Multi-wavelength, en-face photoacoustic microscopy and optical coherence tomography imaging for early and selective detection of laser induced retinal vein occlusion

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Abstract: Multi-wavelength en face photoacoustic microscopy (PAM) was integrated with a spectral domain optical coherence tomography (SD-OCT) to evaluate optical properties of retinal vein occlusion (RVO) and retinal neovascularization (RNV) in living rabbits. The multi-wavelength PAM of the RVO and RNV were performed at several wavelengths ranging from 510 to 600 nm. Rose Bengal-induced RVO and RNV were performed and evaluated on eight rabbits using color fundus photography, fluorescein angiography, OCT, and spectroscopic en face PAM. In vivo experiment demonstrates that the spectral variation of photoacoustic response was achieved. The location and the treatment margins of the occluded vasculature as well as the morphology of individual RNV were obtained with high contrast at a laser energy of 80 nJ, which was only half of the American National Standards Institute safety limit. In addition, dynamic changes in the retinal morphology and retinal neovascularization were administered using PA spectroscopy at numerous time points: 0, 3, 7, 14, 21, 28, and 35 days after photocoagulation. The proposed multi-wavelength spectroscopic PAM imaging may provide a potential imaging platform to differentiate occluded retinal vasculature and to improve characterization of microvasculature in a safe and efficient manner.

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1. Introduction

Retinal vein occlusion (RVO) represents a major cause of vision loss in elderly patients, affecting more than 16 million people worldwide [1–3]. Although RVO is associated with thrombophilias and coagulation abnormalities, hypertension remains the most commonly associated medical abnormality. The treatment of RVO currently involves a thorough eye examination and appropriate medical work-up to identify any underlying systemic cause.

Several imaging platforms have been applied to monitor and evaluate RVO, including fluorescein angiography (FA), indocyanine green angiography (ICGA), optical coherence tomography (OCT), and OCT angiography (OCTA) [4–7]. Each technique has its own strengths and limitations in assessing RVO in vivo. FA and ICGA visualize only the superficial capillaries, but no quantitative depth information of the vasculature can be obtained [5]. In addition, both ICGA and FA are invasive, requiring the injection of an intravenous exogenous dye, and can cause nausea and vomiting in up to 10% of patients and rarely anaphylaxis which can cause death [8]. OCT is a non-invasive technique to detect and...
visualize the retinal anatomy and layers with high resolution. However, OCT provides limited information on the choriocapillaris and choroidal vasculature and blood flow [9]. OCTA provides high resolution images of both the superficial and deep capillary plexus [10]. Kuehlewin et al. have reported that areas of nonperfusion following RVO could be precisely delineated at several retinal levels using FA and OCT [11]. However, OCTA cannot demonstrate leakage, provides limited visualization of microaneurysms, can often have motion artifacts, and has limited ability to visualize the choroid and choriocapillaris microvasculature [10,12].

A more recent development in optical imaging, termed photoacoustic imaging, combines optical and acoustic imaging, and has been investigated to visualize and evaluate various kinds of ocular tissues. A unique advantage of PA imaging is that it can image targeted tissues with high-resolution, high sensitivity, high-contrast, and high depth of penetration [13–19]. In 2010, de la Zerda et al. described photoacoustic imaging for the detection of retinal vasculature [13]. Photoacoustic ophthalmoscopy has been used to evaluate with in vivo imaging the vasculature of the retina in rodents. Liu, Jiao, and Song et al. have described an integrated PAM and OCT to better visualize retinal vessels, choroidal vessels, and the RPE [15,20,21]. Our group reported an integrated photoacoustic microscopy (PAM) and OCT system for evaluating retinal and choroidal blood vessels [22,23] in living rabbits with high temporal and spatial resolution [22,24]. Our multimodal imaging platform performs imaging with high lateral spatial resolution of 4.1 µm for PAM and 3.8 µm for OCT at the focal plane of the objective with a high depth of penetration that allows visualization of both retinal and choroidal vasculature. Thus, PAM and OCT can feasibly be used to better characterize RVO and retinal neovascularization.

Fig. 1. Schematic diagram of the integrated en face photoacoustic microscopy (PAM) and optical coherence tomography (OCT) for multimodal ocular imaging.
The aim of this study is to address the characterization of laser-induced vein occlusion with Rose Bengal by using spectroscopic photoacoustic microscopy. The occluded retinal veins were further monitored and evaluated by OCT, FA, and color fundus photography. Retinal vein occlusion was produced using the intravenous injection of Rose Bengal (5 mg/kg) followed by millisecond duration green laser light (150-300 mW). The location of the occluded retinal vein after laser application was monitored by multimodal PAM and OCT. In addition, spectroscopic photoacoustic microscopy was performed at different wavelengths ranging from 510 to 600 nm to further evaluate the dynamic change in the retinal morphology, to determine the concentration of both oxygenated and deoxygenated hemoglobin of the occluded retinal vessels, and to evaluate the spatial extent of retinal vascular changes.

2. Materials and methods

2.1 Reagents

Rose Bengal was purchased from Sigma-Aldrich (Sigma, St. Louis, Mo, USA). 10% fluorescein was obtained from Akorn (Akorn, Lake Forest, IL, USA). Phosphate-buffered saline (PBS) was ordered from Gibco BRL, Life Technologies (Grand Island, NY, USA). Ketamine was ordered from the University of Michigan Pharmacy from JHP Pharmaceuticals (JHP Pharmaceuticals, Rochester, MI, USA). Xylazine was acquired from MWI Animal Health (Anased Boise, ID, USA). Davidson’s fixative solution obtained from Electron Microscope Sciences (Electron Microscope Sciences, PA, USA), 10% neutral buffered formalin (NBF) was ordered from Avantar (VWR, Radnor, PA, USA). Euthanasia solution was purchased from Intervet (Beuthanasia-D Special, Intervet Inc., Madison, NJ, USA). Alcohol solution was acquired from Fisher Scientific (Fisher Scientific, PA, USA). All chemicals were used as received without further purification.

2.2 Animal model preparation

All animal used in this study were approved the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan (Protocol number: PRO00006486, PI: Y. Paulus), and the animal experiments were performed in accordance with the ARVO (The Association for Research in Vision and Ophthalmology) Statement on the Use of Laboratory Animals in Ophthalmic and Vision Research.

