Tween-20 (polyoxyethylene sorbitan monolaurate)	Bio-Rad	Cas 9005-64-5
Triton-100 (octyl phenoxy polyethoxythanal)	Kodak	Cas 9002-93-1
10× PBS	Fisher BioReagents	BP 3994
Immu-Mount	Thermo Scientific	Ref 9990402
Software and algorithms		
ImageJ software (Fiji)	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
FV10-ASW 4.1	Olympus Life Sciences	https://www.olympus-lifescience.com/en/support/downloads/
MetaMorph 7.7	Molecular Devices	https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy
Experimental models: organisms/strains		
C57BL/6J	The Jackson Laboratory	Stock No: 000664
Other		
Coverslips	Fisher Scientific	12-541-B 22×22–1.5
Variable rocker	LabDoctor by MIDSCI	Serial 17080084
Student Vannas spring scissor	Germany Stainless	Code 91501-09FST
Student Dumont #5 forceps - standard tips, straight, Inox, 11 cm	Dumont Switzerland	Code 91150-20FST
Stemin 305 Microscope	Zeiss	www.zeiss.com/us/microscopy
Olympus IX81 Fluoview 1000 laser scanning confocal microscope	Olympus	www.olympus.com

STEP-BY-STEP METHOD DETAILS
Dissecting mouse corneas: Day 1

🕐 Timing: 1 h

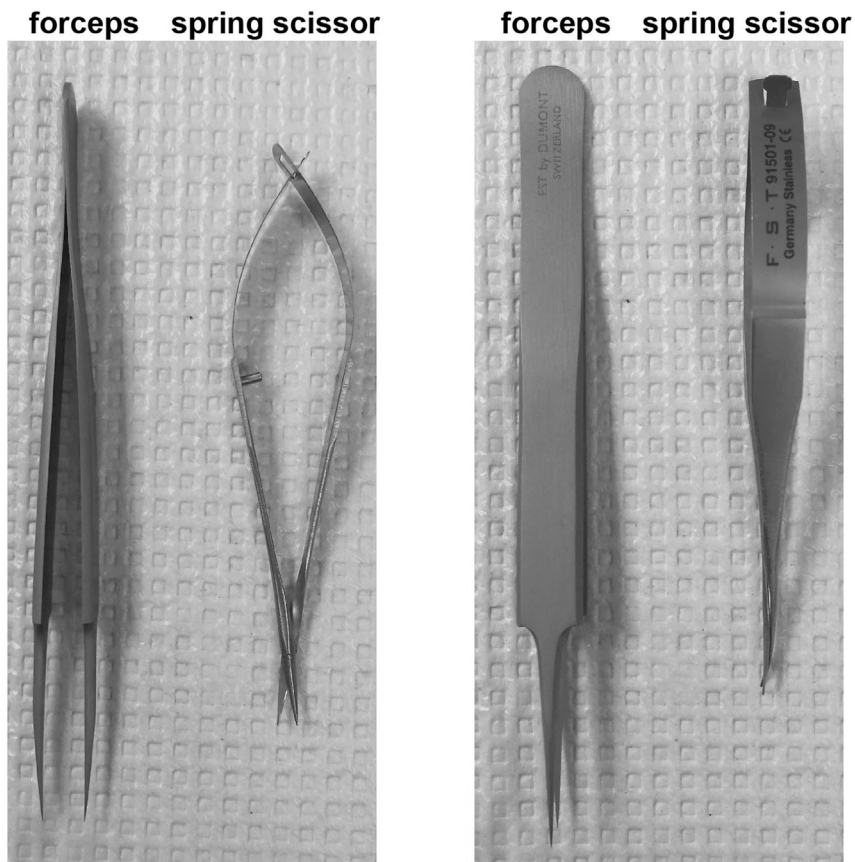forceps   spring scissor   forceps   spring scissor

Figure 3. A Student Vannas spring scissor and a Student Dumont #5 forceps (standard tips, straight, Inox, 11 cm) were used for corneal dissection

1. Dissect the corneas using surgical spring scissors and forceps (Figure 3), under a binocular dissecting microscope (Zeiss stenin 305) (Methods video S1).
2. Use forceps to hold the posterior part of the eye softly.
3. Use the tip of the surgical spring scissors to make the first incision upon the sclera.
4. Enlarge the incision and make it a circular cut to eliminate part of the sclera that is attached to the optic nerve.
5. Gently press the eye ball to push out the lens.
6. Make radial incisions to the central cornea and gently scrape off the retina with the tip of the scissors.
7. Gently scrape off the iris with the tip of the scissors.

