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Non-separate Mouse Sclerochoroid/RPE/Retina Staining and Whole Mount for the Integral Observation of Subretinal Layer

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[Abstract] The subretinal layer between retinal pigment epithelium (RPE) and photoreceptors is a region involved in inflammation and angiogenesis during the procession of diseases such as age-related macular degeneration. The current protocols of whole mounts (retina and RPE) are unable to address the intact view of the subretinal layer because the separation between retina and RPE is required, and each separate tissue is then stained. Non-separate Sclerochoroid/RPE/Retina whole mount staining was recently developed and reported. The method can be further combined and optimized with melanin bleaching and tissue clearing. Here, we describe steps of both non-pigmented and pigmented mouse Sclerochoroid/RPE/Retina whole mount including eyeball preparation, staining, mounting and confocal scanning. In addition, we present the confocal images of RPE, subretinal microglia and the neighboring photoreceptors in Sclerochoroid/RPE/Retina whole mounts.

Keywords: Whole mount, Subretina, Microglia, Retina pigment epithelium, Photoreceptors, Bleaching

[Background] The retina in eyes is surrounded by retina pigmented epithelium (RPE), choroid and sclera. Generally, in whole mount staining, the retina tissue is separate from the choroid/RPE, and each part of the separate retina and choroid/RPE is stained. Thus, the separate whole mount staining of either retina or choroid/RPE does not address integral subretinal information. Recently, a Sclerochoroid/RPE/Retina whole mount protocol was developed (Kim *et al.*, 2016; Ye *et al.*, 2020). The method allows us to observe the integral and intact subretina as well as the neighboring RPE and photoreceptors. The method is also applicable to examine choroidal neovascularization from the choroid to the photoreceptor layer, but there is still a limitation in laser penetration depth of confocal microscopes, restraining the application of this protocol in animals with large eyes, which could be countered by use of light-sheet fluorescence microscope (Renier *et al.*, 2014). Here, we illustrate the details of whole steps for immunofluorescent staining and mounting procedure of Sclerochoroid/RPE/Retina using mouse eyeballs.

Materials and Reagents

1. Kimtech wipers (Yuhan, catalog number: 47201; KimWipes, catalog number: 470224-038)
2. Micro spoon spatula (Scilab, catalog number: SL.Spa7011, L150, Bowl-3x5, Blade-5x50, Stem-Ø2.5mm)

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3. Transfer pipettes
4. 60 mm dish (SPL Life Sciences, catalog number: 10060; Tomas Scientific, catalog number: 1213F04)
5. 48-well plates (Tomas Scientific, catalog number: 1219Z64)
6. Industrial Single Edge Razor Blades (Dorco, catalog number: 4649676382; Polysciences, catalog number: 08410-1)
7. Cover glasses (Marienfeld-Superior, catalog numbers: HSU-0101152-22x60, 40, 32; ThermoFisher, catalog numbers: 12-545-J, C, B)
8. Microscope slides (Marienfeld-Superior, catalog number: HSU-0810001; ThermoFisher, catalog number: 12-544-7)
9. Mouse eye balls*
10. Dulbecco's phosphate buffer saline (D-PBS), pH 7.4 (Welgene, catalog number: LB 001-02; Fisher Scientific, catalog number: 14190250)
11. 4% paraformaldehyde (PFA) in PBS, pH 7.4 (Biosesang, catalog number: PC2031; Thermo Scientific, catalog number: J19943K2)
12. Hydrogen peroxide (H_2O_2) (30%, Sigma-Aldrich, catalog number: 216763)
13. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
14. Sodium Azide (Sigma-Aldrich, catalog number: S2002)
15. Bovine serum albumin (GenDEPOT, catalog number: A0100; Sigma-Aldrich, catalog number: A9647)
16. Antibodies and staining agents to label RPE, microglia and cell nuclei:
    RPE65 (Life Technologies, catalog number: MA1-116578, dilution 1:500)
    CD11b (Bio-Rad, formerly AbD Serotec, catalog number: MCA711, dilution 1:1,000)
    Phallodin-488 (Life Technologies, catalog number: A12379, dilution 1:500)
    Alexa Fluor 568-conjugated secondary antibody (Life Technologies, catalog number: A-11004, dilution 1:1,000)
    Hoechst 33342 (Life Technologies, catalog number: H3570, dilution 1:1,000)
17. Mounting buffer Fluoromount-G (Southern Biotech, catalog number: 0100-01)

