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A Protocol for Immunostaining of Macrophages in Whole-Mount Mouse Cornea, Conjunctiva, and Lacrimal Gland

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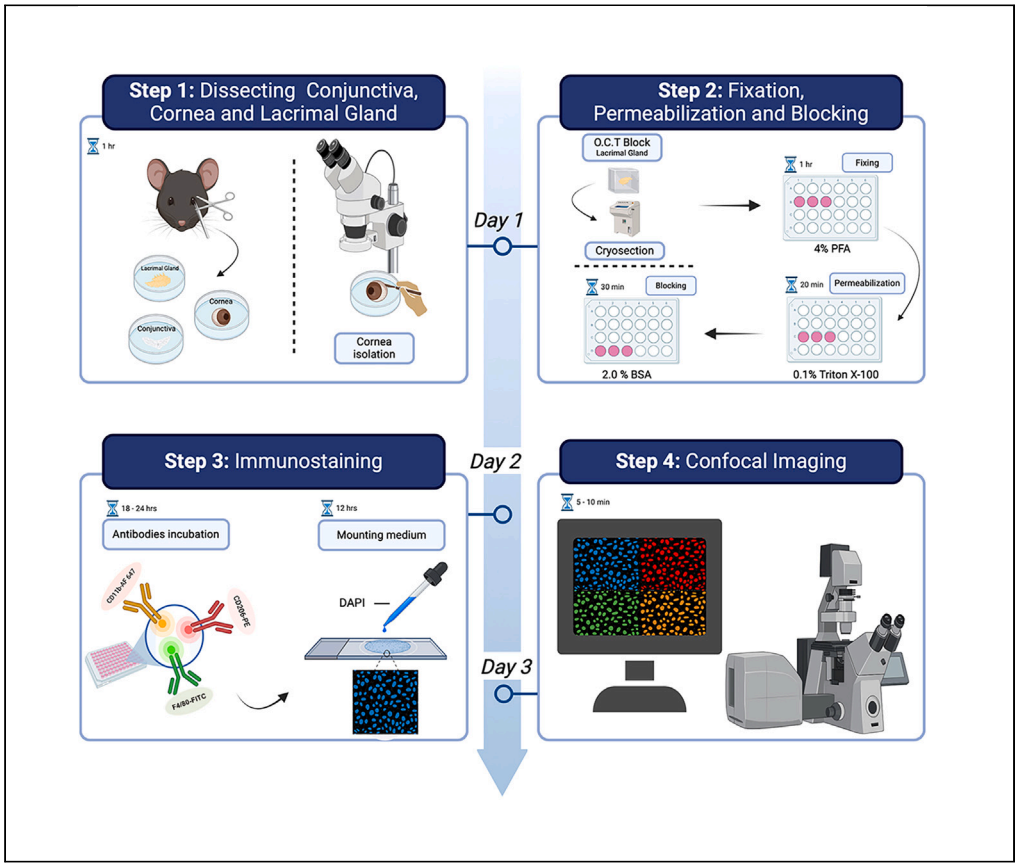

The healthy lacrimal functional unit contains a resident macrophage population. Here, we present a protocol for immunofluorescent staining of macrophage markers, CD11b, F4/80, and CD206, in whole-mount mouse cornea, conjunctiva, and 50-μM-thick lacrimal gland section. We describe steps for dissection, fixation, permeabilization, and blocking. We then detail procedures for the detection and spatial localization of macrophages through immunostaining and confocal imaging. This approach circumvents the need to obtain thin tissue sections and acquire macrophage images from each tissue section.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for surgical isolation of mouse conjunctiva, cornea, and lacrimal gland

Macrophage immunostaining in whole-mount mouse conjunctiva and cornea

Preparation of 50-μm cryosection of lacrimal gland and macrophage immunostaining

The z stack confocal imaging of immunostained tissues

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A protocol for immunostaining of macrophages in whole-mount mouse cornea, conjunctiva, and lacrimal gland

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SUMMARY

The healthy lacrimal functional unit contains a resident macrophage population. Here, we present a protocol for immunofluorescent staining of macrophage markers, CD11b, F4/80, and CD206, in whole-mount mouse cornea, conjunctiva, and 50-μM-thick lacrimal gland section. We describe steps for dissection, fixation, permeabilization, and blocking. We then detail procedures for the detection and spatial localization of macrophages through immunostaining and confocal imaging. This approach circumvents the need to obtain thin tissue sections and acquire macrophage images from each tissue section.

BEFORE YOU BEGIN

The protocol below describes the specific steps for immunostaining the macrophages in whole-mount mouse cornea, conjunctiva, and 50 μM thick section of the lacrimal gland using CD11b, F4/80, and CD206 markers. This protocol aims to provide step-by-step methods for immunostaining for these markers to identify the majority of the macrophage population. The protocol does not require cardiac perfusion since there are no circulating macrophages in these tissues. This protocol is not for identifying different sub-populations of macrophages in these tissues for which other markers such as MHCII, CSFR1, CCR2, CD64, CD163, and LyC6 may need to be used.[1–3]

For this protocol, our research group used C57BL/6 strain male mice (obtained from Charles River, Wilmington, MA) weighing 20 g–25 g and 8–12 weeks of age. The protocol should be applicable to other adult mice strains, independent of sex.

