Optical coherence tomography angiography of stimulus evoked hemodynamic responses in individual retinal layers

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Abstract: Blood flow changes are highly related to neural activities in the retina. It has been reported that neural activity increases when flickering light stimulation of the retina is used. It is known that blood flow changes with flickering light stimulation can be altered in patients with vascular disease and that measurement of flicker-induced vasodilatation is an easily applied tool for monitoring functional microvascular alterations. However, details of distortions in retinal neurovascular coupling associated with major eye diseases are not well understood due to the limitation of existing techniques. In this study, flickering light stimulation was applied to mouse retinas to investigate stimulus evoked hemodynamic responses in individual retinal layers. A spectral domain optical coherence tomography (OCT) angiography imaging system was developed to provide dynamic mapping of hemodynamic responses in the ganglion cell layer, inner plexiform layer, outer plexiform layer and choroid layer before, during and after flickering light stimulation. Experimental results showed hemodynamic responses with different magnitudes and time courses in individual retinal layers. We anticipate that the dynamic OCT angiography of stimulus evoked hemodynamic responses can greatly foster the study of neurovascular coupling mechanisms in the retina, promising new biomarkers for retinal disease detection and diagnosis.

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1. Introduction

Neural activity in a localized brain region leads to rapid blood flow changes in that region. This phenomenon, called neurovascular coupling [1], also exists in the retina, which is part of the central nervous system [2]. The retina consists of multiple functional layers, which contain cell bodies, plexiform layers with axons and dendrites to form the neuronal network that...
preprocesses light-evoked signals before transmission to the brain [3]. The major retinal
diseases, such as retinitis pigmentosa (RP) [4], age-related macular degeneration (AMD) [5],
glaucoma [6], and diabetic retinopathy (DR) [7], or endothelial dysfunction secondary to
systemic diseases [8], are usually localized to one of these layers, leading to and
morphological abnormalities, impaired neural and physiological responses and blood vascular
system damage. However, details of the distortions in retinal neurovascular coupling (i.e.,
coherent interaction between retinal neural physiology and the blood vascular system)
associated with these major eye diseases are not well understood due to the limitation of
existing techniques.

Many techniques have been used to evaluate retinal neurovascular coupling such as
fundus photography [5], blue field simulation [9], color Doppler imaging [10], laser Doppler
velocimetry (LDV) [11], laser Doppler flowmetry [12], and land dynamic vessel analysis
[13]. Each technique has limitations such as operator dependency in fundus photography [14],
the subject’s approach in blue field simulation [15], limited detectable range in color Doppler
imaging [16], complexity of use in LDV [17], Doppler angle dependent detectability for laser
Doppler flowmetry [18], and only large vessel detection for dynamic vessel analysis [19].
Moreover, none of these methods can provide the necessary axial resolution to differentiate
individual retinal layers.

By providing excellent axial resolution, optical coherence tomography (OCT) provides a
noninvasive method for depth-resolved visualization of biological structures with a
micrometer level resolution. OCT has been extensively used for depth-resolved examination
of retinal morphology [20–25] and physiology [26–33]. OCT angiography, as a functional
extension of OCT, allows three-dimensional (3D) and label-free mapping of vascular
structures [34–40]. Several techniques were developed for the quantification of retinal blood
flow using OCT angiography such as speckle variance (SV) [41], phase variance (PV) [42],
optical microangiography (OMAG) [38], split spectrum amplitude-decorrelation (SSAD)
[37], and correlation mapping (CM) [35]. The SV method calculates inter-frame speckle
variance of OCT intensity images. The PV method is similar to the SV method but it uses a
phase signal to calculate the variance between consecutive frames. OMAG is based on
Doppler effect that can isolate blood flow from the static tissue background. SSAD divides
the OCT spectrum into several spectral bands and calculates the decorrelation between the
frames of each divided band, which are then averaged. CM calculates the correlation between
consecutive frames using an M by N window. Unlike conventional Doppler OCT or LDV,
which measures large retinal vessels (arteries and veins) to obtain total retinal blood flow,
OCT angiography is capable of measuring both the macro- and micro-circulation (down to the
capillaries) and angle of individual blood vessels. This allows OCT angiography to evaluate
the microcirculation of specific regions of the retina, which would not be possible using other
techniques that measure total retinal blood flow only.

It has been reported that neural activity increases when the retina is subjected to flickering
light stimulation [5]. It is also known that flickering light stimulation changes blood flow
responses in vascular diseased eyes [7, 43] and that measurement of flicker-induced blood
flow responses is an easily applied tool for monitoring functional microvascular alterations
[7]. In this study, light stimulation was applied to mouse retinas to investigate subsequent
hemodynamic responses in different retinal layers over time. A spectral domain OCT
angiography imaging system was developed to measure blood flow changes correlated with
light stimulation.