Notes:

a. *The protocol was developed at National Eye Institute, National Institutes of Health (US) and has been adopted at ExosomePlus, Inc. (South Korea). We, thus, try to provide commercial catalog numbers used in US and South Korea.*
b. **Any mouse eyeball can be used, but the pigmented eyeballs need melanin bleaching (Kim et al., 2016).*

Equipment

1. Typing Forceps (McPherson, straight 5 mm, smooth 10.8 CM)

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2. No. 5 forceps (Dumont, Swiss, catalog numbers: B-0208-5-PS, B-0108-5-PS; Roboz, catalog number: RS4925)
3. Micro spring scissors (Nopa Instruments, catalog number: AC778/02; Roboz, catalog number: RS5601)
4. Shaker (Lab. Companion, model: SK-300)
5. [Option for bleaching] Heating block
6. Confocal microscope (Carl Zeiss Meditec, model: Zeiss-700)
7. Dissection stereo microscope (Kern-Shhn, model: OZL-45R; Carl Zeiss Meditec, Stemi 305/508)

Procedure

A. Preparation of Permeabilization/Blocking/Antibody dilution stock and working solutions
   1. 10% Triton X-100 in PBS, stock solution: total volume, 50 ml; 5 ml Triton X-100 in PBS.
   2. 10% sodium azide in distilled water (DW), stock solution: total volume, 50 ml; 5 g sodium azide in DW.
   3. 0.01% sodium azide in PBS for the storage of fixed eyeballs: total 500 ml; 1,000x dilution (500 µl) of 10% sodium azide in PBS.
   4. 0.5% Triton X-100, 1% BSA solution in 0.01% sodium azide PBS, working solution for blocking and dilution: total volume, 50ml; 20x dilution of 10% Triton X-100 (2.5 ml), 0.5 g BSA in 0.01% sodium azide PBS.

B. Fixation of Eyeballs
   1. Pull and enucleate eyeballs from mice with typing forceps and fix them in 4% PFA-PBS for 1 h at room temperature.
   2. Rinse them in PBS twice.
   3. Store eyeballs in 0.01% sodium azide PBS solution at 4 °C until use.
   4. [Option for bleaching of pigmented eyeballs] Put the pigmented whole eyeballs into 10% H_2O_2 for melanin bleaching at 4 °C for a week.
      Notes:
      a. *If you find too many bubbles in eyeballs due to the endogenous peroxidase activity, make sure to store fixed eyeballs at 4 °C over one night before H_2O_2 bleaching, and/or put the eyeballs into 1-3% H_2O_2 for 10-20 min first before 10% H_2O_2.*
      b. *Instead of this step, you can use '55 °C for 1-2 h' (see Procedure C).*

C. Preparation of posterior eyeballs-Sclerochoroid/RPE/Retina
   1. Prepare No. 5 forceps, spring scissors, wipers, a spatula, 60 mm dishes, 48-well plates, dissection microscope, PBS and transfer pipettes (Figure 1A).
   2. Remove optic nerve head and muscle tissues from the eyeballs on the PBS-moisten KIMTECH wipers under a dissection microscope, using micro-spring scissors and No. 5 forceps (Figure

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1B; Video 1).

3. Cut a circle at the ora serrata and remove the cornea and lens (Figures 1C, 1D; Video 2).
4. Rinse Sclerochoroid/RPE/Retina in PBS.
5. [Option for bleaching of pigmented eyeballs] Put Sclerochoroid/RPE/Retina into 10% H_2O_2 for melanin bleaching at 55 °C for 1-2 h, and rinse them in PBS.
   Note: Instead of this step, you can use '4 °C for a week' in Procedure B.
6. Before staining, put your Sclerochoroid/RPE/Retina tissues into a 48-well plate with PBS, using a spatula (Figures 1E, 1F).