Institutional permissions

The animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the Chapman University IACUC Committee.

Gather the required surgical instruments and autoclave them (Figure 1).

Figure 1. Surgical instruments for dissecting out the conjunctiva, cornea, and lacrimal gland L to R: Spring scissors, Pointed scissors, Toothed forceps, Curved toothed forceps, Pointed forceps, Curved forceps, #15 surgical blade

Preparation of solutions

Timing: 60 min

1. Prepare 4% paraformaldehyde solution.

⚠ CRITICAL: Prepare the paraformaldehyde solution in the chemical fume hood. Wear gloves and a mask.

a. Heat ~80 mL of deionized water to 55°C–65°C in a 250 mL conical flask in a fume hood.
b. Weigh and add 4 g paraformaldehyde powder and stir using a magnetic stirrer for 5–10 min.

Note: Do not let the solution boil.

c. Add a few drops of 1M NaOH to dissolve the paraformaldehyde completely.
d. Add 10 mL of 10× PBS. Let the solution cool down. Adjust pH with HCl to 7.4. Adjust volume to 100 mL with deionized water.

Note: Paraformaldehyde solution is light-sensitive and susceptible to oxidation. It should be stored in amber-colored or aluminum-wrapped containers, and the container size should be selected to fill up with the volume to minimize air inside the container. The solution can be stored at 4°C for 1–2 weeks, but we typically prepare 10 mL aliquots and store them at −80°C for up to 1 year of use. The aliquots can be thawed immediately before use.

2. Prepare permeabilization buffer (0.1% triton X-100 in 1 × PBS).

Add 10 μL of triton X-100 to 10 mL of 1 × PBS and store at 4°C.

Note: The triton X-100 solution is viscous. Vortex or mix well to dissolve and let the foam subside.

3. Prepare blocking buffer (2% Bovine Serum Albumin, IgG Free in 1× PBS).

Dissolve 200 mg of bovine serum albumin in 10 mL of 1× PBS and store at 4°C.

Note: We typically prepare and store buffers at 4°C for 72 h. These buffers are likely stable for more than 72 h, but we have not tested their storage and stability for > 72 h for this protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD11b Alexa Fluor 647 Anti-mouse/human (1:25 dilution) Clone: M1170, isotype: IgG 2b, k	BioLegend	101218
F4/80 FITC Anti-mouse (1:25 dilution) Clone: BMB, isotype: rat IgG 2a, k	BioLegend	123108
CD206 (MMR)PE Anti-mouse (1:25 dilution) Clone: C068C2, isotype: rat IgG 2a, k	BioLegend	141706
Chemicals, peptides, and recombinant proteins		
Bovine serum albumin	Abcam	ab64009
Triton X-100	Thermo Scientific	327372500
10× PBS	Fisher BioReagents	BP 665-1
DAPI-Aqueous Flouro Shield	Abcam	ab104139
Paraformaldehyde	MilliporeSigma	158127
Optimum Cutting Temperature (O.C.T.) compound	Fisher Scientific	14-373-65
Software and algorithms		
Imaris Viewer	Oxford Instruments	https://imaris.oxinst.com/imaris-viewer
Experimental models: Organisms/strains		
C57BL/6 strain 8–12 weeks of age male mice	Charles River	556NCIC57BL
Other		
1.5 mL tubes	Fisher Scientific	02-682-002
Orbital shaker	BT Lab Systems	MU-E30-1013
3D Rocker	USA Scientific	17020029
Micro-cover glass	Electron Microscopy Sciences	72200-40 22 mm × 50 mm
96 Well flat bottom plate	Fisher Scientific	12565501
24 Multi-well plate	CytoONE	CC7682-7524
Superfrost Plus microscope slides	Fisher Scientific	12-550-15S24 75 × 25 × 1.0 mm
Cryostat	Leica	CM1860
Cryomold	Fisher Scientific	22-363-553
Confocal microscope	Nikon A1	

STEP-BY-STEP METHOD DETAILS

Cornea, conjunctiva, and lacrimal gland dissection: Day 1

Timing: 30 min

This step describes the dissection of mouse conjunctiva, cornea, and lacrimal gland.

