Agilent

Trusted Answers

Visualizing the Mouse Retinal Vasculature using the Agilent BioTek Cytation C10 Confocal Imaging Reader

Introduction

The mouse retina is one of the most widely used *in vivo* systems to study angiogenesis and its associated pathologies. Mice are actually born without a retinal vasculature, however over the course of the first week of life, this vasculature expands radially from the optic opening to the periphery on the surface of the retina (Figure 1A). At this expansion front are specialized tip endothelial cells that use actin-based cellular extensions called filopodia to sense growth factor gradients (Figure 1B). These filopodia can vary in width by a few hundred nanometers. Once the initial vasculature reaches the periphery of the retina, nascent blood vessels start to branch into the retina, ultimately maturing into a tri-layer plexus (Figure 2A).

The retinal vasculature is well-suited for high resolution imaging because it is situated superficially within the first 50 to 60 µm of the retina (Figure 2B). While the accessibility of this vasculature is relatively ideal, the narrow widths of filopodia, and the depth required to obtain images resolved enough to discern the multiple layers of the plexus poses significant challenges for widefield microscopy (Figure 1). This application brief describes an approach to overcome these challenges using the Agilent BioTek Cytation C10 confocal imaging reader, combined with the image processing capabilities of Agilent BioTek Gen5 microplate reader and imager software, to generate a more resolved depiction of the mouse retinal vasculature.
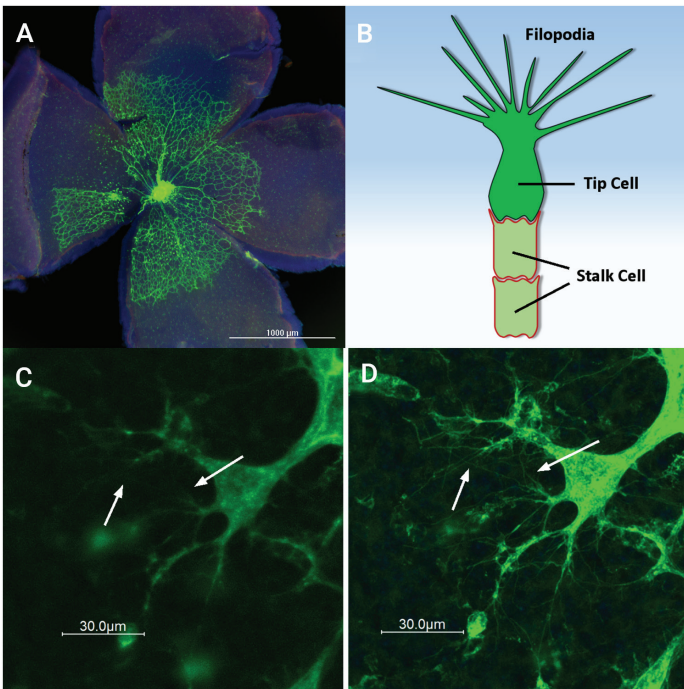
Figure 1. Filopodia in the developing mouse retinal vasculature. (A) Whole mounted neonatal mouse retina (P4) imaged at 4x. Green: isolectin B4-Alexa Fluor 488, blue: Hoechst 33342, and red: Alexa Fluor 546-phalloidin. (B) Artistic depiction of tip endothelial cells sprouting filopodia. (C) and (D) Tip endothelial cell with filopodia (white arrows) at the vascular expansion front imaged with a 40x objective (0.6 NA) in either (C) widefield or (D) confocal mode (60 μm disc). Maximum intensity projections were generated from a 5 μm z-stack. Green: isolectin B4-Alexa Fluor 488.