Eight New Zealand rabbits (weighing 2.2-3.2 kg, 2-4 months of age, both genders) were obtained from the Center for Advanced Models and Translational Sciences and Therapeutics (CAMTrasST) at the University of Michigan Medical School. High power laser light was applied to all of the animals to induced retinal vein occlusion. During in vivo experiments, the animal vitals such as mucous membrane color, temperature, heart rate, and respiratory rate were monitored and recorded using a pulse oximeter (V8400D Capnograph & SpO2 Digital Pulse Oximetry, Smiths Medical, MN, USA) prior to the experiments. Then, each rabbit was anesthetized by intramuscular (IM) injection of ketamine (40 mg/kg IM, 100 mg/mL) and xylazine (5 mg/kg IM, 100 mg/mL). To maintain anesthesia during the in vivo experiments, a vaporized isoflurane anesthetic (1 L/min oxygen and 0.75% isoflurane) (Surgivet, MN, USA) was provided. The pupils of the rabbit were dilated using Tropicamide 1% ophthalmic and phenylephrine hydrochloride 2.5% ophthalmic. Topical tetracaine 0.5% was instilled in the eye for topical anesthesia. Frequent corneal hydration (Systane, Alcon Inc., TX, USA) was applied during the experiment to prevent corneal dehydration.

2.3 Retinal vein occlusion (RVO)

The retinal vein occlusion model was created using dye-enhanced photothrombosis as described previously by Oncel et al. [25]. In brief, a contact lens (Volk H-R Wide Field, laser spot 2x magnification, Volk Optical Inc, Mentor, OH, USA) was mounted on the cornea of
the rabbit eye with Gonak Hypromellose Ophthalmic Demulcent Solution 2.5% (Akorn, Lake Forest, IL, USA) used for coupling. Rose Bengal solution was intravenously injected into the rabbit via the marginal ear vein. 5 seconds after the Rose Bengal injection, the rabbit eye was illuminated with a 532-nm green light laser system (Vitra 532nm, Quantel Medical, Cournon d’Auvergne, France) at a laser power of 150-300 mW. The laser spot size was approximately 75 µm in aerial diameter, and the exposure time was 0.5 s per spot. The laser was illuminated at a distance of a half to one disc diameter from the optic disc margin. Twenty shots of the laser were delivered at the same position on the retinal vein at the laser power of 150 mW until the blood flow was completely stopped and observed in the vein. Then, the laser power was increased to 300 mW and illuminated for further 20 shots to prevent reperfusion of the vein [26].

2.4 RVO monitoring

All treated rabbits were imaged with color fundus photography, fluorescein angiography (FA), Optical Coherence Tomography (OCT), and photoacoustic microscopy (PAM) fifteen minutes after photocoagulation. To monitor the change of vascular after the laser-induced RVO, the treated rabbit was further imaged with photography, FA, OCT, and PAM on days 1, 3, 5, 14, 28, and 35.

2.5 Color fundus photography

The retinal veins were imaged before and after laser illumination using 50 degree color fundus photography (Topcon 50EX, Topcon Corporation, Tokyo, Japan). In addition, the percentage of blood perfusion before and after laser application was obtained from the color fundus images.

2.6 Fluorescein angiography (FA)

Immediately after taking color fundus images, fluorescein angiography (FA) was performed to assess the vascular occlusion. FA was performed using the Topcon TRC-50EX as described by previous studies [22]. 0.2 mL sodium fluorescein solution at a concentration of 10% fluorescein (Akorn, Lake Forest, IL, USA) was injected in the rabbit marginal ear vein. The FA images were subsequently taken immediately after injection. Late phase fluorescence photos were acquired at least every minute for a period of at least 15 minutes.

2.7 Integrated en face photoacoustic microscopy (PAM) and optical coherence tomography (OCT) imaging system

The schematic diagram of a custom-built integrated en face PAM and OCT system is shown in Fig. 1 [22]. For PAM, laser pulses with a pulse width of 3~5 ns and a pulse repetition rate of 1 kHz were generated from an optical parametric oscillator (OPO) pumped by a diode-pumped solid-state laser (NT-242, Ekspla, Vilnius, Lithuania, tunable wavelength range 405 – 2600 nm) and served as the illumination source. The laser light output from the OPO was delivered through a half-wave plate attenuator and collimated to 2 mm in diameter by a beam collimator. The collimated light was then transmitted to a scanning head and raster-scanned by a two-dimensional galvanometer, which is a shared component with the spectral domain (SD)-OCT system. The scanned beam traveled through a telescope consisting of a scan lens (focal length 36 mm, OCT-LK3-BB, Thorlabs, Inc., Newton, NJ) and an ophthalmic lens (OL, focal length 10 mm, AC080-010-B-ML, Thorlabs) and was finally focused on the fundus by the rabbit eye optics. The laser-induced acoustic signals were detected by a custom-made 27.0 MHz needle-shaped ultrasound transducer (Optosonic Inc., Arcadia, CA, USA). The transducer was mounted in contact with the conjunctiva and aligned to enable accurate alignment with the illuminating laser light. The generated PA signal was amplified by a low-noise amplifier (gain 57 dB, AU-1647, L3 Narda-MITEQ, NY). Then, the signals were digitized and recorded using a high-speed digitizer at a sampling rate of 200 MS/s.
The recorded data was then used to reconstruct two-dimensional (2D) or three-dimensional (3D) images of the retinal blood vessels. For image reconstruction, a single laser pulse excitation at a fixed position creates the acoustic signal, which is recorded and converted into 1D depth-resolved PA image along the Z axis, referred to as A-lines. By implementing horizontal scanning lines along the x-axis on the sample, the two-dimensional depth-sensitive PA image is acquired. To obtain 3D volumetric PA image, each sample is scanned along x- and y-directions optical-scanning galvanometer with a resolution of 7.5 x 7.5 µm². For a 1.5 x 1.5 mm² field of view, the acquisition time was approximately 40 s. For quantitative evaluation, the PA amplitudes at the different region of interest (ROI) will be measured and compared [27–29]. In addition, spectroscopic PA imaging is performed at various optical wavelengths ranging from 510 to 600 nm to determine the concentration of hemoglobin, to quantify the suitable excited wavelength for detection and evaluation of photothrombosis; and to distinguish between normal to occluded vessels.