1. Dissecting the Conjunctiva.

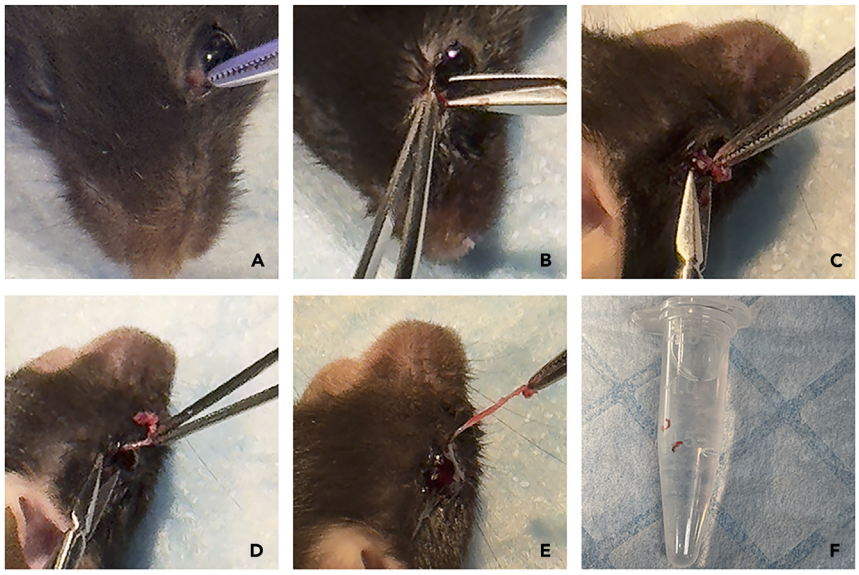

Figure 2. Steps for dissection of conjunctiva

Note: This step describes the dissection of mouse conjunctiva.

a. Using an American Veterinary Medical Association (AVMA)-approved CO_2 meter, euthanize the mouse by CO_2 exposure followed by the secondary method of euthanasia (e.g., cervical dislocation).
b. Using toothed forceps in one hand, grab the conjunctiva at the inner canthus of the conjunctival sac. Make a small incision using curved micro scissors with the other hand (Figures 2A and 2B).
c. While gently pulling the tissue with the forceps, cut along the junction of the conjunctiva and cornea and the outer margin of the upper eyelid (Figure 2C).
d. Once you reach the outer canthus, rotate the mouse and continue cutting along the conjunctiva, cornea, and outer margin of the lower eyelid (Figures 2D and 2E).