   Note: Ensure that pressure is on the sclera and not the cornea as it could damage cellular morphology.

   Note: The dissection of the corneas should be performed in a petri dish filled with 1× PBS to prevent the corneas from drying out

8. Cut off the sclera, making sure to allow for a generous margin at the limbus to include nerves at the corneal/limbal interface.

Primary antibody incubation: Day 1

⏱ Timing: 5–7 h

9. Using a 96-well plate for staining, place one cornea per well and wash the corneas with 1× PBS 5 times/5 min at 20°C–25°C.

   Note: Both Eppendorf tubes and 96-well plates can be used for the staining. Keep one cornea in one tube or one well.

   Note: Thorough washing of the cornea in this step decreases nonspecific background signal. See troubleshooting: problem 3.

10. Pipette off 1× PBS after the last wash and add permeabilization buffer (1% Triton-X100 in 1× PBS).Then, keep the plate on a low-speed orbital shaker at 20°C–25°C for 60 min.
11. Transfer the corneas using fine-tip forceps into new wells and add in 20% serum in blocking buffer (0.3% Triton-X-100/0.1% Tween-20 in 1× PBS) and incubate at 20°C–25°C for 30 min.

   Note: Only touch the residual sclera to ensure that mechanical disruption of corneal epithelium and endothelium does not take place.

   Note: For the staining illustrated in this protocol, we use goat serum because our secondary antibody is goat specie (Cedarlane, Burlington, NC, USA); however, the serum used in the blocking buffer should match the species of the secondary antibodies you are going to use. See troubleshooting: problem 3.

12. Transfer the corneas into new wells and add a 100 μL cocktail of primary antibodies diluted in blocking buffer to each well. For this study we used, a pan-neuro marker, rabbit anti-mouse βIII tubulin at a dilution of 1:200.
13. Incubate at 20°C–25°C for 2 h, followed by an additional incubation for 12–16 h at 4°C.

   Note: Place the plate or tubes on a low-speed orbital shaker for all the washing steps and incubation steps.

   ⚠ CRITICAL: If it is necessary to add BSA to the blocking buffer when making the antibody cocktail, make sure to use BSA with the highest purity, as BSA with low purity causes high background.

Secondary antibody incubation: Day 2

   ⏲ Timing: 4–5 h

14. Wash the corneas in washing buffer (0.1% Tween-20 in 1× PBS) 5 times/5 min
15. Transfer the corneas into new wells (or new tubes) and add a 100 μL cocktail of secondary antibodies diluted in blocking buffer (1:500 dilution) into each well. Incubate at 20°C–25°C for 2 h.
16. Following five 10-min washes with washing buffer, mount the corneas on slides with the epithelium apposed to the #1.5 coverslip (170 μm). There should be only one cornea per slide.

   Note: For the staining illustrated in this protocol, we use Alexa Fluor 488 goat anti-rabbit IgG(H+L) (Dilution 1:500) as secondary antibody.

   Note: Sensory nerve endings and the fine subbasal plexus are more prone to bleaching compared to the thick neuronal trunks in the stroma. Therefore, scanning should always begin with the epithelium and end at the endothelium. See troubleshooting: problem 1.

17. Dry the slides at 4°C for at least 12 h before imaging (Figure 4).

Figure 4. A mouse cornea was mounted on a slide with a #1.5 thickness coverslip

Note: Drying times may vary with different types of mounting media. We use Immu-Mount from Thermo Scientific (Ref 9990402).

⚠ CRITICAL: Examining the corneas with a confocal microscope before the mounting medium is fully dried will compromise the imaging; the corneas will not lie flat and the acquired z-stack image mosaic will be difficult to stitch because of the objective lens of the microscope may disrupt the contact between the coverslip and cornea during scanning acquisition (Figure 5). See troubleshooting: problem 4.