For in vivo experiments, the laser pulse energy on the eye used to obtain images was less than 80 nJ, which is half of the American National Standards Institute (ANSI) eye safety limit of 160 nJ at these settings as described previously [22]. The ultrasound transducer yields PAM lateral and axial resolutions of 4.1 and 37.0 µm, respectively. To visualize the margin of the blood vessel, 3D image reconstruction was performed using Amira software (FEI, Hillsboro, OR). Further, image segmentation was also performed to classify the positions of arteries, veins, choroidal vasculature, and neovascularization. The major retinal arteries and veins were identified from FA images (see A1). As shown in Fig. 10, delay in the appearance of the FA dye into the central retinal vessels indicate the position of retinal veins. On the other hand, slight up of dye into the central artery is completed within one or two second, which was approximately 2 to 4 seconds faster than in the retinal vein.

Spectral domain Optical Coherence Tomography (SD-OCT) was modified from a commercial OCT system (Ganymede-II-HR, Thorlabs, Newton, NJ) by adding the ocular lens after the scan lens and a dispersion compensation glass in the reference arm [22]. A combination of two superluminescent light emitting diodes with center wavelengths of 846 nm and 932nm (see A2) was used to excite the tissue. A single mode fiber delivered the light from the light source and split it into sample and reference arms. The OCT light source was coaxially aligned with the PAM system. The lateral and axial resolutions for OCT are 3.8 µm and 3 µm, respectively. A-line acquisition rate was 36 kHz. The scanning areas was 3.5 x 2.5 mm², and high-resolution OCT images were captured, where 512 x 1024 A-lines were recorded per image with an image acquisition time of 0.103 seconds. To achieve 3D OCT image, 512 x 512 B-scan were sequentially acquired. The total acquisition time was approximately 53 s.

2.8 In vivo en face PAM/OCT for retinal vein occlusion

To evaluate the coagulated lesion after laser-induced RVO models, all the treated animals were imaged by dual PAM and SD-OCT. The en face PAM and OCT images of the retinal vessels were acquired before and after laser-induced retinal ischemia and RVO. Both the PAM and OCT share a galvanometer to obtain raster scanning of the normal and occluded vessels. The imaging systems were controlled by Matlab 2016 software (MathWorks, Massachusetts, USA). For in vivo PA imaging, the rabbits were placed on the imaging platform after anesthetic injection. To minimize breathing and other motion artifacts, the head and body of the rabbit were placed on two different custom-made stabilization platforms. The temperature of the rabbit was maintained during the experiment using a water-circulating blanket (TP-700, Stryker Corporation, Kalamazoo, MI). The regions of interest (ROI) were first imaged with color fundus photography, FA, and OCT. Then, a needle ultrasonic transducer was mounted in the eye chamber, allowing it to move freely in 3D on the rabbit eyes to acquire PAM images. After acquiring the baseline images, Rose Bengal-induced RVO
was performed on the rabbit. The rabbit retinas after laser treatment were imaged with PAM, OCT, fundus photography, and FA at 15 minutes in addition to 1, 3, 5, 14, 28, and 35 days after RVO to monitor the status of the blood vessels (i.e., occluded, non-perfusion, re-perfusion, presence of retinal neovascularization, size of retinal vessels, and stability of RNV). After the in vivo experiments, the rabbits were placed in a recovery area with a heat blanket, and vitals such as mucous membrane color, rectal temperature, heart rate, and respiratory rate were observed and documented until the rabbit was fully recovered. Then, the rabbit was returned to the animal facility and the body weight was monitored every day for 7 days.

2.9 Histological analysis

All rabbits with the RVO model were sacrificed thirty five days after photocoagulation, and their eyes and other organs were extracted for histological analysis. The rabbits were euthanized by injection of intravenous injection of pentobarbital (Beuthanasia solution, 0.22 mg/kg I.V, 50 mg/ml). The eyeball and select tissues were harvested aseptically from the euthanized rabbits. The isolated tissues were cut and fixed in 10% NBF for a minimum of 48 hours. To avoid retinal detachment, the isolated eyeball was fixed in Davidson’s fixative solution for 24 hours. The sample was transferred to 50% of alcohol solution for an additional 24 hours. Finally, the sample was changed to 70% alcohol solution and kept at room temperature for 24h prior to embedding in paraffin. The fixed tissues were cross-sectionally cut into 5 mm sections and embedded in paraffin. Subsequently, the tissues embedded in paraffin were sectioned into a thickness of 4 µm and stained with hematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) for standard histopathological examination using a Leica autostainer XL (Leica Biosystems, Nussloch, Germany) under standard conditions. Images of the slides were captured using a Leica DM600 light microscope (Leica Biosystems, Nussloch, Germany) to detect the position of retinal neovascularization. Digital images were achieved with the BF450C camera. In addition, the retinal thickness was also quantitatively measured using LAS X software (Leica Biosystems, Nussloch, Germany).

2.10 Statistical analysis

Data are presented as the mean ± standard deviation (SD). Student’s t-tests were performed to compare two experimental conditions using the Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA), and $P \leq 0.05$ was considered as statistically significant.
Fig. 2. Visualization of retinal blood vessels in rabbits: (a) color fundus photography of the retina. Black arrow denotes the position of arteries, blue arrow reveals the position of the vein, white and yellow arrows represent the location of the optic nerve and choroidal vessels, respectively. White dotted lines display the selected scanning regions for OCT. (b) and (c) early phase and late phase fluorescein angiography of the eye, respectively. (a1-a4) B-scan OCT images of the retinal and choroidal vessels obtained along the selected lines from a. The OCT image showing choroidal vessels (CVs), retinal vessels (RVs), Ganglion cell layer (GCL), nerve fiber layer (NFL) and retinal layers. (d) and (e) 3D volumetric OCT images of retinal and choroidal vessels, respectively.
3. Results

