Assessment and characterization of hyaloid vessels in mice

Zhongxiao Wang¹, Chi-Hsiu Liu¹, Shuo Huang¹, Jing Chen^{1,2}

¹Department of Ophthalmology, Boston Children's Hospital, Harvard Medical School, Boston, MA, US.

²The Manton Center for Orphan Disease Research, Boston Children's Hospital, Harvard Medical School, Boston, MA, US.

Abstract

In the eye, the embryonic hyaloid vessels nourish developing lens and retina, and regress when the retinal vessels develop. Persistent or failed regression of hyaloid vessels can be seen in diseases such as persistent hyperplastic primary vitreous (PHPV), leading to obstructed light path and impaired visual function. Understanding the mechanisms underlying the hyaloid vessel regression may lead to new molecular insights in the vascular regression process and potential new ways to manage diseases with persistent hyaloid vessels. Here we describe the procedures for imaging hyaloid in live mice with optical coherence tomography (OCT) and fundus fluorescein angiography (FFA) and detailed technical protocol of isolating and flat mounting hyaloid *ex vivo* for quantitative analysis. Low-density lipoprotein receptor-related protein 5 (LRP5) knockout mice were used as an experimental model of persistent hyaloid vessels, to illustrate the techniques. Together these techniques may facilitate thorough assessment of hyaloid vessels as an experimental model of vascular regression, and studies on the mechanism of persistent hyaloid vessels.

SUMMARY:

This protocol describes both *in vivo* and *ex vivo* methods to fully visualize and characterize hyaloid vessels, a model of vascular regression in mouse eyes, using optical coherence tomography (OCT) and fundus fluorescein angiography (FFA) for live imaging and *ex vivo* isolation and subsequent flat mount of hyaloid for quantitative analysis.

Keywords

Eye; hyaloid vessels; vascular regression; flat mount; development; LRP5; Wnt

INTRODUCTION:

The blood supply in the eye is essential to ensure the normal development of the retina and surrounding ocular tissues, and to equip a proper visual function. There are three vascular

Corresponding Author: Jing Chen at jing.chen@childrens.harvard.edu.

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beds in the eye: the retinal vasculature, the choroid, and a transient embryonic circulatory network of hyaloid vessels. Development of the ocular vasculature requires spatial and temporal coordination throughout embryogenesis and tissue maturation. Among the three vascular beds, the hyaloid vasculature is the first functional blood supply system to provide nutrition and oxygen to the newly-formed embryonic lens and the developing retina. Hyaloid vessels regress at the same time that the retina vasculatures develop and mature¹. The regression of hyaloid vasculature is pivotal to allow a clear visual pathway for the development of visual function; hence this vascular regression process is as important as the growth of retinal vasculature. Impaired hyaloid regression may lead to eye diseases. Moreover, regression of hyaloid vessels provides a model system to investigate the cellular and molecular mechanisms involved in the regulation of vascular regression, which may have implications for angiogenic regulation in other organs as well.

The hyaloids vasculature, derived from the hyaloid artery (HA), is composed of vasa hyaloidea propria (VHP), tunica vasculosa lentis (TVL), and pupillary membrane (PM). It provides nourishment to the developing retina, the primary vitreous, and the lens during embryonic development². Arising from HA, VHP branches anteriorly through the vitreous to the lens. The TVL cups the posterior surface of the lens capsule, and anastomoses to the PM which connects to the anterior ciliary arteries, covering the anterior surface of the lens^{2,3}, resulting in the formation of a network of vessels in the PM³⁻⁵. Interestingly, there are no veins in the hyaloid vasculature, and the system makes use of choroidal veins to accomplish venous drainage.

In the human embryo, the hyaloid vasculature is nearly complete at approximately the ninth week of gestation, and starts to regress when the first retinal vessels appear, during the fourth month of gestation². Beginning with atrophy of the VHP, regression of the capillary networks of the TVL, the PM, and lastly the HA occurs subsequently^{2,3}. Meanwhile, the primary vitreous retracts; and the secondary vitreous starts to form, composed of the extracellular matrix components, including collagen fibers. By the sixth month of gestation, the primary vitreous is reduced to a small transparent canal extending from the optic nerve disc to the lens, called the Cloquet's canal or hyaloid canal, and the secondary vitreous becomes the main component of the posterior segment^{2,3}. The hyaloid circulation vanishes mostly at 35 to 36 weeks of gestation, just before birth³.