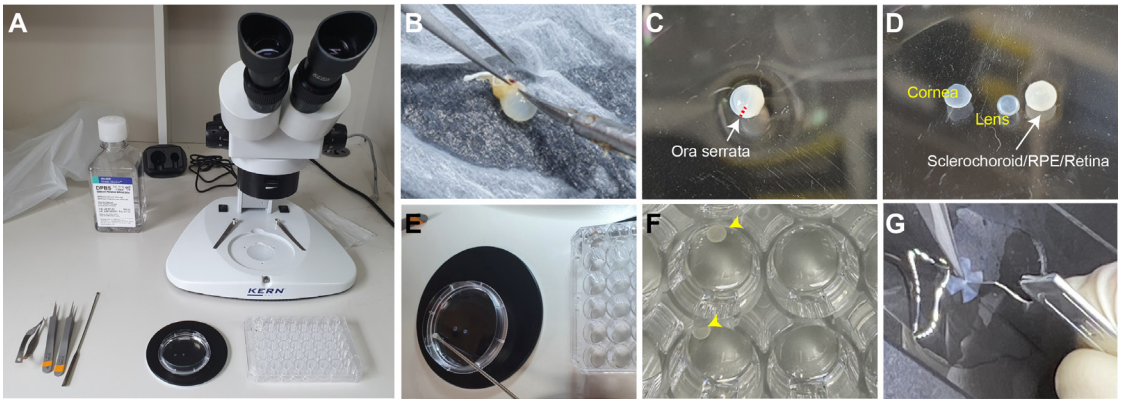
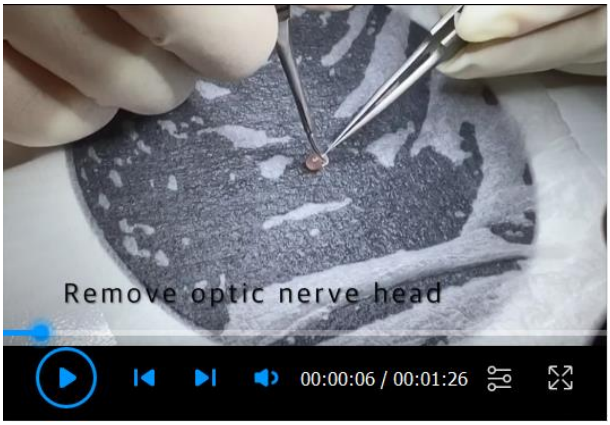


Figure 1. Preparation, staining and mounting of the Sclerochoroid/RPE/Retina. A. Essential items for the preparation of the Sclerochoroid/RPE/Retina. B. An eyeball on PBS-moisten KIMTECH wiper under a dissection microscope to remove optic nerve, muscles, cornea and lens (Video 1). C. Ora serrata. D. Cornea, lens and Sclerochoroid/RPE/Retina. E. Sclerochoroid/RPE/Retina tissues are transferred using a spatula. F. Sclerochoroid/RPE/Retina tissues in a 48-well plate during the staining (arrow heads). G. A quadrant mount of Sclerochoroid/RPE/Retina on a slide glass using a blade. RPE, retinal pigment epithelium.



Video 1. Eyeball preparation

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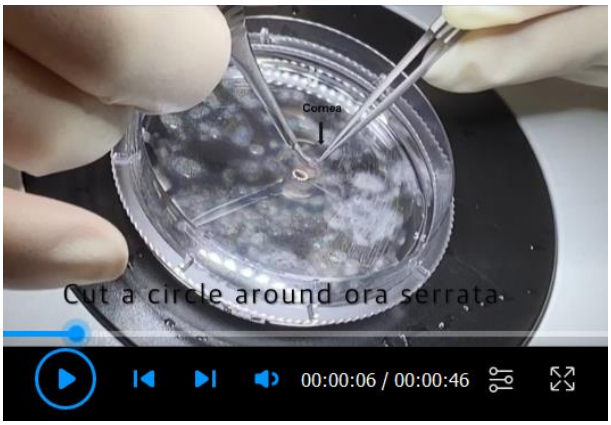

Video 2. Remove cornea and lens

D. Staining protocol

1. Permeabilize Sclerochoroid/RPE/Retina tissues in blocking solution at RT shaking (85 rpm) for 1-2 h.
2. Incubate Sclerochoroid/RPE/Retina in blocking solution containing primary antibodies (*e.g.*, RPE65, CD11b) at RT shaking (85 rpm) for 2 h to 6 days.
3. Wash Sclerochoroid/RPE/Retina three times in PBS at RT, shaking (85 rpm) for 10-15 min each.
4. Incubate Sclerochoroid/RPE/Retina in blocking solution containing fluorescent labeled secondary antibodies and other reagents such as Phallodin-488 and Hoechst 33342 at RT shaking (85 rpm) for 2 h to 6 days.
5. Wash Sclerochoroid/RPE/Retina three times in PBS at RT shaking (85 rpm) for 10-15 min each.
6. Put the stained Sclerochoroid/RPE/Retina on a glass slide with ganglion cell (GC) layer against the glass, cut into quadrants (Figure 1G) and mount with a coverslip.