The change of retinal vessels in rabbits after photocoagulation were monitored and evaluated using various imaging modalities, including fundus photography, FA, and OCT imaging platforms. Baseline images of major retinal blood vessels were acquired before laser-induced retinal vein occlusion (RVO) as shown in Fig. 2. Figure 2(a) represents a color fundus photograph of the retina obtained before in vivo OCT, PAM, and laser-induced RVO model. Blue arrow indicates the location of major retinal veins, black arrow exhibits the location of the major retinal artery, whereas white and yellow arrows depict the position of the optics nerve and choroidal vessels, respectively. White dotted lines show the selected scanning areas for OCT imaging. Note that the retinal veins and arteries were distinguished from sequential FA images (see A1). Figures 2(b)-2(c) show FA images acquired at different times: early phase and late phase, respectively. The FA image demonstrates the superficial retinal vessels such as retinal and capillaries and was used to assess blood perfusion after RVO. In addition, no leakage was observed from the late phase FA image, indicating that the retinal vessels are normal. Figures 2(a1)-2(a4) illustrate the cross-sectional OCT image obtained from the dotted lines 1-4 in Fig. 2(a). These B-scan OCT images were acquired along the retinal and choroidal vessels. Obviously, the retinal and choroidal vessels were laid in different layers and show intact retinal layers (Figs. 10, 11, and 12). As shown in Fig. 2(a4), various layers of retinal tissues were easily observed such as the ganglion cell layer (GCL), RPE, choroidal vessels, and sclera. Figures 2(d)-2(e) represent 3D OCT volumetric images of the retinal and choroidal blood vessels. The OCT volumetric images clearly illustrate a healthy branching network of retinal vasculature.
Fig. 3. In vivo en face spectroscopic photoacoustic microscopy analysis of the retina and choroid. (a) maximum intensity projection (MIP) PAM images of retinal vessels acquired along the selected scanning area at different wavelengths ranging from 510 to 600 nm. White arrows represent the position of retinal vessels. White dotted arrows illustrate the location of retinal vessels. (b) corresponding en face PAM image of choroidal vessels. The en face PAM image demonstrates individual morphology, high resolution and high contrast of retinal microvasculature. Note that both the retinal and choroidal vessels were visualized and demonstrate higher contrast at the wavelengths of 563, 570 and 580 nm in comparison with shorter and longer wavelengths.

Figure 3 shows the en face maximum intensity projection (MIP) PAM of both retinal and choroidal vessels in the xy-plane (top view on the tissue, with in each pixel the maximum value found in all voxels below that pixel). The color scaling in all images related to the same
absolute value. The \textit{en face} PAM image was obtained along the selected scanning areas as shown in Appendix Fig. 12 at different wavelengths ranging from 510 to 600 nm to quantify the optical properties of blood vessels such as the concentration of oxygenated hemoglobin (HbO2) and deoxygenated hemoglobin (Hb). The \textit{en face} PAM image exhibits the single structure of the retinal and choroidal vessels with high resolution and high contrast. As shown in these PAM images, both retinal and choroidal vessels were clearly observed at the wavelengths of 532 nm, 563 nm, 570, and 580 nm due to strong laser light absorption at these optical wavelengths (e.g. $\mu_{HbO2} = 236.93$, 183.144, 240.28 and 270.56 cm$^{-1}$ at 532, 563, 570 and 580, respectively). In contrast, these retinal vessels were blurred and lack of contrast at shorter and longer wavelengths due to low optical absorption coefficients (e.g. $\mu_{HbO2} = 108.19$, 130.69, 77.76, and17.28 cm$^{-1}$ at 510, 520, 590, and 600 nm) [30]. In addition, the three-dimensional (3D) images of the vessels were rendered by incorporating time-of-flight (TOF) of the photoacoustic signal and shown in Figs. 3(c)-3(d). The major retinal vessels, retinal microvessels, and choroidal vessels were better visualized in depth and the shadows of vessels were observed, indicating the capacity of \textit{en face} PAM for detection of individual blood vessels \textit{in vivo} (see Visualization 1). In addition, from the acquired photoacoustic signal and counting TOF, the thickness layer between retinal and choroidal vessels was estimated to be 200-300 µm and the diameter of retinal vessels was approximately 70-200 µm. This finding is consistent with previously reported by Barton and Wong \textit{et al}. [31–33].

The laser-induced RVO model was performed on the rabbit retina after acquiring baseline images. The targeted vessels were selected and the desired treatment site was identified from the FA images. Then, the targeted vessels were illuminated using visible laser system (Zeiss SL 130, Carl Zeiss Meditec, Jena, Germany). All the rabbit models were monitored by different imaging modalities (color fundus photography, FA, OCT, and PAM) to evaluate the change of retinal vessels immediately following laser irradiation to 35 days afterward. Figure 4 and Fig. 5 illustrate the dynamic changes of occluded vessels monitored by color fundus, FA and OCT. Column A shows color fundus images of the retina after RVO. The white dotted arrows indicate the treatment site. At the treatment site, the color intensity changed from pink to bright pink color, indicating the significantly reduced intravascular blood volume. There was no hemorrhage after laser irradiation at day 0 and day 1. However, the hemorrhage was observed at day 3 and resolved on day 4.

![Fig. 4. Multimodal color fundus photography, FA, and OCT imaging analysis of retinal vein occlusion immediately following RVO to 7 days afterwards. Column A shows color fundus images of the retina performed at different time (15 min after laser treatment, day 1, 3, and 7) after laser-induced RVO. White dotted arrows show the position of treated sites. Dotted lines represent the selected OCT scanning areas. Column B-C display early phase and late phase FA images. White arrows show the location of occluded vessels. Columns A1-A3 exhibit cross-sectional B-scan OCT image acquired along the dotted line in color fundus images (column A). White arrows show the position of retinal detachment after laser treatment. Note that severe retinal detachment appeared at day 1 post treatment and resolved at 3 days after laser exposure. Column D shows 3D rendering of OCT images.](image-url)
To confirm the interruption of blood flow, FA images were also acquired. As shown in column B and C, no blood flow was observed at the treatment site (white arrows) from day 0 to 7, which implied that the retinal blood vessels were completely blocked. The white dotted circles indicate the position of capillary non-perfusion. There was no evidence of retinal edema or swelling in the areas with capillary non-perfusion on FA images. However, the retinal blood vessels were perfused at the area near the optic nerve. Interesting, retinal neovascularization (RNV) was observed on day 14 and the new blood vessels were increased over time and stabilized up to day 35. White arrows depict the position of new blood vessels, whereas red arrows represent the leaked of fluorescent dyes. This implies that RNV was developed around the laser lesions. Columns A1-A3 display the cross-sectional OCT image obtained along the dotted line 1-3 shown in column A. These OCT images present the change in retinal layers. As shown in the OCT images, a localized retinal detachment was detected (white arrows) with the accumulation of subretinal fluid. The serous retinal detachment was observed in the first 15 min following laser irradiation and 1 day after treatment. Then, the retinal detachment was gradually reduced and completely revolved by 3 days after treatment. The yellow arrows indicate the location of formed RNV. Column D shows the 3D OCT images of the treated vessels. These 3D OCT images demonstrate the structure of retinal vasculature. Pseudo-color bar shows that the retinal and choroidal vessels lay at different depths.