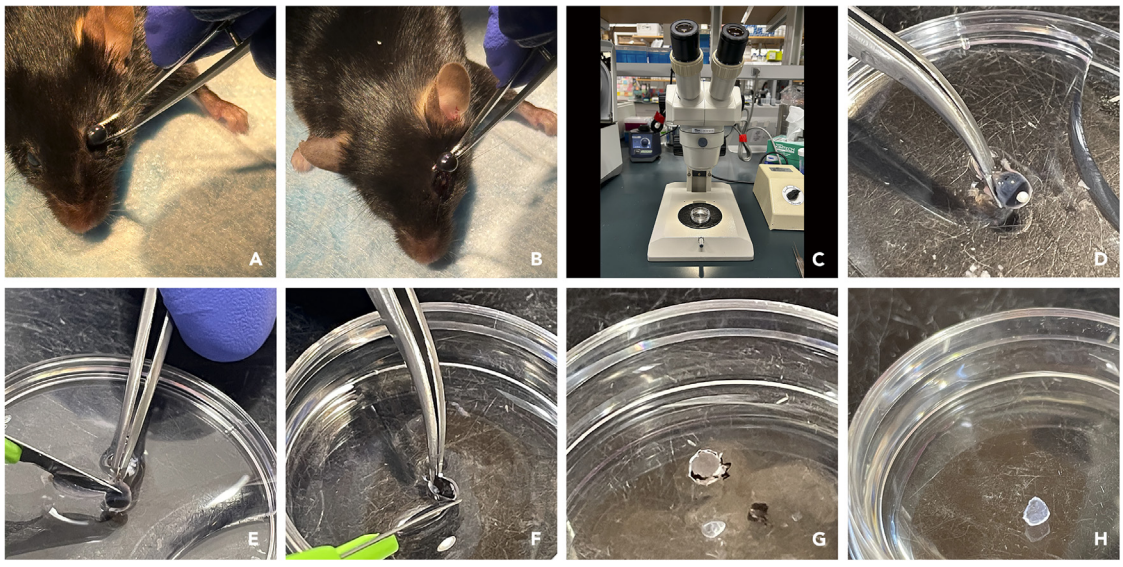

Figure 3. Steps for dissection of cornea

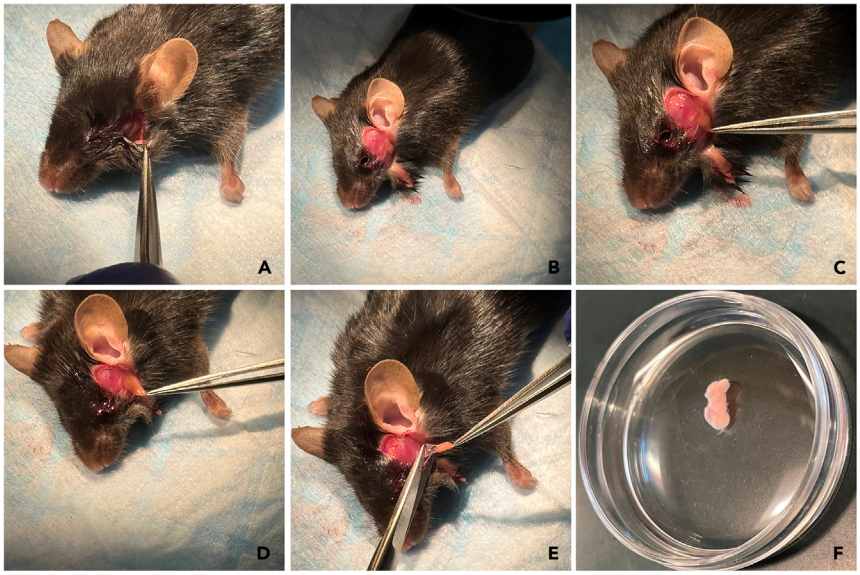

Figure 4. Steps for dissection of the lacrimal gland

e. Rinse the excised conjunctiva ice-cold 1× PBS to remove any blood.
f. Place the isolated conjunctiva in an Eppendorf tube containing 1× PBS on the ice (Figure 2F).

2. Dissecting the Cornea.

Note: This step describes the removal of the eyeball and dissection of the mouse cornea.

a. Once the conjunctiva has been excised, apply gentle pressure around the eyeball using curved forceps to extrude the eyeball (Figure 3A). Grab the optic nerve and pluck the eyeball out using the curved forceps (Figure 3B).
b. Wash the enucleated eyeball with ice-cold 1× PBS to remove blood (Figure 3C).
c. Carefully hold the posterior portion of the eye with the forceps (Figure 3D).
d. Make an initial incision with the tip of a #15 surgical blade into the sclera and let the vitreous drain out (Figure 3E).
e. Expand this incision further along the sclera to make the cut bigger (Figure 3F).
f. Using forceps, apply pressure to the eyeball away from the cornea and push the lens out (Figure 3G).

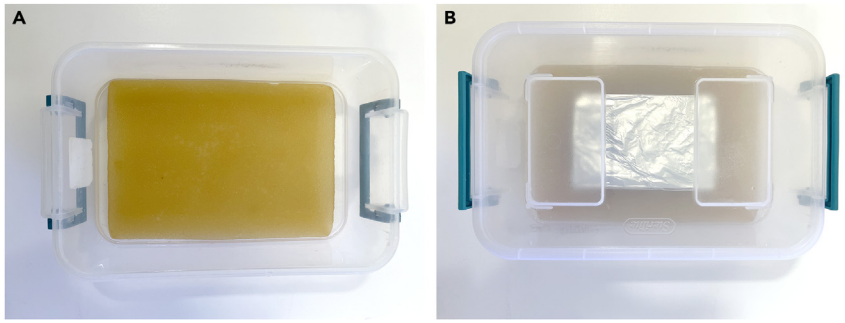

Figure 5. A plastic moisture chamber for incubation of tissues for immunostaining
Panel A shows the box without the lid and the 96-well plate, and panel B shows the lid-covered box with a 96-well plate containing the tissue and the antibodies. The aluminum wrap is to protect the fluorescent-conjugated antibodies from light.

g. Once the lens has been removed, use the tip of the scissors to make a circular incision around the sclera and isolate the cornea (Figure 3H).
h. Using scissors or a #15 blade, remove any iris still attached to the cornea.
i. Place the isolated cornea in the dish containing 1× PBS on the ice.

⚠ CRITICAL: While isolating the conjunctiva or cornea, do not let the tissue dry, and keep it moist by adding a few drops of 1XPBS. To preserve the cornea morphology, avoid pushing the lens towards the cornea and instead direct the pressure to the edge of the sclera towards the retina.

3. Dissecting the lacrimal gland.

Note: This step describes the dissection of the mouse lacrimal gland.

a. Pick the skin under the ears using toothed forceps and make a small incision. Cut along both sides for a U-shaped incision (Figures 4A and 4B).
b. Pick the skin flap to locate the lacrimal gland. The lacrimal gland can be identified by the transparent lacrimal ducts connected to it. Using pointed forceps, free up the tissue surrounding the lacrimal gland (Figures 4C and 4D).
c. Gently remove the lacrimal gland with toothed micro forceps and rinse it in 1× PBS to remove any blood (Figure 4E).
d. Place the isolated lacrimal gland in the dish containing 1× PBS on the ice (Figure 4F).