   Notes:

   a. *Use enough volume of staining solution (over 250 µl), and washing solution (over 500 µl PBS).*
   b. *The incubation time of staining agents can be optimized. 6 days incubation is recommended for strong labeling intensity. At an antibody dilution of 1:1,000, fluorescence labeling intensities in Sclerochoroid/RPE/Retina staining are not saturated before 6 days (data not shown). Long incubation and strong fluorescence labeling allow us to easily observe the RPE layer in Sclerochoroid/RPE/Retina mounts using a fluorescent microscope before confocal scanning.*
   c. *In whole mount staining, the labeling intensity is stronger in the peripheral areas than center, due to different thickness and density (Figure 2)*
   d. *Use RT condition instead of 4 °C to save time (Figure 3).*
   e. *Do not apply Pressure-Related Efficient and Stable Transfer of Macromolecules into Organs (PRESTO) method (Lee and Sun, 2016). PRESTO (600 x g) has no benefit over RT shaking in this staining. We suspect that molecular penetration and diffusion will be different between brain and retina (Figure 4).*

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f. If you want to scan the inner retina such as ganglion cell layer, mount the stained Sclerochoroid/RPE/Retina on a glass slide with sclera layer against the glass.

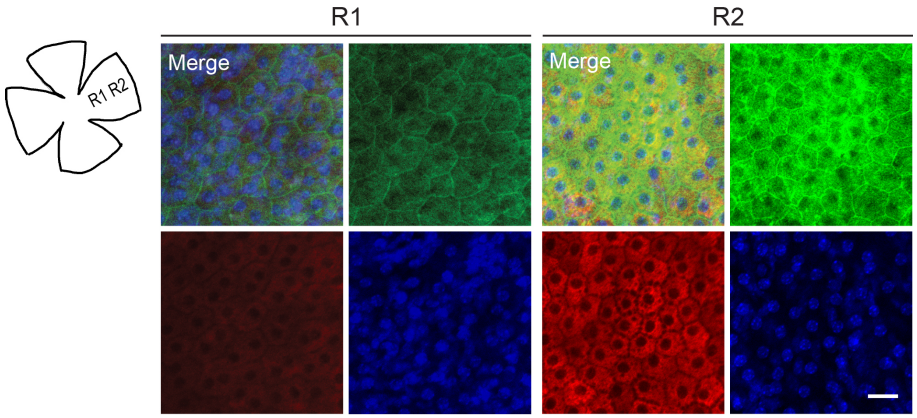

Figure 2. Different labeling intensity depending on the whole mount location. Images in RPE layer were scanned from center (R1) and peripheral (R2) regions of the quadrant whole mount (left). Sclerochoroid/RPE/Retina tissues were stained by phallodin-488 (top right), RPE65 antibody (bottom left) and Hoechst 33342 (bottom right) at RT, shaking for 2 h. RPE, retinal pigment epithelium. Scale bar, 20 μm.