Unlike humans, in whom hyaloid vasculature is completely regressed at birth, the mouse hyaloid vascular system starts to regress after birth. As the mouse retina is born avascular, and retinal vessels develop postnatally, hyaloid vessels regress concurrently from postnatal day (P) 4 and complete mostly by day 21⁶ (Figure 1). The PM disappears first between P10 and P12, and the VHP disappears between P12 and P16, while a small number of TVL and HA cells remain even at P16, and by P21 the hyaloid vascular system regression is almost complete⁶. In the meantime, retinal vasculature begins developing after birth. The superficial layer of vascular plexus fully extends to the peripheral retina at P7-P8, the deep layer (located in the outer plexiform layer) develops from P7-P12, and finally the intermediate plexus in the inner plexiform layer develops, between P12 and P15⁷. As the retinal vasculature develops, it gradually replaces the function of concomitantly regressing hyaloid vessels, providing nutrition and oxygen to the developing eye. The postnatal occurrence of

hyaloid vessel regression in mice provides an easily accessible experimental model to observe and study the hyaloid vasculature as well as the molecular basis governing vascular regression processes under both physiological and pathological conditions⁸.

Failure of hyaloid regression can be seen in diseases such as persistent hyperplastic primary vitreous (PHPV), which is a rare congenital developmental anomaly of the eye resulting from failed or incomplete regression of the embryological, primary vitreous and hyaloid vasculature⁹. The mechanisms regulating the regression process of hyaloid vasculature are complicated and broadly studied. One major molecular pathway essential for normal regression of hyaloid vessels is the Wnt signaling pathway¹⁰, as genetic mutations in this pathway affecting both Wnt ligand and receptors have been linked with PHPV in humans⁹. Experimental studies identified a Wnt ligand, Wnt7b, which is produced by macrophages around hyaloid vessels in the developing eye to mediate this regression process. Wnt7b activates Wnt signaling by binding with the receptors frizzled4 (FZD4)/LRP5 in adjacent endothelial cells to initiate cell apoptosis, and leading to the regression of hyaloid vessels¹⁰. As a result, *Wnt7b* deficient mice show persistence of hyaloid vessels¹⁰. Similarly, a nonconventional Wnt ligand, Norrin (encoded by *Ndp* gene), also binds to FZD4/LRP5 to induce the hyaloid vessel regression during development. *Ndp*^{+/−}, *Lrp5*^{−/−}, and *Fzd4*^{−/−} mice all display postponed hyaloid vessel regression, support a critical regulatory role of Wnt signaling^{11–16}. Moreover, another Wnt co-receptor LRP6 overlaps with LRP5 in their function on modulating the Wnt signaling pathway in hyaloid vascular endothelial cells¹⁷. Other factors that may also contribute to hyaloid regression include the hypoxia-inducible factor^{18,19}, vascular endothelial growth factor^{20,21}, collagen-18^{22,23}, Arf²⁴, angiopoietin-2²⁵, and bone morphogenetic protein-4²⁶. In this paper, we used *Lrp5*^{−/−} mice as a model of persistent hyaloid vessels to demonstrate the techniques of assessing and characterizing hyaloid vasculature through both *in vivo* and *ex vivo* methods.

Visualization of hyaloid vasculature *in vivo* and *ex vivo* is essential for studying the mechanisms of hyaloid vessel regression. Current methods to observe hyaloid vasculature mainly focus on visualizing and analyzing VHP and HA, including optical coherence tomography (OCT) and fundus fluorescein angiography (FFA) images, eye cross section, and hyaloid flat mount. OCT and FFA are powerful *in vivo* imaging tools, allowing longitudinal observation in live animals after eye opening. Moreover, isolated hyaloid flat mount provides visualization of the whole hyaloid vasculature and a means to achieve accurate quantification of the vessel numbers. Yet the delicate and fragile nature of hyaloid vessels and resulting technical difficulties of its isolation may have limited its use in eye research somewhat^{10,17,27}. In this paper, we provide a detailed protocol of visualization of hyaloid vessels combining both *in vivo* live retinal imaging and *ex vivo* isolated hyaloid flat mount to enhance the feasibility of these techniques, which was adapted with modification and expansion from previous publications on *in vivo* method of live fundus and OCT imaging²⁸ and *ex vivo* method of isolated hyaloid flat mount¹¹.