To evaluate the characterization and to optimize the PAM wavelength for identifying the thermally occluded retinal vessels after photothrombosis, the treated retinal vessels were imaged by a PAM platform at different laser wavelengths ranging from 510 to 600 nm as shown in Fig. 6. The \textit{en face} PAM was acquired along the scanning areas extracted from the color fundus photography (see A4). The \textit{en face} PAM image emphasizes the pathologic variations in the retinal vessels. The spectroscopic PAM quantitatively demonstrated that the wavelength of 532, 563, 570, and 580 nm achieved a higher contrast compared to shorter and longer wavelengths. The baseline PA images of the retinal vessels before treatment was acquired and displayed in Fig. 3(a1)-3(a8). At day 0 post treatment, \textit{en face} MIP PAM images show that the treatment vessels were occluded (white arrows) and achieved a lower photoacoustic image contrast compared to the untreated sites (green arrows). Yellow arrows depict the change of abnormal vessels. It was noted that the retinal vessels were larger at this
position in comparison to other position due to the accumulation of red blood cells and Rose Bengal. The white dotted arrows demonstrate the detected RNV with high image contrast. The RNV developed at day 21 after laser-induced RVO and gradually increased and stabilized by day 35. Apparently, RNV was clearly observed at the wavelengths of 563, 570, and 580 nm with higher density in comparison to 532 nm. In contrast, no RNV was detected at the wavelengths of 510, 520, 590 and 600 nm.

Fig. 6. Spectroscopic photoacoustic microscopy of retinal neovascularization. The en face MIP PAM images of the retina acquired at various wavelength (510-600 nm) at different times after laser treatment: 15 min after treatment (day 0), days 3, 5, 7, 14, 21, 28, and 35. White arrows show the detected position of the retinal vein occlusion as shown on Day 0. Yellow arrows indicate the position of abnormal retinal vessels at day 7 after treatment. White dotted arrows depict the position of retinal neovascularization. The retinal neovascularization developed at day 21 after laser treatment.

To demonstrate the potential of en face PAM for visualization of RNV, 3D PAM image reconstruction was performed using Amira software. Figures 7(a)-7(c) illustrate the 3D volumetric reconstruction image of the treated vessels at day 28. The 3D images were reconstructed from the raw photoacoustic signals acquired at the wavelengths of 532, 570 and 580 nm, respectively (see Visualization 2). The structural features of the vascular networks were better visualized and the margin of individual RNV were readily distinguished. The type of vessels such as RVs, CVs, RNV were classified from FA images as shown in Fig. 14. The white arrow depicts the revascularized retinal vessels, the white dotted arrows exhibit the features of neovascularization, and the yellow arrows indicate the choroidal vessels. This reveals that PAM can possibly visualize and detect the formation of RNV.
To precisely classify the margin of each retinal vessel and to discover the dynamic changes in vessel diameter, image segmentation was employed and displayed in Figs. 6(d)-6(f). As shown in these images, the boundary of the retinal arteries, vein, choroidal vessels (CVs), and RNV was segmented. The red color shows the position of arteries, blue color indicates the veins, green denotes the RNV and white color marks for CVs. The entire structure, morphology, and location of RNV are vividly classified. Figure 6(g) shows the quantitative measurement PA amplitudes from the region of interests (ROIs) of various treatment groups: Pre (control), day 0, 3, 7, 14, 21, 28 and 35 measured at eight different optical wavelengths ranging from 510 to 600 nm. For the control group (red line), the spectroscopic PA amplitude demonstrates that the PA amplitudes increased with the wavelength and then achieved a peak value at 570 nm and 580 nm (e.g., PA amplitudes = 0.88 ± 0.01 (a.u.) for 570 nm and PA amplitudes = 0.92 ± 0.07 (a.u.) for 580 nm). Then the PA amplitude reduced to 0.21 ± 0.01 (a.u.) at 600 nm. It was noted that the PA amplitude at 580 nm exhibits 4.4 and 4.8 times higher than those at shorter or longer wavelengths (e.g., PA amplitudes = 0.92 ± 0.07 (a.u.) at 570 nm vs. PA amplitudes = 0.21 ± 0.02 (a.u.) at 510 nm and PA amplitudes = 0.19 ± 0.01 (a.u.) at 600 nm).