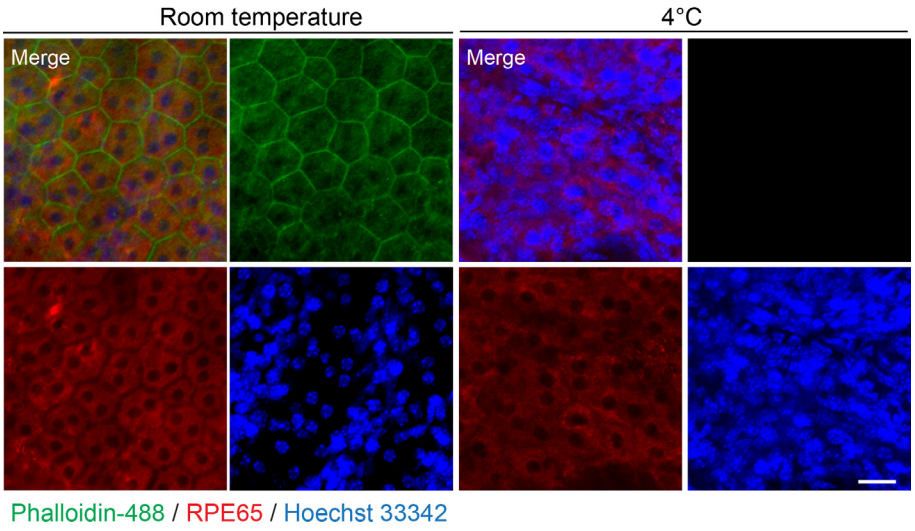

Figure 3. Different labeling intensity depending on the staining temperatures. Sclerochoroid/RPE/Retina tissues were stained by phallodin-488 (top right), RPE65 antibody (bottom left) and Hoechst 33342 (bottom right) at RT shaking for 6 days. Images in RPE layer were scanned from center. RPE, retinal pigment epithelium. Scale bar, 20 μm.

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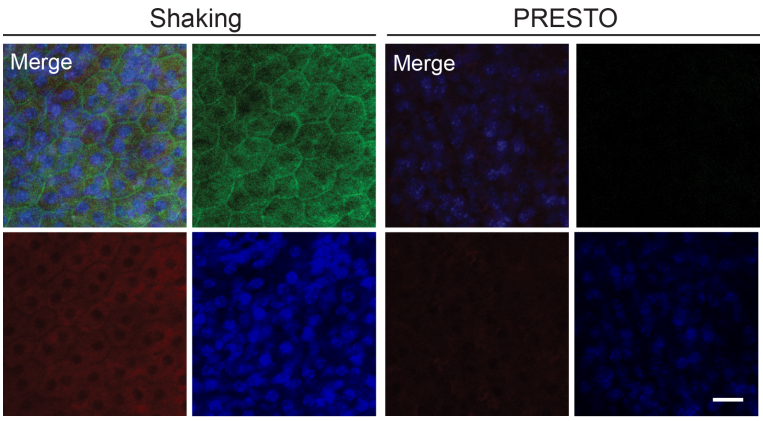

Figure 4. No benefit of PRESTO in Sclerochoroid/RPE/Retina staining. Sclerochoroid/RPE/Retina tissues were stained by phallodin-488 (top right), RPE65 antibody (bottom left) and Hoechst 33342 (bottom right) at RT, either shaking or using PRESTO (600 g), for 2 h. Images in RPE layer were scanned from center region. PRESTO, Pressure-Related Efficient and Stable Transfer of Macromolecules into Organs; RPE, retinal pigment epithelium. Scale bar, 20 μm.

E. Imaging

Scan horizontal view of any layer in Sclerochoroid/RPE/Retina whole mount: Single, z- and tiled-scan images can be captured under any objective lens of confocal microscope. 20x objective lens will be generally acceptable for this purpose.

Notes:

1. *This protocol has merit to observe the subretinal microglia or macrophages (Figure 5), which are separated to retina and/or choroid/RPE whole mounts in the general protocol. See additional resources for a demonstration of subretinal microglia and extended processes (Kim et al., 2016).*
2. *The protocol is recommended to be applied for the subretinal observation and to see the neighboring layers, but it could also address any retina layer, from sclera to GC layer (Figure 6).*