PROTOCOL:

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research for animal experiments, following the Guidelines of the

NIH regarding the care and use of animals for experimental procedures and the regulations set forth by the Institutional Animal Care and Use Committee (IACUC) at Boston Children's Hospital. *Lrp5*^{-/-} mice (stock no. 005823; Jackson Laboratory, developed by Deltagen Inc.) and its wild type (WT) control C57BL/6J mice (stock no. 000664; Jackson Laboratory) were used for this study.

1. Part I: *In vivo* imaging of hyaloid vessels using a rodent retinal imaging system

1.1 Preparation of mice: anesthesia and pupil dilation.

1.1.1. Select mice:

1.1.1.1. Use mice that are older than P12 (after eye opening) for retinal imaging; both sexes are suitable.

1.1.1.2. As desired, image mutant mice with delayed hyaloid regression (and their WT controls) throughout adulthood. WT mice older than 3 weeks may not exhibit much detectable remaining hyaloid vessels, hence P12-P21 is ideal for imaging remaining visible WT hyaloid.

1.1.2. Prepare ketamine/xylazine mixture for anesthesia:

1.1.2.1. Take 2.3 ml ketamine stock solution (100 mg/ml), 0.7 ml xylazine stock solution (20 mg/ml), and add 20 ml sterile saline (0.9% sodium chloride) to making a working solution of ketamine/xylazine mixture (10 mg/ml ketamine and 0.6 mg/ml xylazine).

1.1.3. Anesthetize mice by intraperitoneally injecting ketamine/xylazine mixture in the volume of 8 times mouse body weight, e.g., a 20-gram mouse needs 160 μ l ketamine/xylazine working solution mixture to achieve a working dose of ketamine (80 mg/kg body weight) and xylazine (4.8 mg/kg body weight) mixture.

1.1.4. Apply one drop of pupil-dilating drug solution (see Table of Materials) per eye to dilate mouse pupils immediately following anesthesia.

1.1.5. Assess level of anesthesia by pedal reflex (firm hind limb toe pinch). Wait until the mouse is sufficiently anesthetized (no pedal reflex) and its pupils are widely dilated.

1.2. Optical coherence tomography (OCT) imaging of hyaloid vessels.

1.2.1. Moisturize mouse corneas with artificial tears, then place the mouse on the position stage.

1.2.2. Gently contact mouse cornea with the lens of OCT probe with. Adjust the mouse and probe so that the optic nerve head is in the center of the visual field in the fundus image, to guide the OCT imaging.

NOTE: For more details on the general setup of rodent retinal imaging system (see Table of Materials) and adjusting mouse position, please refer to Gong et al. 2015²⁸.

1.2.3. Adjust focus to achieve the optimal images of OCT.

1.2.4. Adjust angle of indicated line in the OCT imaging software (see Table of Materials) to reveal persistent hyaloid vessels and then take images.

1.3. Fundus fluorescein angiography (FFA) imaging of hyaloid vessels.

1.3.1. Prepare fluorescein solution: add 9 ml sterile PBS to 1 ml fluorescein solution (stock concentration: 100 mg/ml). The concentration of final working solution is 10 mg/ml.

1.3.2. Intraperitoneally inject fluorescein working solution (5 μ l/g body weight).

1.3.3. Moisturize mouse cornea with artificial tears, then place the mouse on the position stage.

1.3.4. Position the lens of Micron IV retinal imaging microscope to make direct gentle contact with the mouse cornea. Adjust the alignment slightly to position the optic nerve head in the center of the view field.