Fixation of the harvested tissue in 4% paraformaldehyde solution: Day 1

Timing: 90 min

4. This step describes the paraformaldehyde fixation of the isolated cornea, conjunctiva, and lacrimal gland.
   a. Transfer the isolated cornea, conjunctiva, and lacrimal gland into one well each in a 24-well plate containing 500 μL of 4% paraformaldehyde solution.
   b. Place the plate on an orbital shaker at 4°C for 60 min. After that, remove the paraformaldehyde solution and add 500 μL 1× PBS to each well.
   c. Place the sample on the orbital shaker at 20°C–25°C to wash the tissues for 5 min. Remove the 1× PBS, and repeat this step of washing five times.

Pause point: The paraformaldehyde-fixed tissues after the washing can be stored in 1× PBS at 4°C for 2–4 day.

   d. Place the cornea on a glass slide and cut it into 4 pieces using a # 15 surgical blade.
   e. The cornea and conjunctiva are ready for the next step, which is permeabilization, blocking, and immunostaining.

Preparation of cryomold for lacrimal gland: Day 1

Timing: 1 h

5. This step describes the preparation of cryomold for the lacrimal gland to obtain 50 μm tissue sections.
   a. Place the lacrimal gland in a plastic mold containing Optimum Cutting Temperature (O.C.T.) solution. The gland should be placed flat in the mold to obtain longitudinal tissue sections.

b. Prepare a 2-methylbutane bath on liquid nitrogen and hold the plastic mold on the cold 2-methylbutane using a hemostat (Methods video S1). The O.C.T. block should freeze in about 40–50 s. The block should be ready to obtain 50 μm thick tissue sections on a glass slide using a cryostat.

Pause point: The tissue in cryomold can be stored at −80°C upto several months for later use.

Note: Touch the base of the plastic mold to the cold 2-methylbutane, but do not let the 2-methylbutane enter into the O.C.T solution.

c. Thaw the tissue sections at 20°C–25°C for about 5–10 min and wash them with 1× PBS. The lacrimal gland sections will be ready for the permeabilization, blocking, and immunostaining step.

Permeabilization, blocking, and immunostaining: Day 1

Timing: 18–24 h

6. This step describes the permeabilization, blocking, and immunostaining of the cornea, conjunctiva, and lacrimal gland.
   a. After the last wash, add 500 μL of permeabilization buffer to 3 wells of a 24-well plate. Place the cornea, conjunctiva, and section of the lacrimal gland into one well each. Place the plate on the orbital shaker for 20 min at 20°C–25°C.
   b. Wash the tissues with 500 μL of 1× PBS for 5 min at 20°C–25°C.
   c. After the wash, add 500 μL of blocking buffer to each well containing the tissue and place the plate on the orbital shaker for 30 min at 20°C–25°C.
   d. Prepare a cocktail of antibodies by adding 9 μL each antibody to 225 μL of 1× PBS. This study used CD11b Alexa Fluor 647, F4/80-FITC, and CD206-PE at a final dilution of 1:25 for each antibody.
   e. Transfer 75 μL of the antibody cocktail each to 3 wells of a 96 well-plate. Using forceps, transfer each tissue into each of the three wells. Cover the plate with aluminum foil.
   f. Prepare a moist chamber using an airtight plastic container and keep a water-soaked sponge or filter paper at the bottom (Figure 5A).
   g. Place the plate in the moist chamber (Figure 5B) and keep the moist chamber on an orbital shaker to incubate for 18–21 h at 20°C–25°C.

Washing and mounting: Day 2

Timing: 90 min

7. This step describes the washing step after the immunostaining and mounting tissue in a DAPI-containing anti-fade medium.
   a. The next day, transfer the tissues from the 96-well plate back to the 24-well plate.
   b. Wash the tissues by adding 500 μL of 1× PBS to each well containing the tissue and place it on an orbital shaker for 10 min at 20°C–25°C. Repeat this washing step five times.
   c. After the last washing, remove the tissues from the plate and gently dab off the excess 1× PBS by touching the tissue corners with a Kim wipe.
   d. Place the tissues on a glass slide and add a drop of an aqueous mounting medium containing DAPI.
   e. Place the coverslip and leave the slides at 4°C in a refrigerator at least for 5 h for DAPI to penetrate and stain the nuclei.

Note: This study used a DAPI-Aqueous Fluoro Shield mounting medium. Alternative commercially available mounting media can be used.

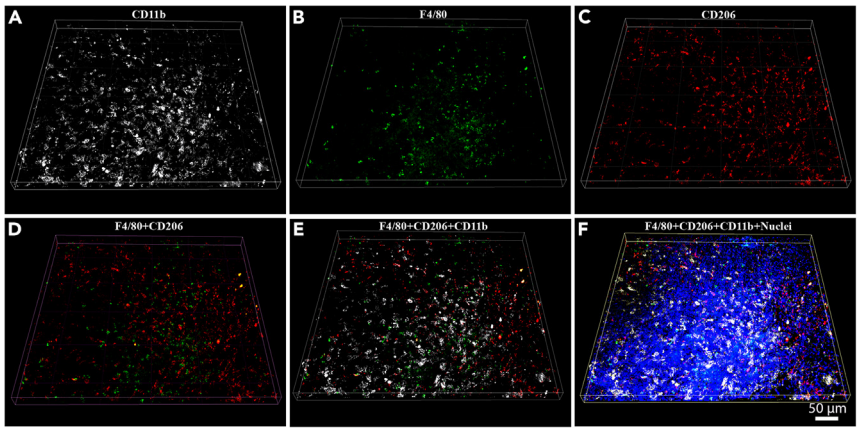

Figure 6. The confocal microscopy Z-stack projection images of mouse conjunctiva stained for macrophage markers