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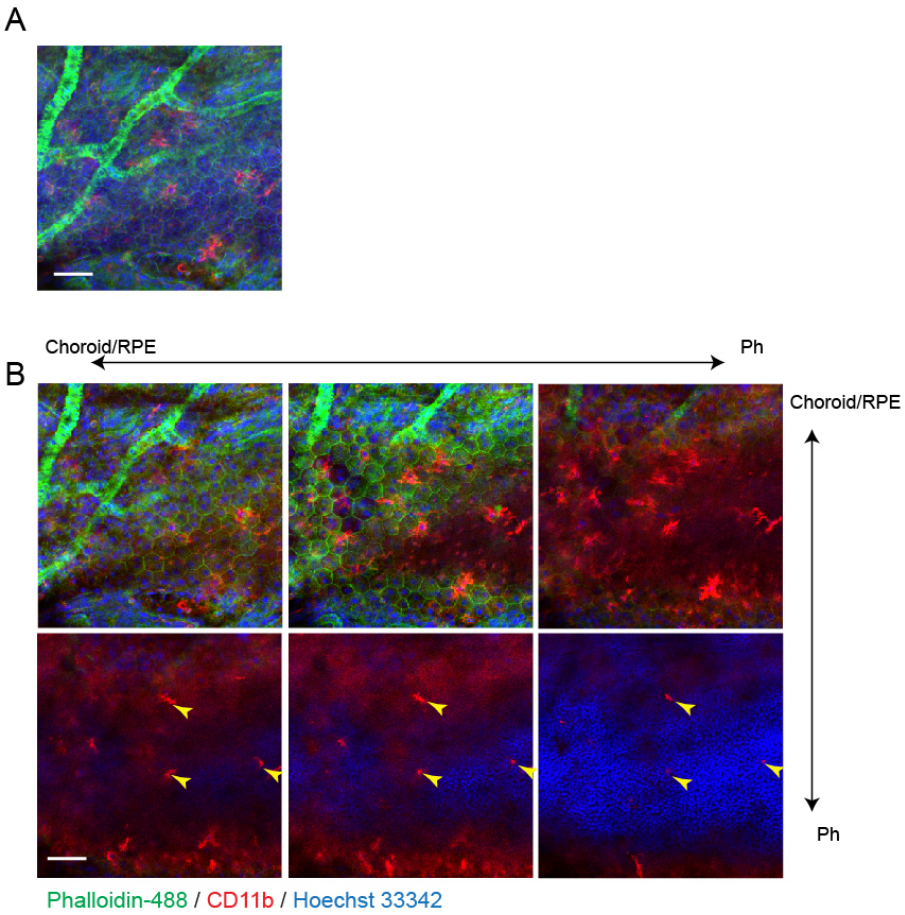

Figure 5. Subretinal microglia in Sclerochoroid/RPE/Retina whole mount. Sclerochoroid/RPE/Retina tissues are labeled by phallodin-488 (green), CD11b antibody (red) and Hoechst 33342 (blue). Images were captured from choroid/RPE to the upper level of photoreceptor layer, every 2 μm z-interval. A. A projected image. B. Images arranged at 4 μm interval. Arrow heads indicate migrating/extending processes of microglia from the photoreceptor layer to the subretina. Ph, photoreceptors; RPE, retinal pigment epithelium. Scale bar, 50 μm.

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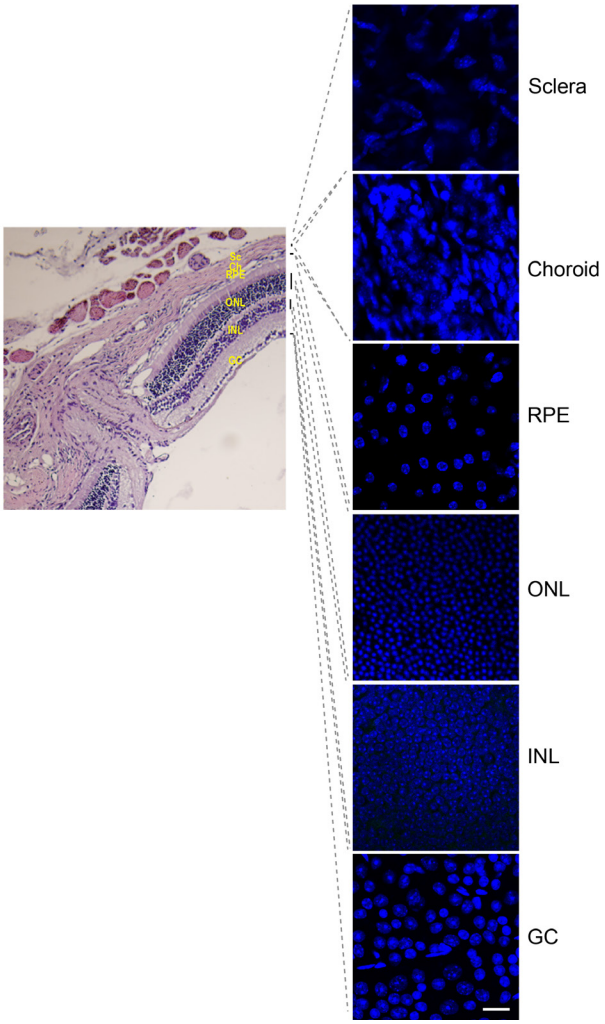

Figure 6. Cell nuclei in retinal layers from sclera to ganglion cell layer, stained by Hoechst 33342. Sc, Sclera; Ch, Choroid; RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GC, ganglion cell. Scale bar, 20 μm.

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Competing interests

No conflict of Interests.

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