1.3.5. Change microscope filter to green fluorescent channel.

1.3.6. Focus on persistent hyaloid vessels to take images.

1.3.7. Take multiple images after 1 min, 3 min, 5 min and 10 min (no more than 10 min) post-injection to determine the best time point (signal/background ratio) for observing hyaloid vessels. It is ideal to complete the FFA procedure within 10 min, after which the fluorescein may become too diffused and make vessels invisible.

1.4. Recovery of mice from anesthesia:

1.4.1. After procedures, keep mice on a warm heating pad.

1.4.2. Wait until the mice are mobile again to return to the mouse cage.

2. Part II: *Ex vivo* visualization of hyaloid vessels

2.1. Preparation of fixed mouse eyes.

2.1.1. Mice selection and age consideration: Neonatal mice are typically sacrificed at postnatal day 8 (P8) for hyaloid dissection. Both sexes are suitable. Mutant mice with delayed hyaloid regression (and their respective WT controls) may be dissected and analyzed throughout adulthood.

2.1.2. Euthanize mice by exposure to CO₂.

2.1.3. Enucleate the mouse eye by blunt dissection.

2.1.4. Open the eyelids wide with microsurgery forceps to allow access to the eyeball. Place curved microsurgery forceps under the globe in the orbit to grasp the optic nerve, without squeezing the eye ball. Gently pull and remove the eye ball with the forceps.

2.1.5. Alternatively, dissect the eyeball by using microsurgery scissors to carefully cut parallel to the globe from the four sides towards the back of orbit and making the globe separate from surrounding connective tissues.

2.1.6. Immerse eyes in 4% paraformaldehyde in phosphate-buffered saline (PBS) buffer for 30 min at room temperature for fixation.

2.1.7. Transfer fixed eye balls to ice-cold PBS buffer. The eye balls may be stored in PBS at 4°C for up to 1 week.

2.2. Embedding hyaloid vessels with gelatin injection.

2.2.1. Prepare 5% (w/v) gelatin solution.

2.2.1.1. Weigh 50 mg gelatin.

2.2.1.2. Dissolve gelatin in 1 ml distilled water.

2.2.1.3. Incubate gelatin solution in 37°C water bath until fully dissolved. Keep the solution in 37°C water until use. A larger batch of solution may be prepared and stored at 4°C as aliquots. Every time before use, the solution needs to be warmed in 37°C water bath to achieve a clear consistency.

2.2.2. Under a dissection microscope, inject 50 µl of gelatin solution into the vitreous body at the limbus; repeat injection 3 more times at different sites to make a total of 4 injections, evenly spaced around limbus (Figure 2A).

2.2.3. Incubate the eye balls in 4 °C refrigerator or on ice for 30 min to solidify injected gelatin in the vitreous space.

2.3. Dissection and isolation of hyaloid vessels (Figure 2B–E).

2.3.1. Place eye balls in a petri dish of PBS to keep tissue from drying. Make an incision with microsurgery scissors at limbus and remove cornea.

2.3.2. Snip and remove optic nerve.

2.3.3. Under a dissection microscope, use two pairs of forceps to peel off and discard sclera, choroid and retinal pigment epithelium (RPE) layers and remove iris.

2.3.4. With optic nerve side of the retina facing up and lens side down, inject 50 µl of PBS just beneath the retina cup to allow accumulation of the PBS buffer between gelatinized vitreous body and the retina.

2.3.5. Gently peel off and remove retina cup and ciliary body from the vitreous body with microsurgery forceps.

2.3.6. Turn the rest of tissue (lens/hyaloid) over, so the lens side is facing up.

2.3.7. Lift lens and gently loosen the connection between lens-TVL and VHP, then cut HA with microsurgery scissors to remove lens-TVL. Keep the VHP part of hyaloid cup for flat mount.

2.4. Flat mounting and staining of hyaloid vessels.

2.4.1. Transfer gelatin cup containing hyaloid tissue immersed in PBS onto a microscope slide using transfer pipette.

2.4.2. Gently rinse hyaloid cup with PBS on the slide to remove all dissection debris.

2.4.3. Make sure hyaloid cup is floating in adequate PBS solution on the slide.

2.4.4. Gently arrange and adjust position of gelatinized hyaloid cup with microsurgery forceps, with the rim of cup facing down on the slide, to achieve optimal appearance after melting.