For the treatment group, the quantified spectroscopic PA amplitude within the retinal blood vessels reveals that the PA amplitudes was immediately decreased following laser treatment (black line) and then, the PA amplitudes were gradually increased afterwards. Compared to the control group, PA amplitude at the treated site was 3.7 times lower than that of the control at 570 nm (e.g., PA amplitudes = 0.88 ± 0.01 (a.u.) for control vs. PA amplitudes = 0.24 ± 0.01 (a.u.) for day 0 post treatment). The PA amplitude was suddenly decreased at day 3 after treatment (blue line) and exhibited 4.4 times lower as that from control (e.g., PA amplitudes = 0.88 ± 0.01 (a.u.) for day 3 post treatment). Then the PA amplitudes were gradually increased from day 7 (brown line) and reached the peak PA amplitudes at day 21 (gray line), day 28 (violet line) and day 35 (dark yellow line). Thus, the decrease of PA image amplitude could be used to differentiate and identify treated vessels.
To further quantify the effect of optical wavelength to the RNV detection, the morphology and vessel density of RNV were characterized and measured. Figure 7(h) illustrate the quantification of vessel density extracted from PA images acquired at day 28 and day 35. Overall, the estimated vessel density initially increased with the incident optical wavelength. Then, the largest vessels density was detected at 570 nm (e.g., vessel density = 61.82 ± 3.38%, and 62.10 ± 3.51% at day 28, and 35, respectively) and 580 nm (e.g., vessel density = 63.21 ± 1.10%, and 64.06 ± 1.79% at day 28, and 35, respectively). Then, the vessel density decreased afterwards. Both the detected vessel densities at 570 and 580 nm are comparable to the development of the RNV (~63%). The spectroscopic PA amplitudes as well as the vessels density characterization confirmed that both 570 and 580 nm could detect and visualize the position and margin of the occluded blood vessels after application of the laser, and estimate the growth of RNV due to strong laser light absorption by the oxygenated hemoglobin and
deoxygenated hemoglobin. The effects of photocoagulation to vascular were quantitatively evaluated at various times after laser irradiation for a period of 5 weeks, as shown in Fig. 7(i). As shown in this figure, the change of vessel size before and after RVO was measured from PAM, color fundus photographs, and OCT images to minimize the measurement error. Apparently, these measurement presented similar results, indicating that the measurement results are consistent. From the acquired PAM, quantitative measurements validated that the measured vasculature diameter significantly decreased from approximately 113.89 ± 8.13 µm (pre-occlusion) to 70.00 ± 8.40 µm (day 0 post-occlusion). Then, the vessel diameter gradually reduced to 61.96 ± 5.81 (day 21 post-occlusion) and became stability from day 28 to 38 (i.e., 28.56 ± 3.88 for day 28 post-occlusion and 28.04 ± 2.57 for day 35 post-occlusion). Similarly, the retinal diameters estimated from OCT images were 160% thinner after occlusion than that of pre-occlusion (i.e., vessel diameter = 115.33 ± 10.54 (pre-occlusion)). Then the vessels diameter also gradually decreased (i.e., 47.95 ± 5.14 (day 3 post-occlusion) vs 32.04 ± 7.34 (day 21 post-occlusion)) and stabilized from day 20 to 35 (i.e., 29.02 ± 3.91 (day 28 post-occlusion) vs 29.53 ± 5.65 (day 35 post-occlusion)); (p < 0.01). The measured vessel size from the fundus photography was also not significantly different. The major vessel diameter was calculated to be 118.57 ± 9.81 µm (pre-occlusion); p < 0.003, which was approximately 2.80%, and 4.1% different from OCT and PAM measurements, respectively. Fundus photography got consistently slightly enlarged estimated vessel sizes at all times, whereas PAM and OCT were very similar.

The spectroscopic PAM was also performed to evaluate the dynamic change of choroidal blood vessels (CVs) in rabbits after laser-induced RVO. Figure 8(a) shows the color fundus of CVs after laser-induced RVO at day 35. The white rectangle and dotted line indicate the scanning areas. Figures 8(b)-8(c) illustrate the early phase and late phase FA images. The FA image is clearly visible to the morphology of CVs. The white dotted arrows identify the region of formed choroidal neovascularization. Figure 8(d) shows a B-scan OCT image acquired along the dotted line as shown in Fig. 8(a). The OCT reveals that the retina after laser treatment is thinner than that of the control OCT image (Fig. 2(a4)). By using ImageJ, the retinal thickness was estimated to be 290.83 ± 2.24 µm and 98.29 ± 2.41 µm; p < 0.001 before and after laser treatment (day 35).

Figures 8(e)-8(l) illustrate the corresponding en face MIP PAM images of the CVs acquired from the white rectangle shown in Fig. 8(a) at various wavelengths. The margin of CVs easily visualized from the en face PAM images. According to the spectroscopic PAM image, the wavelength of 570 and 580 nm obtained high image contrast in comparison with other wavelengths. To visualize CVs in three dimensions, 3D image reconstruction was implemented and shown in Fig. 8(m). Apparently, the 3D images show that CVs network was clearly defined and visualized (see Visualization 3). Acquired PAM volumetric data helped to estimate the size and shape of the CVs precisely. Based upon the 3D image, the CVs vessels diameter was estimated to be 76 ± 2.94 µm, which is 261.84% thinner than that of the CVs before treatment (199 ± 8.37 µm); p <0.001. Quantitative PA amplitudes measurements in Fig. 8(n) also exhibits that the wavelength of 570 and 580 nm achieved the highest PAM amplitudes due to the maximum light absorption by the blood vessels in comparison with other wavelengths. In contrast, the PA amplitude significantly reduced at shorter or longer wavelengths. This result is similar to the spectroscopic PA signal measured from the retinal vessels (Fig. 7(g)).
Fig. 8. Spectroscopic PAM image of choroidal vessels after laser photocoagulation. (a) color fundus image of choroidal vessels after laser-induced RVO at day 35. The color fundus showing the change in structure and morphology of choroidal vessels. Due to the effect of laser, some of the choroidal vessels are absent as indicating by white arrows. White dotted line and white rectangle exhibit the selected region of interest (ROI). (b) and (c) represent the FA images at early phase and late phase. The white arrows indicate the position of development of neovascularization. (d) cross-sectional b-scan OCT images. White arrows show the location of neovascularization. (e-l) corresponding en face spectroscopic PAM imaging of abnormal choroidal vessels. The PAM images show clearly the structure of individual choroidal vessels. (m) 3D rendering PAM image.