2. Materials and methods

2.1 Animal preparation

The study followed the Association for Research in Vision and Ophthalmology statement for
the use of animals in ophthalmic and vision research. All experiments were performed
following the protocols approved by the Animal Care Committee (ACC) at the University of
Illinois at Chicago. Wild type mice (strain C57BL/6J, acquired from the Jackson Laboratory) with a body weight of 30 g to 35 g were used in this study. Anesthesia was induced by a mixture of 60 mg/kg ketamine and 3 mg/kg xylazine, injected intraperitoneally. A custom designed animal holder with an ear bar and a bite bar were used to minimize movement during imaging. Ophthalmic mydriatics were applied to the eye and a cover glass (12-545-80; Microscope cover glass, Fisherbrand) along with gel (Severe; GenTeal, Novartis) was placed on the eye ball to work as a contact lens to improve image resolution and image quality by reducing optical aberrations of the mouse eye.

2.2 Spectral domain OCT angiography imaging system

Figure 1 shows a schematic diagram of the custom-designed spectral domain OCT. The light source was a near infrared superluminescent diode (SLD) with a central wavelength $\lambda$ of 850 nm and a bandwidth $\Delta \lambda$ of 100 nm (D840, Superlum). A fiber coupler with a splitting ratio of 75:25 divided the light coming from the source into the sample and reference arms. The reference arm contained a variable neutral density (ND) filter and a glass plate for balancing dispersion due to optical components between the sample and reference paths and matching ocular dispersion. In the sample arm, the light source was collimated through a collimating lens, and it then passed through two scanning mirrors (6231H, Cambridge Technology) to produce two dimensional OCT images. Finally, the light illuminated the eye through optical lenses. The scanning mirror was conjugated to the pupil of the eye to minimize the vignetting effect. Three achromatic lenses were used in both the sample and reference arms; L1 and L2 were used for beam expanding and L3 was used for focusing the light beam. A custom-designed spectrometer was constructed to image the interference spectrum returning from the interferometer. The line CCD camera (AViiVA EM4, E2v Technologies) provided a line rate up to 70,000 Hz. The speed used for this study was 57,000 Hz and 8,600 Hz, corresponding to a B-scan frame speed of 200 frames/s (Fig. 4) and 30 frames/s (Figs. 5, 6 and 7), respectively. The axial and lateral resolutions of the system were theoretically estimated at 3 µm and 12 µm in air, respectively. The axial and lateral resolutions can be estimated as $0.44 \cdot \lambda^2 / \Delta \lambda$ and $0.61 \cdot \lambda / NA$, respectively, where $\lambda$ is the central wavelength, $\Delta \lambda$ is the bandwidth of the SLD and NA is the numerical aperture in this system. The blood vessel diameter in the mouse retina is in the range of $30.0 \pm 6.7$ µm for arterioles and $46.5 \pm 16.5$ µm for venules [44]. Because the thickness of the mouse retina is about 200 ~250 µm, the depth of field (DOF) of the OCT system was designed at 300 µm and was calculated using $2 \pi \omega^2 / \lambda$, where $\omega$ is the radius of the beam at the retina and $\lambda$ is the central wavelength of the SLD. The oversampling factor (OF) of the tomograms was calculated for high speed imaging to minimize in-frame image blur and between-frame displacement, and thus to minimize the effect of eye movements to enable robust observation of transient blood flow responses correlated with retinal light stimulation [5]. The OF was defined as OF = w · N/d where w is the spot size, N is the number of sampling points, and d is the geometric width of the tomogram. In this study, we used $N = 200$ sampling points (number of A-line) for an OCT B-scan image, and a scan width of ~1.2 mm, leading to an OF of 2. The power of the probe beam incident on the mouse’s cornea was measured to be around 0.95 mW. A green light emitting diode (LED; peak $\lambda = 505$ nm, M505L3, Thorlabs) was coupled into the imaging system with a beam splitter (CM1-BP2, Thorlabs) as a retinal light stimulator.
Fig. 1. Optical diagram of spectral domain optical coherence tomography. BM: beam splitter; CL: collimation lens; L1, L2, L3: lens; PC: polarization controller; SLD: super luminescent diode. The retinal stimulator consists of a narrow-band LED light ($\lambda = 505$ nm).