2.4.5. Place slide with hyaloid cup immersed in PBS onto a slide warmer at 37°C, wait until the gelatin melts and hyaloid flattens, and remove slide when barely dry (Do not over-dry).

2.4.6. (Optional) Immunostaining of hyaloid vessels:

2.4.6.1. Make blocking and penetrating buffer (e.g., 5% BSA and 0.1% Triton X-100 in PBS buffer). Add 50 µl blocking buffer onto a flat mounted hyaloid isolation, incubate at room temperature for 30 min.

2.4.6.2. Apply 50 µl primary antibody (e.g.: CD31) (1:100 dilution in blocking buffer) onto a flat mount of hyaloid isolation, then incubate in a wet box at 4°C overnight.

2.4.6.3. Gently rinse with PBS three times, for 5 min each time.

2.4.6.4. Apply 50 µl secondary antibody (e.g., goat anti-rabbit secondary antibody-Alexa 488, 1:100 dilution) onto the hyaloid flat mount, and incubate at room temperature for 1 hour.

2.4.6.5. Gently rinse with PBS three times, for 5 min each time.

2.4.7. Add a drop of anti-fade mounting medium with DAPI (4', 6-diamidino-2-phenylindole) to stain the nuclei in hyaloid vessels.

2.4.8. Gently place a coverslip on the hyaloid to complete the flat mount.

2.5. Imaging and quantification of flat mounted hyaloid vessels.

2.5.1. Image the DAPI staining of hyaloid vessels with a fluorescent microscope and make sure the central HA is visible (exclude the samples without the HA).

2.5.2. Identify vessel branches directly derived from the HA and manually count the number of vessel branches for quantification.

2.6. Visualization of hyaloid vessels in cross-section of eyes.

2.6.1. Embed fixed eye balls into optimal cutting temperature compound in a suitable tissue mold, then freeze the embedded eyes on dry ice or store in -20°C freezer.

2.6.2. Use cryostat microtome to make cross-section of embedded eyes into sections of 12 µm thickness at -20°C.

2.6.3. Collect eye cross-sections onto a room temperature microscope slide by gently touching the tissue sections, which will adhere to the slide.

2.6.4. Air dry to dehydrate tissue sections for ~30 min, which can then be stored in -20°C freezer until ready for staining.

- 2.6.5. Rinse the slide with PBS once to remove the remaining optimal cutting temperature compound on the slide.
- 2.6.6. (Optional) Immerse slide into an appropriate fixative buffer (e.g., 4% paraformaldehyde in PBS) for 15 min at room temperature for additional fixation if needed.
- 2.6.7. Permeabilize tissue with 0.1% triton X-100 in PBS for 30 min at room temperature.
- 2.6.8. Prepare isolectin-IB₄ staining buffer for blood vessel staining.
- 2.6.9. Dissolve and reconstitute isolectin-IB₄ (500 µg) powder with 50 mL PBS in a 50 mL centrifuge tube.
- 2.6.10. Slowly add 50 µl of 1M CaCl₂ solution, drop by drop, into the 50 mL PBS/isolectin-IB₄ mixture. This step needs to be performed slowly to avoid precipitation. Final concentration of CaCl₂ is 1 µM. Presence of Ca²⁺ is required for isolectin-IB₄ staining.
- 2.6.11. Apply 30 µl isolectin-IB₄ staining buffer onto eye cross-sections on slide and incubate in a wet box at 4°C overnight.
- 2.6.12. Rinse with PBS three times, 5 min each time.
- 2.6.13. Add a drop of anti-fade mounting medium with DAPI to stain the nuclei in the sections.
- 2.6.14. Gently place a coverslip on the tissue section to complete the flat mount.
- 2.6.15. Check under microscopy to visualize presence of hyaloid vessels within eye cup.