Figure 9 demonstrates standard histological staining with hematoxylin and eosin (H&E) images of the retinal tissues after 35 days of laser treatment. Control histological images show no evidence of the cellular morphology changes, and anatomic organization of the retinal tissue was easily observed (Fig. 9(a)). The black arrows indicate the position of major retinal vessels, whereas the blue arrows identify the appearance of capillaries. On the other hand, there were several significant changes in the retinal tissue after the application of laser such as the thinning of the retina, the morphology of tissue structure, and the formation of new retinal
vessels as shown in Figs. 9(b)-9(d). The thickness of retinal tissue after RVO was estimated to be 31.46 ± 2.34 µm, which was approximately 3.9-fold thinner than the thickness without laser treatment (e.g., 123.65 ± 3.14 µm). The current finding also exhibits a good agreement with the thickness measurement from OCT images. As shown in Figs. 9(c)-9(d), new retinal blood vessels were easily visualized around the optic nerve. The black arrows indicate the region of retinal neovascularization. The blue arrows showed the major retinal vessels. Apparently, the major retinal vessels were filled with clot (blue arrows). The histology image demonstrated that laser-induced retinal vascular occlusion has the potential to create retinal neovascularization in rabbits, which will be an important step for studies of RVO. In addition, TUNEL assay was also employed to further evaluate the potential effect of illumination light to the eye. As shown in Fig. 9(g), no significant TUNEL staining can be observed in any of the tissues after PAM and OCT, demonstrating the safety and lack of cellular toxicity with the exposure of laser light.

Fig. 9. Histological analysis of the rabbit retina after laser-induced RVO. (a) and (b) show the H&E-stained images of RVO achieved from control and treatment groups, respectively. Black arrows demonstrate the position of retinal vessels, whereas blue arrows illustrate the appearance of retinal capillaries. The retinal thickness of the treated groups is thinner than that of the one from control group. The RNV development were detected at: (c) × 20 and (d) × 40. Black arrows represent the location of RNV. Blue arrows show the major position of retinal vessels after treatment. (e-g) TUNEL staining assay were carried out to evaluate the potential effect of laser exposure after PAM. Figure 9(e) and Fig. 9(f) are positive and negative control, respectively. Note that brown color indicates the position of apoptotic cells. No significant pathological changes can be observed in the eye tissue (Fig. 9(f)).

4. Discussion

The current study successfully developed an integration of multi-wavelength PAM and OCT imaging system for selective monitoring and detection of retinal vein occlusion and retinal neovascularization in the rabbit retina. This is the first study demonstrating spectroscopic PAM to visualize retinal vascular occlusion in large animal eyes. To achieve maximum PA intensity, spectroscopic PAM was performed at multiple wavelengths. Thus, the optimal
wavelength can be identified, leading to better characterize and assess the retinal vasculature. This is important because the optical absorption changes in the retinal microvasculature at different time point can be estimated and adjusted. Although near-infrared (NIR) laser light could provide deeper imaging than green and blue laser light due to low level of hemoglobin absorption, it require apply higher illumination energy to achieve high contrast image [34]. In contrast, hemoglobin absorbs strongly laser light at visible light. Thus, the dose of exposure could be reduced and high contrast image could be achieved within ANSI limit for the eye.

PAM is new ocular imaging technique based on the optical absorption contrast of endogenous absorbers (e.g., hemoglobin, RPE, melanin, lipids, and water) and exogenous absorbers (e.g., photosensitizer agents). Recent studies have demonstrated that spectroscopic PAM can be applied to detect blood flow, the concentration of hemoglobin, and oxygen saturation due to the different between the absorption spectra of oxygenated-hemoglobin and deoxygenated-hemoglobin [19,34]. In addition, PAM provides better spatial resolution and achieves depth information of choroidal and deep vasculature than OCT and OCTA. Although OCT is widely used to detect retinal vasculature, RVO, and RNV models, OCT is based on the scattering contrast of tissue, resulting in limited it penetration depth [4].

As a non-invasive imaging method, OCTA can generate angiographic imaging by employing motion contrast imaging to high-resolution volumetric blood flow information. Following the principle that movement in the back of the eye presents the blood flowed, the motion artifacts cannot be avoided in OCTA imaging. The minimum detectable flow velocity is determined by the time between the two sequential OCT B-scans, which will make it miss some areas with slow blood flow. This is critically clinically since slow blood flow is what interests clinicians most, namely visualization of leakage from angiogenesis and microaneurysms. OCT and OCTA can provide high resolution structurally and some functional information of the retina, but they have limited visualization of the choroid and molecular imaging capabilities remain rudimentary. The seeing neurons called photoreceptors receive their blood supply by a deeper vascular supply called the choroid particularly in the central vision, the fovea, where there is a foveal retinal avascular zone. OCT, OCTA, and other optical techniques have difficulty penetrating to this depth to visualize the choroidal vasculature with high resolution and instead report a choroidal thickness measurement, which serves as a poor biomarker for disease. Thus, the current study integrated OCT and PAM to overcome the limitation of OCT and to better observe the retinal vascular network. Structural and functional information of retinal vasculature was quantitatively identified using spectroscopic PAM. In addition, the current imaging system has excellent lateral and axial resolution of 4.1 and 37 µm for PAM and 3.8 and 3.0 µm for OCT, enabling visualization of microvasculature.

The acquired PAM images in Fig. 6 demonstrate that the treated retinal veins were well defined with high contrast. PA image contrast typically represents the intensity of the acquired PA signal, which is related to the optical energy absorbed by oxygenated hemoglobin and deoxygenated hemoglobin in blood vessels. Thus, the reduced contrast indicated lower light absorption by the targeted tissue. As shown in Fig. 6, the PA image contrast at the treated areas was significantly decreased in comparison to the surrounding tissue. This may occur due to the decreased in optical absorption of hemoglobin. For example, the optical absorption coefficient of oxygenated-hemoglobin is about 1.4-fold higher than that of deoxygenated-hemoglobin at 580 nm (e.g., µa = 270.56 cm\(^{-1}\) for oxygenated-hemoglobin vs. µa = 199.91 cm\(^{-1}\) for deoxygenated hemoglobin at 580 nm) [30,35]. According to Fig. 6(g), the measured PA signals at day 0 to day 7 were extremely lower than that of PA signal from the control group (red line), indicating that the retinal vessels were still occluded. In contrast, the measured PA signal at day 21 to 35 is similar to the control. This implies that the retina has revascularized. This finding is well registered with FA images shown in Figs. 4 and 5. Note that the PA image at day 3 and day 7 have more background noise in comparison with the quality images acquired at others days. The
background may possibly occur due to several facts: the cornea of the eye develops superficial punctate keratopathy and edema after laser illumination, resulting in increasing scattering on the eye surface and distribution of Rose Bengal particles beneath the retinal vessels, resulting in increasing the light absorption of the background.