Confocal microscopy acquisition: Day 3

18. Turn on the upright Olympus IX81 Fluoview 1000 laser scanning confocal microscope equipped with a 20× oil (numerical aperture 0.85) objective lens and an automated stage. Make sure the system is set up to do ZDC stitched mosaics with 10% overlap of the images to assist in good registration. Engage Multi-Area Time Lapse (MATL), and then go to Multi-Area Time Lapse Controller and select Mosaic Outline. Define the boundary of the cornea in the mosaic outline, allowing generous margins around the cornea.

Alternatives: We use an Olympus IX81 Fluoview 1000 laser scanning confocal microscope; however, any confocal microscope should be sufficient to capture detailed images of corneal nerves.

Note: We use a 20× oil objective for our studies; however, we have imaged corneal nerves with 10×, 40×, 60×, and digital 360×.

19. Focus the objective and set up the start and stop points of the z-scan spanning from the epithelium to the endothelium. Check the z settings at multiple locations in the tissue to ensure that the entire cornea will be included in the stacks.
20. Apply all the points in the Mosaic Outline.
21. Go to Registered Point List, check Display Current Area in Center of Stage Map.
22. Go to File and Folder, save the scanning files, check "Auto create sub folder every experiment."
23. Make sure to check "Set Track info to FV with position movement" and Re-Register to include all the points selected in step 19.
24. Check Ready button and then start scanning.
25. Save images in the native Olympus Image Binary (OIB) format or an OIF format. After acquisition images will be ready to be stitched together using FV10-ASW 2.0 software (Olympus Life Science, Tokyo, Japan).
26. To stitch mosaic: go to MATL Viewer under Devices, then click Open MATL-Log File, choose the MATL Mosaic file from the data folder, and then press the Select Action button, select the areas that need to be stitched and click Stitch.

Note: Stitching can also be performed using other software like Fiji.

DAPI
beta III Tubulin

Not Fully Dried (4% PFA/1 hour)　　　　Fully Dried (4% PFA/1 hour)
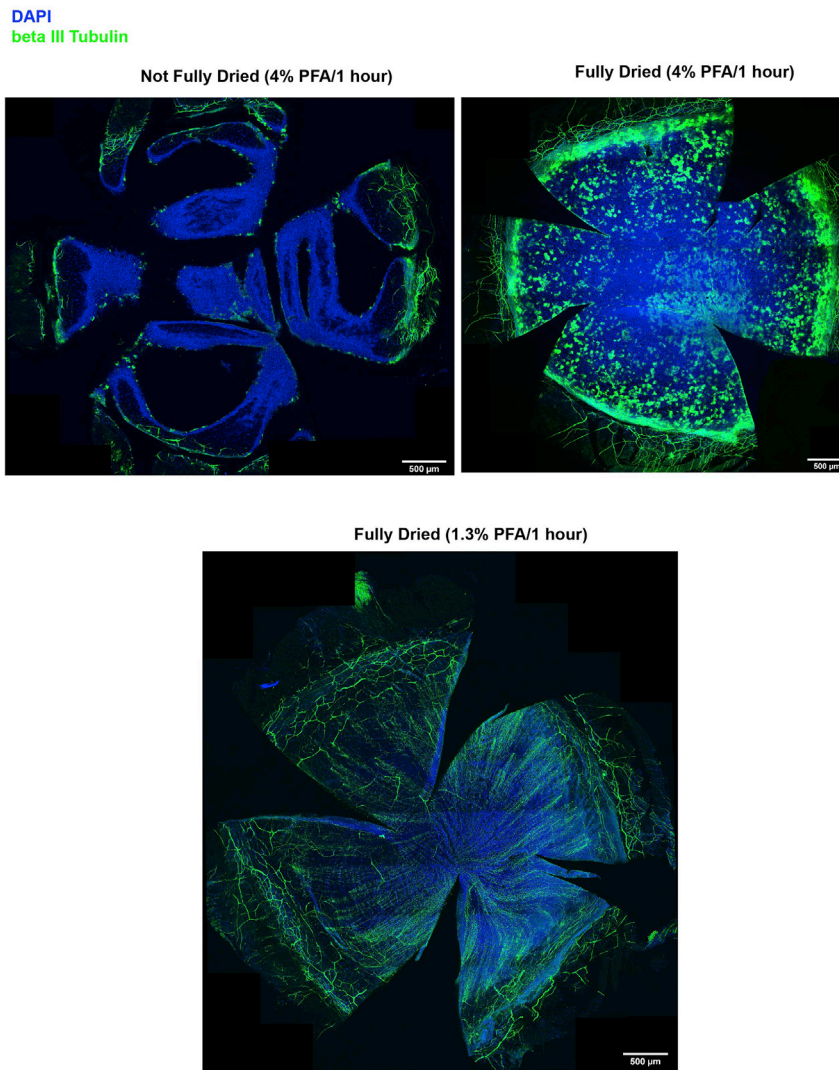
Fully Dried (1.3% PFA/1 hour)