REPRESENTATIVE RESULTS:

***In vivo* imaging of hyaloid vessels in live mice.**

Figure 3A reveals cross-sectional views of OCT images for the retina and hyaloid tissues in 3-month-old WT and *Lrp5*^{-/-} mice, an animal model with persistent hyaloid. WT eye shows absence of hyaloid tissue, whereas *Lrp5*^{-/-} eye shows two persistent hyaloid vessels derived from optic nerve head. Figure 3B displays FFA images of persistent hyaloid vessels (green) in the fluorescent field in 6-week-old *Lrp5*^{-/-} mice. WT mouse shows no remnant of hyaloid vessels, and *Lrp5*^{-/-} mouse shows eight branches of hyaloid vessels in the vitreous body.

***Ex vivo* visualization of hyaloid vessels.**

Figure 4A&B demonstrates isolated hyaloid vessels as visualized in flat mounts, where vascular cells and associated macrophages were stained by their nuclei with DAPI staining (blue). HA is at the center of each image, and hyaloid vessels are revealed by the DAPI-derived discontinuous lines. Each line represents one vessel of VHP. In *Lrp5*^{-/-} mice, higher numbers of remaining hyaloid vessels were observed at P8 in flat mounts (Figure 4A). WT mice have an average of 12 branches of hyaloid vessels at P8, whereas age-matched *Lrp5*^{-/-} pups show around 25 branches of hyaloid vessels, demonstrating significantly impaired

regression of hyaloid vasculature (Figure 4B). In addition, delayed and incomplete retina vascular development, another characteristics often associated with persistent hyaloid vessels, was also observed in *Lrp5*^{-/-} pups (Figure 4C&D). Figure 5 shows remaining hyaloid vessels in cross sections of *Lrp5*^{-/-} eyes at P8, whereas the WT eyes do not display hyaloid vessels.

DISCUSSION:

Techniques to assess and characterize hyaloid vessels are intuitive and necessary procedures to observe the hyaloid vessel regression in animal models, to allow studies on the mechanisms underlying the vascular regression during development. While the *in vivo* retinal imaging allows longitudinal observation of hyaloid regression in the same animal, access to a rodent fundus imaging system for OCT and FFA may be a limiting factor. In addition, *in vivo* imaging in live mice is not feasible before eye opening. Therefore this methodology is not applicable during eye development in the neonatal stage. On the other hand, while imaging cross-sections of isolated eyes may be used for any age of the mouse and has a low technical barrier, it is not quantitative when there are only a few visible hyaloid vessels, and the chance of obtaining an ideal image depends largely on the angle of sectioning (Figure 5). In comparison, isolation of hyaloid vessels for flat mount visualization allows complete imaging of the whole hyaloid vessel and accurate quantification, yet technical challenges may exist due to the delicate and fragile nature of hyaloid vessels. We hope the protocol detailed in this paper helps overcome these challenges.

Several critical steps in the hyaloid isolation protocol include injection of gelatin solution, removal of retina, and final flat mounting. The technical difficulty of handling hyaloid vasculature lies in its delicate nature, the almost liquid-like state of vitreous where hyaloid vessels reside, and its close connection with adjacent lens and the retina. Injecting gelatin solution intravitreally is key to solidifying the vitreous body containing hyaloid vessels and thereby making their texture firmer for easier dissection and handling. Gelatin is a gelling agent forming transparent elastic thermoreversible gels. By injecting the liquid form of gelatin (room temperature or 37°C) into the vitreous space and cooling to lower temperature (4°C), the vitreous body transforms into a firmer gel cup. Performing multiple gelatin injections into the eye allows the full dispersion of sufficient amount of the gelatin into the vitreous space, to form a uniform and round gelatinized hyaloid tissue cup. Another technical difficulty is in removing the retina without damaging hyaloid tissue. Unlike the choroid, which is relatively easy to dissect apart, the gelatinized vitreous body containing hyaloid vessels is challenging to separate from the nearby retina without damaging the hyaloid. We found that injecting PBS into the space between the vitreous body and retina generated a liquid buffer layer, making it much easier to separate these two tissues. This is somewhat similar to the hydrodissection technique used in cataract surgery to separate capsule and cataract cortex. The final flat mounting is the last technically difficult step of the protocol. Warming the gelatinized hyaloid tissue in a PBS droplet on a slide warmer converts it back to more liquid-like form to allow easier flat mounting. Proper arrangement and orientation of the hyaloid gel cup is still essential to ensure its even flattening after melting and drying.