Immunostaining for CD11b (Panel A pseudo-color gray), F4/80 (Panel B green), and CD206 (Panel C red) alone. Panel D is the F4/80 and CD206 merged image showing that a small number of macrophages stained both for F4/80 and CD206 (yellow), a few stained for F4/80 only (green), and a large number stained for CD206 only (red). Panel E is CD11b, F4/80, and CD206 merged images showing that both F4/80 and CD206 positive macrophages are also positive for CD11b. Panel F shows nuclei stained blue with DAPI along with CD11b, F4/80, and CD206 staining. Scale bar = 50 µm.

Confocal microscopy to capture images of immunostained tissues: Day 3

Timing: 10 min/image

8. This step briefly describes capturing the Z-stack confocal images of DAPI-mounted immunostained tissues.
   a. This study used a Nikon A1 model confocal microscope and captured Z-stack images using a 10× and 20× objective lens.

EXPECTED OUTCOMES

The macrophages in the whole mount conjunctiva, cornea, and 50 µm thick lacrimal gland can be detected by double immunostaining for CD11b and F4/80 or CD206 (Methods videos S2–S7). The number of F4/80-stained macrophages in mouse conjunctiva (Figure 6 Panel B, Methods video S4) and lacrimal gland (Figure 9 Panel B, Methods video S7) are lower than those with CD206 staining (Figure 6 Panel C, Methods video S3; Figure 9 Panel C, Methods video S6). These macrophages also show double-positive staining for CD11b (Figures 6 and 9, Panel E). A few macrophages (yellow Figures 6 and 9, Panel D) show triple-positive staining for CD11b, F4/80, and CD206. Our immunostaining data agrees with the previously published single-cell RNA-Seq data that also demonstrated that mouse conjunctiva contains a higher number of regulatory macrophages that show expression of markers such as CD206 or CD209. Furthermore, the conjunctiva and lacrimal gland macrophages also show Adgre1 expression (gene encodes for F4/80).[4,5] Most of the macrophages in the cornea are triple positive for CD11b, F4/80, and CD206 (Figure 7). Previous studies have shown that macrophages in the cornea are primarily located around the periphery in proximation to the limbus.[6] Our immunostaining data also indicates that most of the macrophages in the cornea are localized mainly within a short distance from the limbus (Figure 8).

QUANTIFICATION AND STATISTICAL ANALYSIS

The images were merged, and Z-stack projections were generated using Imaris Software. Stitching of cornea images was performed using Adobe Photoshop.

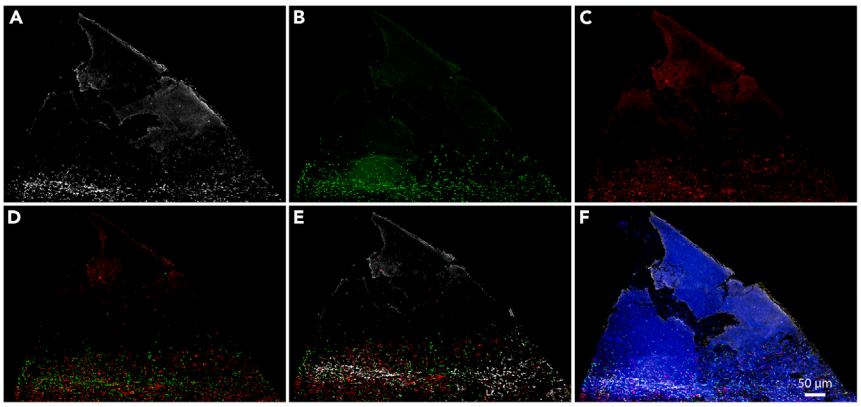

Figure 7. The confocal microscopy Z-stack projection stitched images of a quarter piece of mouse cornea stained for macrophage markers

Immunostaining for CD11b (Panel A pseudo-color gray), F4/80 (Panel B green), and CD206 (Panel C red) alone. Panel D is the F4/80 and CD206 merged image showing that most of the macrophages stained for both F4/80 and CD206 (yellow), with a smaller percentage showing either F4/80 (green) or CD206 (red) only staining. Panel E is CD11b, F4/80, and CD206 merged images showing that both F4/80 and CD206 positive macrophages are also positive for CD11b. Nuclei are stained blue with DAPI (F). Scale bar: 50 µm.