Sample preparation for imaging the mouse retina

Adult and P4 mouse eyes were purchased from BrainBits, LLC (Springfield, IL) and shipped live overnight in Hibernate A complete medium. Upon arrival, the cornea was removed and eyes were immediately fixed for two hours with 4% paraformaldehyde (in PBS). Following fixation, four anterior-to-posterior incisions were made to the eye cup, then the retina was gently peeled away from the sclera using forceps. Because of the spherical shape of the eye, the incisions are necessary to lay it flat between a coverslip and slide. Next, the retina was permeabilized in 0.5% Triton X-100 for 1 hour, then blocked in 5% BSA. Blocked retinas were then immunostained overnight at 4 °C with a rabbit anti-GFAP antibody, catalog number RPCA-GFAP, from EnCor Biotechnology, Inc. (Gainsville, FL) as well as Alexa Fluor-conjugated isolectin B4, catalog number 121411, from Thermo Fisher Scientific (Waltham, MA).
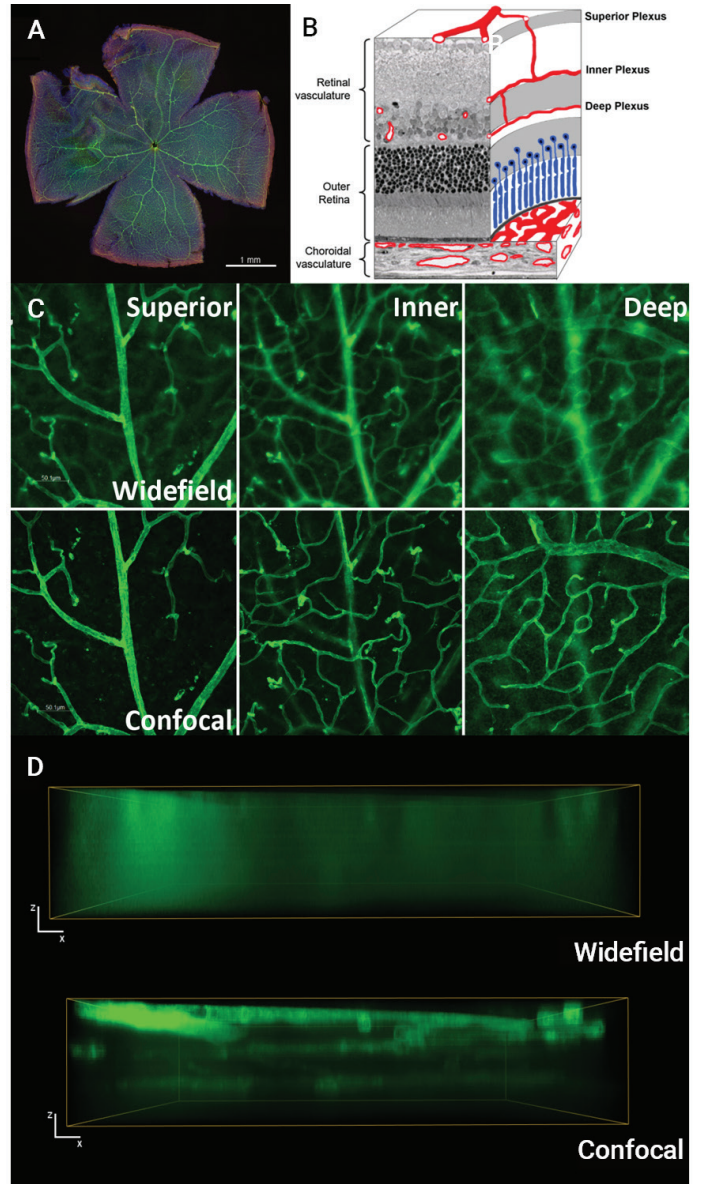