The protocol of isolating hyaloid vessels may be further modified in several ways. The concentration of gelatin solution we used is 5%, yet higher or lower concentrations may also work, depending on the preference of the researcher to achieve firmer or softer texture for handling. DAPI staining of cellular nuclei may also be modified with isolectin-IB4 or other endothelial cell or macrophage markers, to better distinguish vascular endothelial cells from macrophages. A word of caution: the flat mounted hyaloid vessels are very delicate and only loosely adhered to the slide, hence the hyaloid slides need to be handled very gently and carefully if rinsing is needed during staining. Limitations of this isolation protocol also exist, including the complexity of performing the fine dissection and the difficulty of long-term preservation of samples due to the nature of fragile tissue. Nevertheless, isolating and flat mounting hyaloid vessels is still the most comprehensive and intuitive way to study hyaloid vasculature, to promote ocular and angiogenesis studies^{10,17,27,29–31}.

The hyaloid vasculature provides an excellent experimental model of studying developmental vessel growth and regression, relevant for research fields in ophthalmology, angiogenesis and programmed cell death, with which this paper aims to assist. Future modification of the isolation protocol may be directed at improving the feasibility of technical dissection procedure. Overall, combined *in vivo* imaging and *ex vivo* isolation of hyaloid vessels are advantageous methods to allow full assessment and characterization of hyaloid vessels in mice, as a useful model of vascular regression.

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Figure 1: Schematic diagram depicting the developmental regression of hyaloid vasculature in mouse eyes.

Hyaloid vessels and branches, including VHP, TVL and PM, are derived from HA, and occupy much of the space between lens and immature retina at birth (P0). Hyaloid involution in mice starts from regression of PM capillaries as early as P4. At P8, PM, VHP and TVL layers are continuously regressing, coinciding with complete formation of superficial layer of the retinal vessels. By P12, the involution of the PM layer is complete, whereas atrophy of VHP and TVL is still in progress. In the meantime, a deep layer of retinal vessels starts to form during P7 to P12. By P16, the regression of hyaloid system is partially completed (with remaining TVL vessels and HA left), and intermediate layer of retinal vascular plexus continues to develop. The retinal vasculature is fully mature by P21 and takes over the role of nourishing retinal tissues from hyaloid vessels, which are now mostly regressed. At P21 the vitreous in the absence of hyaloid shows a clear visual pathway. Dashed red lines represent the regressing vessels. VHP, vasa hyaloidea propria; TVL, tunica vasculosa lentis; PM, pupillary membrane; HA, hyaloid artery.

Figure 2: Schematic diagram depicting the sequential procedure of hyaloid vessel isolation for *ex vivo* flat mounting visualization.

A: Injection of gelatin through four injecting points (red dots) at the limbus into the enucleated eye to gelidify the vitreous body. **B:** Removal of cornea and optic nerve, careful dissection of iris, and peeling off sclera-choroid-RPE complex. **C:** Flipping remaining retinal cup with lens to make the retina side facing up, then injecting PBS between the retina and vitreous body to separate them, followed by peeling off the retinal layer. **D:** Flipping again upside down the rest of tissue containing lens with surrounding hyaloid. Lifting the lens slightly to loosen the connection of TVL and VHP. **E:** Cutting at the HA between TVL and VHP and removal of lens and TVL. Flat mounting VHP layer. VHP, vasa hyaloidea propria; TVL, tunica vasculosa lentis; PM, pupillary membrane; HA, hyaloid artery.

Figure 3: *In vivo* imaging of hyaloid vessels with optical coherence tomography (OCT), and fundus fluorescence angiography (FFA).

A: Representative OCT images of WT and *Lrp5*^{-/-} mice at 3-month-old showing persistent hyaloid vessels in the vitreous space in *Lrp5*^{-/-} eyes. **B:** Representative FFA images of WT and *Lrp5*^{-/-} mice at 6-weeks-old. Mice were injected with 1mg fluorescein sodium in 0.1ml saline per mouse after anesthesia, and images were taken 5 min after injection, focused on hyaloid vessels in *Lrp5*^{-/-} mice or vitreous body in WT (in the absence of hyaloid vessels). Persistent hyaloid vessels were visualized in *Lrp5*^{-/-} but not WT eyes. Red arrows indicate hyaloid vessels. Both scale bars: 100 μm (A).