The morphology of the retinal structure (size, shape, and vessel density) are able to be characterized from the OCT and PAM image. Based upon Figs. 3, 6 and 8, the diameter of treated retinal and choroidal vessels were estimated to be 79 ± 8.98 µm, and 76 ± 2.94 µm, which is 143.04, 261.84% times thinner than that of the normal vessel diameters (e.g., vessel diameter = 113 ± 5.35µm for RVs and 199 ± 8.37µm for CVs). Another advantage of the spectroscopic quantification is that the density of RNV detected at 580 nm was approximately 1.4 and 2.7 times higher than that of the density of RNV acquired at 532 nm and 590 nm, respectively due to strongly absorption laser light of hemoglobin. For example, hemoglobin absorbs approximately 250% and 348% greater amounts of light than hemoglobin at 510 nm and 590 nm, respectively (e.g. \(\mu_a = 270.6\, \text{cm}^{-1}\) for 580 nm vs. \(\mu_a = 108.19\, \text{cm}^{-1}\) and \(\mu_a = 77.76\, \text{cm}^{-1}\) for 532 and 590 nm, respectively [30]). In addition, the current PAM system can quickly achieve high-resolution images in less than one minute, which is limited by the laser repetition rate of 1 kHz of the OPO. However, the acquisition time can be improved by increasing the laser repetition rate. For example, a reported by Liu and Song et al. described that the acquisition time is about 2.7 s to achieve a raster-scan region of 2 x 2 mm using optical scanning method [15,34,36]. Another challenge of the PAM system in the eye is the illumination dose of laser light. Although the current PAM modality could non-invasively, non-ionizing, and label free distinguish individual retinal neovascularization at low laser exposure energy of 80 nJ, a half of the ANSI safety limit, long-term effect should be examined and monitored. Recently, Hariri et al. developed a PAM system using LEDs as an alternative illumination source [37]. The use of LEDs illumination can reduce the risk occur on the eye during performing PAM. In addition, LEDs is inexpensive and can be used to develop portable photoacoustic system.

Retinal vein occlusion in rodents has been investigated by several groups [4,38,39]. However, these studies induced retinal neovascularization in small animals such as mice and rats. The drawback of these studies is that the eyeball of the small animal is much smaller than human eyes (e.g., ~3 mm for mice, ~6 mm for rats vs. ~23 mm for human), resulting in difficulty for translation to clinical applications particularly for the ultrasound portion of PAM. In contrast, this study has successfully developed the RNV model in rabbits. The RNV model was developed at day 14 after laser-induced RVO and stabilized from days 21 to 35. The advantage of this RNV model in the rabbit is that the eyeball of the rabbit is similar to the human eyeball axial length (~18mm for rabbits), which is an important step for studies of RVO with PAM. Future work may incorporate oxygen saturation measurement for diagnosis of the abnormal blood flow in retinal vessels. In addition, the most important requirement for PAM imaging of the eye is to rapidly achieve high contrast and high resolution images of the eye without affecting or damaging sensitive neural tissue. Although the current study applied laser dose below ANSI safety limit, long-term thermal damage, thermoacoustic damage, and photochemical damage can be injured the retina [40,41]. Thus, further safety evaluation will also perform to evaluate the potential long-term effect of illumination light to the eye before translate this imaging technique to clinical application.

5. Conclusion

This study has successfully developed a multimodal spectroscopic PAM and OCT system for evaluation of the retinal vein occlusion and investigated the wavelength dependence of PA amplitudes for evaluation of the retinal neovascularization in rabbits. Experimental results demonstrate that the interruption of local blood perfusion can create retinal neovascularization in rabbits. The retinal neovascularization can be observed and evaluated using spectroscopic PAM and OCT in vivo in the rabbit. The combination en face PAM and
OCT imaging system have a high potential for detection of microvessels with high resolution and high contrast. The system has excellent lateral and axial resolutions (e.g., lateral resolutions are 4.1 um and 3.8 um and axial resolution are 37.0 um and 4.0 um for PAM and OCT, respectively). Importantly, the system could achieve label free imaging of microvasculature without administration of exogenous contrast agents, which was extremely desirable for clinical applications. The PAM system uses laser energy of 80 nJ to acquire the PA image which is well below the ANSI safety limit. The OCT system could provide useful information to quantify RNV and classify different layers of RNV, RPE, choroid, and sclera that will supplement the information provided by PAM. Both en face PAM and OCT can visualize the dynamic change in retinal thickness. In addition, spectroscopic PAM image helps to select the optimal wavelength for monitoring and quantification of the RNV structure. Therefore, the spectroscopic imaging system can serve as an efficient and safe manner for detection and characterization of the retinal neovascularization diseases.

Appendix

Fig. 10. Sequential fluorescein angiography images. The red arrow shows the position of veins, whereas blue arrow depicts the location of the artery. The time on the top right indicates the number of seconds after intravenous fluorescein dye injection.
Fig. 11. OCT spectral distribution

Fig. 12. Color fundus photography. White rectangles indicate the selected scanning areas for PAM

Fig. 13. Color fundus photography of the retina after laser-induced RVO. White rectangles indicate the selected scanning regions for PAM
Fig. 14. Fluorescein angiography (FA) images of retinal vein occlusion acquired at various time post-treatment. (a) FA images acquired at day 14, 17, 21 and 28 post laser photocoagulation. The green arrows indicate the position of the developed new retinal neovascularization (RNV). Note that the RNV increased over time and achieved peak at day 28. (b) sequential FA images. The red arrows indicate the region of retinal arteries. The blue arrows show the area of veins. The green and yellow arrows exhibit the areas of RNV and CVs, respectively. The time on the top right indicates the number of seconds after intravenous fluorescein dye injection.

**Funding**

National Eye Institute 1K08EY027458 (YMP), 4K12EY022299 (YMP), Fight for Sight-International Retinal Research Foundation FFSGIA16002 (YMP), unrestricted departmental support from Research to Prevent Blindness, and the University of Michigan Department of Ophthalmology and Visual Sciences. This study utilized the Core Center for Vision Research funded by P30 EY007003 from the National Eye Institute.

**Acknowledgments**

We thank Dr. Yuqing Chen for the generous donation of wild type New Zealand white rabbits for the experiments

**Disclosures**

The authors declare that there are no conflicts of interest related to this article

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