Figure 2. Vascular plexus of the developed mouse retina. (A) Whole mounted adult mouse retina (3mo) imaged at 4x. Stitched image was generated from a 5x5 montage. Green: Isolectin B4-Alexa Fluor 488, blue: Hoechst 33342, and red: Alexa Fluor 546-phalloidin. (B) Artistic cross-section of the fully developed retina, illustrating the tri-layered vascular plexus, which consists of three layers: superior, inner, and deep plexus.[1] (C) "Zoned" maximum intensity projections isolating the three plexus of the developed retinal vasculature imaged at 40x (0.6 NA) in either widefield (upper panels) or confocal (lower panels; 60 μm disc). Extracted from a z-stack encompassing the entire tri-layer plexus. (D) Orthogonal view (xz) of the developed vascular plexus imaged at 40x (0.6 NA) in either widefield or confocal mode. Z-stacks were rendered with the 3D viewer in the Agilent BioTek Gen5 microplate reader and imager software.

After three washes in PBS containing 0.1% Tween 20, retinas were incubated in a Goat anti-Rabbit secondary conjugated to Alexa Fluor 555, catalog number A21428, from Thermo Fisher Scientific (Waltham, MA). Lastly, retinas were mounted between a slide and a #1.5 glass coverslip using Fluoromount-G, catalog number 0100-35, from Southern Biotech (Birmingham, AL).

Imaging procedure and processing

Confocal and widefield z-stack images of both P4 (Figure 1) and adult mouse retina (Figures 2 and 3) were captured at 40x using the Cytation C10 confocal imaging reader and Gen5 software in Manual Mode (IMM). Once a region of interest was identified, the dynamic range of each channel was maximized by selecting the "show saturated pixels" button. The exposure settings for each channel were adjusted such that they were within range, and there were no saturated pixels. The z-range was determined by toggling below and above the sample until the structure of interest (in this case, blood vessels) faded in intensity. The z-step size was set using Nyquist sampling recommendations; for imaging Alexa Fluor 488 using the 40x air objective (NA = 0.6), steps sizes were determined to be 1 µm for widefield, and 0.6 µm for confocal. Raw images were then subjected to five iterations of deconvolution using a point spread function based on the objective. Deconvolved images were then preprocessed to reduce background signal. The rolling ball size for background reduction for both IB4-AF488 (GFP channel) and αGFAP-AF555 (TRITC channel) was set to 50 µm. For the P4 retina, filopodia were visualized by applying Maximum Intensity Projection encompassing the entire z-stack (Figure 1C and 1D). For the adult retina, zoned z-projections were generated that isolated each of the three matured retinal plexus (superficial, inner and deep plexus) (Figure 2C). Lastly, to better visualize the tri-plexus layers of the adult vasculature, 3D renders were generated using the 3D viewer in Gen5. Improved z-resolution is demonstrated by rotated the 3D rendered z-stacks to show the orthoganol x,y orientation (Figure 2D and 3B).

Figure 3. Astrocytes interact with the superior plexus. (A) Whole mounted adult mouse retina (3mo) immunostained with αGFAP (Alexa Fluor 555 secondary), as well as isolectin B4-Alexa Fluor 488, Hoechst 33342, and Alexa Fluor 647-phalloidin. Z-stacks were captured at 40x (0.6NA) in either widefield or confocal mode (40 µm disc) and maximum intensity projections generated. (B) Orthogonal view (xz) of the entire vascular plexus imaged at 40x (0.6 NA) in either widefield or confocal mode. Astrocytes localize specifically within the superior plexus. Z-stacks were rendered with the 3D viewer in Agilent BioTek Gen5 microplate reader and imager software.

Conclusion

The mouse retina is a well-established *in vivo* system to study angiogenesis, as well as vascular pathologies. To assess pharmacological effects or genetic perturbations to the vasculature, it is crucial to be able to gain as clear of an image as possible. This application brief demonstrates that the Agilent BioTek Cytation C10 confocal imaging reader is capable of improving the resolution of such tissue, compared to widefield microscopy.

References

1. Simx, D. and Fruttiger, M. Ophthalmology: Keeping Blood Vessels Out of Sight. *eLife* **2013**;2:e00948 DOI: 10.7554/eLife.00948.