Figure 4: Visualization of delayed hyaloid vessel regression and delayed retinal vasculature development in *Lrp5*^{-/-} mice.

A: Representative images of flat-mounted hyaloid vessels stained with DAPI (blue) in WT and *Lrp5*^{-/-} eyes at P8. Arrows (white) indicate the hyaloid artery. **B:** Quantification of the numbers of hyaloid vessels branching from the hyaloid artery in WT and *Lrp5*^{-/-} eyes. **C:** Representative flat-mounted retinas stained with isolectin-IB4 (red) for vasculature in WT and *Lrp5*^{-/-} eyes at P8. White dashed lines indicate retina edge. Yellow lines indicate the vascularized area edge. **D:** Quantification of vascular coverage of the superficial retinal vascular plexus in WT and *Lrp5*^{-/-} eyes. $n = 8-12/\text{group}$. All scale bars: 1 mm (**A**, **C**). Data are shown as mean $\pm$ SEM. Two-tailed Student's *t*-test was used for statistical analysis. $**P < 0.01$. This figure was modified with permission from Wang et al., Am J Pathol, 2016²⁷.

Figure 5: Visualization of hyaloid vessels in cross sections of *Lrp5*^{-/-} eyes.

Representative images of cross-sections of eyes isolated from WT and *Lrp5*^{-/-} mice at P8. Eyes were enucleated and embedded in optimal cutting temperature, and sections were cut using cryostat. Cross sections were stained with DAPI (blue) to show the nuclei and isolectin B4 (red) to show the blood vessels. White arrows indicate hyaloid vessels. Scale bar: 500 μ m. Figures were adapted with permission from Chen et al., PLoS One, 2012¹⁶.

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
AK-Fluor® (fluorescein injection, USP)	Akorn	17478-253-10	
Anti-CD31 antibody	Abcam	ab28364	
Antifade mounting medium	Thermo Fisher	S2828	
Antifade Mounting Medium with DAPI	Vector Laboratories	H-1200	
Artificial tear eyedrop	Systane	N/A	
Bovine serum albumin (BSA)	Sigma-Aldrich	A2058	
C57BL/6J mice	The Jackson Laboratory	Stock NO: 000664	
Calcium chloride (CaCl ₂)	Sigma-Aldrich	C1016	
Cryostat	Leica	CM3050S	
Cryostat	Leica	CM3050 S	
Cyclopentolate hydrochloride and phenylephrine hydrochloride eyedrop	Cyclomydril	N/A	
Gelatin	Sigma-Aldrich	G9382	
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher Scientific	A-11008	
Heating board	Lab-Line Instruments Inc.	N/A	
Isolectin GS-IB4, 594 conjugate	ThermoFisher Scientific	I21413	
Ketamine hydrochloride injection	KetaVed	NDC 50989-996-06	
<i>Lrp5</i> ^{-/-} mice	The Jackson Laboratory	Stock NO. 005823	Developed by Deltagen Inc., San Mateo, CA
Micron IV and OCT	Phoenix Research Labs	N/A	Imaging software: InSight
Microscope	Zeiss	discovery v8	
Microsurgery forceps	Scanlan International	4004-05	
Microsurgery scissors	Scanlan International	6006-44	
Optimal cutting temperature compound	Tissue-Tek	4583	
Optimal cutting temperature compound	Agar Scientific	AGR1180	
Paraformaldehyde (16%)	Electron Microscopy Sciences	15710	
Peel-A-Way disposable embedding molds (tissue molds)	Fisher Scientific	12-20	
Phosphate-buffered saline (PBS) buffer (10X)	Teknova	P0496	
Slide cover glass	Premiere	94-2222-10	

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Superfrost microscope slides	Fisherbrand	12-550-15	
Triton X-100	Sigma-Aldrich	X100	
Xylazine sterile solution	Akorn: AnaSed	NDC: 59399-110-20	