Figure 5. Scanning the corneas before the mounting media was completely dried led to curved images
Here we showed the images of the same cornea (4% PFA/ 1 h fixation) before and after the mount medium was fully dried, and in addition a cornea fixed with 1.3% PFA for 1 h (blue, DAPI; green, βIII Tubulin).

27. Check all the channels that need to be stitched, and check Correction and Smoothing, and then press the Show button.
28. After the "tiling" (stitching) process is finished, save the file with the original name in the native OIB format, and make sure to check Apply to Series.

   Note: Make sure to click all the options mentioned in this step, otherwise, the raw data might not be saved correctly and might be lost after stitching. See troubleshooting problem 5.

EXPECTED OUTCOMES

The image of the whole nerve architecture spanning from the epithelium to the endothelium can be acquired via confocal microscope. In a healthy cornea, the sensory endings in the epithelium (Figure 1), the nerve plexus in the epithelium-stroma interface (Figure 2), and the nerve trunks in the stroma are labeled with pan-neuronal marker βIII Tubulin. Under pathological conditions,

sympathetic nerves that invade the cornea can be detected by anti-tyrosine hydroxylase antibody (Yun et al., 2016). The clean staining of the nerve fibers allows the quantification of nerve density. As mentioned above, nerves begin to deteriorate in a short period of time if the corneas are not fixed immediately, and the concentration of the fixation buffer as well as the fixation time both have critical impact on the staining. Aspects of this protocol likely extend to other tissues such as the skin where the intermingling of sensory, sympathetic, and parasympathetic nerves are more apparent. The fixing and whole mounting procedure described above preserves the nerve architecture more closely than previously described methods. The method we described should be employed to ensure proper delineation of specific nerve populations within peripheral tissues.

QUANTIFICATION AND STATISTICAL ANALYSIS

For the quantification of corneal nerve density, representative corneal regions can be selected from each stitched volume. To ensure that the location of the selected regions is consistent in all corneas, a selection journal should be generated in MetaMorph 7.7.8 (Molecular Devices, LLC, Sunnyvale, California, USA) or in Fiji. Define a consistent region size and copy representative areas from the peripheral cornea in each quadrant as well as the central area (for example, all regions located in the peripheral cornea 500 μm away from the limbus, and in the central cornea, with sampled regions each being 400×400 pixels):

To quantify the nerve density in a certain area:

1. Save the spliced channels as 8-bit images.
2. Start the plugin from the Fiji Plugins ⟩ Segmentation ⟩ Simple Neurite Tracer menu option, Open the image in XY, ZY and XY view.
3. Choose to see a 3D representation of the tracings, and leave the Resampling factor as the default.
4. Enable cursor snapping within XY and Z.
5. Enable the option for using Hessian-based analysis of images.
6. Click on the point in the image stack where you want to start the tracing and then select the next point in the path, complete the path after picking out a few more points along the path.
7. Save the files with the original filename with the extension changed to ".traces".
8. Select the paths from the path list and select "Fill Out Path(s)" in the interface.
9. Check Create Image Stack from Fill, and save the file of "filled neuron." For a detailed walk-through of this plugin and how to adjust the fill threshold reference: https://imagej.net/SNT:_Step-By-Step_Instructions.html#II.Adjusting_the_Fill_Threshold
10. Make sure to choose the file of "filled neuron," then go to Fiji Plugins ⟩Skeleton ⟩Skeletonize.
11. Go to Fiji Plugins ⟩Skeleton ⟩Analyze Skeleton, choose the Prune cycle method and the Results and Output options.
12. Confirm the name of the original image which was analyzed
13. Save the Result file which provides the number of branches and the average and maximum length of branches in the corresponding units, and the file of Branch information which includes skeleton ID and calibrated branch length for further analysis.