LIMITATIONS

This protocol describes immunostaining for mouse macrophage cell surface markers, CD11b, F4/80, and CD206, primarily expressed on M0 or regulatory macrophages. The majority of the macrophages in healthy disease-free mouse cornea, conjunctiva, and lacrimal glands can be identified by these markers based on our experience and personal communication with other investigators who are lacrimal functional unit immunology experts. If the goal is to identify different subpopulations of macrophages, immunostaining for additional markers such as MHCII, CSFR1, CCR2, CD64, CD163, and LyC6 may be needed.[1–3] Mouse models of ocular surface diseases or injury may show an increase in proinflammatory macrophages or monocyte-derived macrophages.[3,7,8] The proinflammatory macrophages or monocyte-derived macrophages express a different set of cell surface markers, e.g., CD80, CD86, or Ly6C, and thus will require immunostaining of those markers for their detection. Furthermore, antibodies conjugated to a different set of fluorochromes can be used for the immunostaining, e.g., Alexa 488 conjugated F4/80 instead of FITC and instead of PE either Alexa 555 or Alexa 594. It may require an optimization of antibody concentration and incubation time. Furthermore, to identify the spatial localization of these macrophages within each tissue such as epithelium or stroma, hematoxylin and eosin staining can be performed to identify these structural tissue details. Finally, acquiring

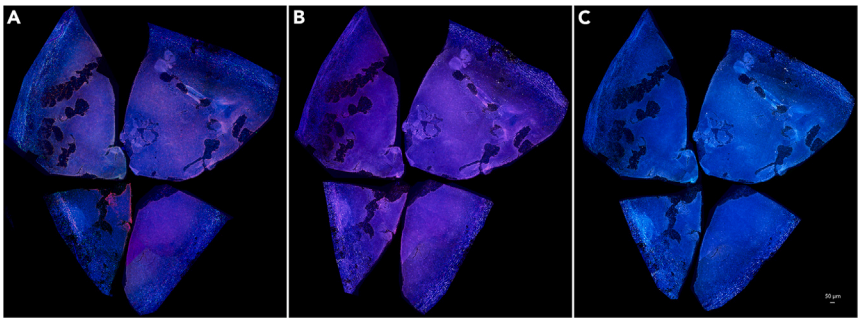

Figure 8. The whole mouse cornea confocal microscopy Z-stack projection stitched and merged images of mouse corneas stained for macrophage markers

The immunostaining for F4/80+CD206 is Panel A, CD11b & CD206 is Panel B, and F4/80 & CD11b is Panel C. Scale bar: 50 µm.

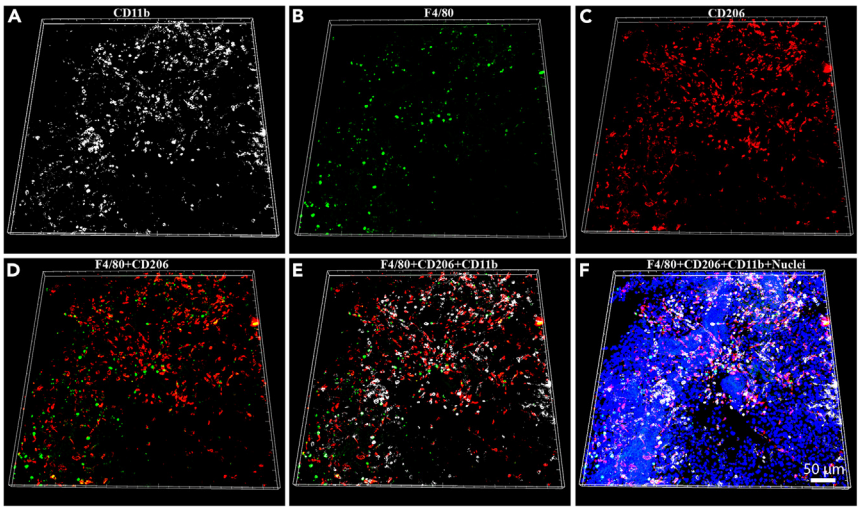

Figure 9. The confocal microscopy Z-stack projection images of mouse lacrimal gland stained for macrophage markers

Immunostaining for CD11b (Panel A pseudo-color gray), F4/80 (Panel B green), and CD206 (Panel C red) alone. Panel D is the F4/80 and CD206 merged image showing a small number of macrophages stained both for F4/80 and CD206 (yellow), a few stained for F4/80 only (green), and a large number stained for CD206 only (red). Panel E is CD11b, F4/80, and CD206 merged images showing that both F4/80 and CD206 positive macrophages are also positive for CD11b. Panel F shows nuclei stained blue with DAPI along with CD11b, F4/80, and CD206 staining. Scale bar = 50 µm.

several images from different areas of the cornea, conjunctiva, and lacrimal gland may be necessary to quantify macrophage count. Using flow cytometry instead of immunostaining may provide more rapid, less laborious, and accurate data if obtaining the macrophage count is the primary goal.

TROUBLESHOOTING

Problem 1

Bleeding during the dissection of conjunctiva and cornea (Pertaining to steps 1 &2).

Potential solution

Bleeding during dissection and the subsequent deposition of clotted blood on the conjunctiva or cornea may interfere with the quality of immunostaining. If bleeding occurs during the dissection, irrigate the tissue with 1× PBS and dab it with a gauze pad.

Problem 2

Presence of high autofluorescence (Pertaining to step 8).

Potential solution

This problem occurs if the tissue becomes dry while transferring from one step to the next or during the mount. Ensure a quick transfer of the tissue between dishes from one step to the next. Do not completely dry the tissue before mounting, and be prompt while mounting.

Problem 3

Low fluorescence signal while acquiring images using the confocal microscope (Pertaining to step 8).

Potential solution

It is important to incubate the tissue in the antibody for at least 18–24 h to obtain a good signal. Also, avoid re-imaging the same area to prevent bleaching due to laser exposure.

Problem 4

The presence of tiny debris particles that auto-fluoresce (Pertaining to step 8).

Potential solution

Filter the PBS and BSA solution through a 2 μm filter.

Problem 5

Suboptimal nuclear staining in the deeper layers of the tissue mount (Pertaining to step 8).

Potential solution

Allow DAPI to penetrate for at least 5 h before imaging.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Ajay Sharma (sharma@chapman.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Saleh Alfuraih (salfuraih@chapman.edu) and Ajay Sharma (sharma@chapman.edu).

Materials availability

No additional reagents or materials were generated for this protocol.

Data and code availability

No new data set or codes were generated for this protocol.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

S.A. optimized the immunostaining method, performed the experiments, and acquired the confocal images. D.P., W.C., and K.R. assisted in conducting experiments and in obtaining images for surgical steps of dissections. R.A. provided inputs for methods optimization. A.S. was responsible for experimental design, troubleshooting, supervising all study aspects, performing image analysis, creating the images for the results, writing the manuscript, and acquiring grant funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103444.

REFERENCES

1. Liu, J., Xue, Y., Dong, D., Xiao, C., Lin, C., Wang, H., Song, F., Fu, T., Wang, Z., Chen, J., et al. (2017). CCR2- and CCR2+ corneal macrophages exhibit distinct characteristics and balance inflammatory responses after epithelial abrasion. Mucosal Immunol. *10*, 1145–1159.

2. Saylor, J., Ma, Z., Goodridge, H.S., Huang, F., Cress, A.E., Pandol, S.J., Shiao, S.L., Vidal, A.C., Wu, L., Nickols, N.G., et al. (2018). Spatial Mapping of Myeloid Cells and Macrophages by Multiplexed Tissue Staining. Front. Immunol. *9*, 2925.

3. Alam, J., de Paiva, C.S., and Pflugfelder, S.C. (2021). Desiccation Induced Conjunctival Monocyte Recruitment and Activation - Implications for Keratoconjunctivitis. Front. Immunol. *12*, 701415.

4. Alam, J., Yazdanpanah, G., Ratnapriya, R., Borcherding, N., de Paiva, C.S., Li, D., and Pflugfelder, S.C. (2022). Single-cell transcriptional profiling of murine conjunctival immune cells reveals distinct populations expressing homeostatic and regulatory genes. Mucosal Immunol. *15*, 620–628.

5. Delcroix, V., Mauduit, O., Lee, H.S., Ivanova, A., Umazume, T., Knox, S.M., de Paiva, C.S., Dartt, D.A., and Makarenkova, H.P. (2023). The First Transcriptomic Atlas of the Adult Lacrimal Gland Reveals Epithelial Complexity and Identifies Novel Progenitor Cells in Mice. Cells *12*, 1435.

6. Wu, Y.F., Chang, N.W., Chu, L.A., Liu, H.Y., Zhou, Y.X., Pai, Y.L., Yu, Y.S., Kuan, C.H., Wu, Y.C., Lin, S.J., and Tan, H.Y. (2023). Single-Cell Transcriptomics Reveals Cellular Heterogeneity and Complex Cell-Cell Communication Networks in the Mouse Cornea. Invest. Ophthalmol. Vis. Sci. *64*, 5.

7. Hadrian, K., Willenborg, S., Bock, F., Cursiefen, C., Eming, S.A., and Hos, D. (2021). Macrophage-Mediated Tissue Vascularization: Similarities and Differences Between Cornea and Skin. Front. Immunol. *12*, 667830.

8. Lamy, R., Wolf, M., Bispo, C., Clay, S.M., Zheng, S., Wolfreys, F., Pan, P., and Chan, M.F. (2022). Characterization of Recruited Mononuclear Phagocytes following Corneal Chemical Injury. Int. J. Mol. Sci. *23*, 2574.