LIMITATIONS

This protocol provides clean tissue/nerve staining for a single antibody; however, we have used this protocol to scan up to four different antibodies. In our experience, more than four combined antibodies will require a longer scanning time and may cause bleaching of the fluorescence, leading to false-negative staining.

The quantification of nerve density using Simple Neurite Tracer can only be completed manually, which is very time-consuming. We have found that different subsets of nerve markers degrade at different rates after sacrifice. Specifically, signals for sensory nerves are lost rather quickly compared

to signals for sympathetic nerves. This speaks to the critical timing and nature of fixation after mouse sacrifice.

TROUBLESHOOTING

Problem 1

Sensory nerve endings and the nerve plexus are not apparent or difficult to see (before you begin step 8, secondary antibody incubation- day 2 step 16).

Potential solution

The time from sacrifice to fixation is critical as it has been reported (Stepp et al., 2017) that sensory endings begin to deteriorate within minutes of sacrifice. Therefore, one can reduce the time between sacrifice and fixation by only sacrificing one mouse at a time, becoming proficient at enucleating eyes(or other tissues), and having all tubes prepared in advance of sacrificing the mice. Furthermore, if the detection of sensory neurite markers is the priority of the designed experiment, try to stain corneas as soon as possible after fixation, and avoid using long stored samples.

Another potential solution could be to reduce the amount of time that corneas are exposed to confocal lasers. The sensory endings and nerve plexus are quite sensitive to bleaching, so limiting the number of analyzed parameters and ensuring that the epithelium is imaged first will help ensure appropriate analysis of sensory endings.

Problem 2

The nerve endings look patchy (before you begin step 8).

Potential solution

We recommend always using freshly diluted low concentrations of PFA to fix the eyes and keep the fixation time as short as possible (Figures 1 and 2). Our experiments showed that higher concentrations of PFA and longer fixation time cause high background and patchy staining in the epithelium, making it impossible to distinguish sensory endings. Moreover, the signal of nerve plexus in the epithelial-stroma interface gradually diminished and eventually turned off when the concentration of PFA and fixation time continually increased.

Problem 3

There is high background in the confocal images (steps 9 and 11).

Potential solution

First, one could try washing the cornea after dissection more thoroughly. Also, the serum used for the blocking buffers should be the same host species as the labeled secondary antibodies to decrease non-specific staining and high background. Through various optimization procedures, we have found that 30 min of blocking is the optimal blocking time. If blocking is done for less than 30 min, there is high background; however, longer blocking times can lead to a decreased signal.

Problem 4

Confocal images are mismatched after stitching and are obscured and curvy (secondary antibody incubation- day 2, step 17).

Potential solution

First, check the settings before the mosaic scanning to make sure there is at least 10% overlap between each adjacent image. Second, the slides need to be dried out at least for 12 h before image acquisition. The undried mount medium will be pushed to the periphery under the cover slip when the oil objective moves toward the bottom of the slide during z stack scanning, thus the scanning

depth will also change. These will affect the stitching and cause mismatch, and the images will be obscure and curvy.

Problem 5
Raw data are lost after stitching the mosaic scanned confocal images (confocal microscopy acquisition - day 3, steps 27 and 28).

Potential solution
First, before acquiring the images, make sure to check the following saving options: 1, create a folder in data drive and save the individual scanning folder there; 2, check "Auto create sub folder every experiment"; 3, check "Add a time stamp".

When stitching the images, make sure to save the file in the native OIB format and click the "Apply to Series" button and then click the save button, the stitched images should then be saved, and the raw individual images will be kept in the original folder.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, [Anthony J. St. Leger] (Anthony.stleger@pitt.edu).

Materials availability
No new materials were generated in this study.

Data and code availability
No data sets or code were developed during this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100734.

AUTHOR CONTRIBUTIONS

A.J.S. and H.Y. designed the experiments. H.Y. and K.L.L. performed and analyzed experiments. K.L.L. created the selection journal in MetaMorph and established the whole work flow for image acquisition. A.J.S., H.Y., and K.L.L. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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