2.3 Experimental protocol

The experiment was conducted in a dark room with the ambient light blocked. The mice were dark adapted for 2 hours prior to the experiment. A 10 Hz light stimulation was applied to the retina (mean illuminance of 930 lux at the cornea, 100% contrast). The OCT measurements were carried out at a distance of about 2 mm from an optic nerve head (ONH) and the selected area was carefully maintained by a microtranslational stage in the animal holder in each experiment. The total OCT measurement time was 60 s in each session, including a 5 s pre-stimulation phase, a 15 s light stimulation phase and a 40 s post-stimulation phase. The OCT measurements were performed continuously during the entire session.

2.4 Data analyses

The OCT images were processed to obtain differential M-scan tomograms and the angiographic changes of individual retinal layers were averaged as a graph with a custom software written in Matlab. Figure 2 shows the data processing flow chart. The acquired OCT spectrum was reconstructed to obtain the B-scan image. The image registration algorithm was applied to compensate bulk motion between the sequential images [45]. Following bulk motion compensation, the images were geometrically corrected using an image flattening algorithm to flatten the OCT B-scan images. A Gaussian filter was applied to the B-scan image to get a smoothed image and a binary image was created using the smoothed image. The maximum value location at each A-line was detected using edge detection and each A-line was aligned along the vertical direction to match the maximum value of each detected location in the same horizontal location. The OCT angiographic images were computed using the speckle variance (SV) calculation method with the following equation [35]:

$$SV_{ij} = \frac{1}{N} \sum_{i}^{N} [I_{ijk}(x,z) - \frac{1}{N} \sum_{i}^{N} I_{ijk}(x,z)]^2 = \frac{1}{N} \sum_{i}^{N} [I_{ijk} - (I_{mean})_{jk}]^2 \quad (1)$$
where \( i, j \) and \( k \) are indices of frame, lateral and depth pixel of the OCT B-scan, respectively. \( N \) is the number of frames used in the calculation. \( (I_{mean})_{jk} \) is the averaged frame of \( N \) frames over the same pixel. Most previous SV angiographic image-related studies calculated SV using repeated discrete frames at different sample locations to create an en-face image [41, 42, 46]. This method was not suitable for this study due to the lack of temporal resolution. Instead of using the conventional SV calculation, which uses discrete image frames for different SV angiographic images, we used a consecutive SV calculation method (see Fig. 3 for the difference between the conventional and consecutive SV calculations). Each SV angiographic image was calculated using 30 frames \((N)\) of OCT B-scan images, with two consecutive angiographic images sharing 29 frames of OCT B-scan images (i.e., the starting frame of an angiographic image was one frame later than that of the immediate previous angiographic image). These two methods shared the same calculation equation but the frames used for calculation were different. This consecutive calculation method can promise high temporal resolution of the SV angiographic images compared to conventional SV calculation methods. To detect the blood vessel in each image, we used the thresholding method to obtain the mask image. A differential OCT angiographic image was generated as \( \Delta SV/SV \), where \( SV \) was an averaged SV determined from the pre-stimulation depth scans and \( \Delta SV \) was the difference between the speckle variance intensity of each frame and the averaged SV determined from the pre-stimulation depth scans. The differential OCT angiographic image indicated the SV change rate to baseline (pre-stimulation time). Then differential OCT angiographic images and mask images obtained from the OCT angiographic images were combined to extract signals from vessel regions only. A differential M-scan tomogram along the elapsed time was calculated to improve the visibility of the stimulus-induced blood flow changes. Every frame of the masked differential OCT angiography was averaged toward the column direction and combined to generate a differential M-scan tomogram. A differential M-scan tomogram, reflecting SV changes relative to the pre-stimulation baseline along the elapsed time, was calculated to improve the visibility of the stimulus-induced blood flow changes. Every frame of masked differential OCT angiography was averaged toward the column direction and combined to generate a differential M-scan tomogram. The hemodynamic responses at each retinal layer were averaged. Individual functional layers were segmented manually to plot averaged angiographic changes. First, each individual layer was confirmed approximately using OCT images and then each blood vessel in each individual layer was found using SV images. The blood vessels in SV images were not dense and easy to differentiate and it was therefore possible to segment accurately.

3. Results
The OCT B-scan images clearly showed differentiated retinal layers (Figs. 4(A1) and 4(B1)) and the ONH (Fig. 4(A1)). Some large blood vessels were observed in the NFL/GC layer. The
projection fundus images enabled visualization of large blood vessels (Figs. 4(A2) and 4(B2)). The OCT en-face angiographic images showed depth-resolved vascular information for both large vessels when imaged near the ONH and more dense small blood vessels when imaged at a 2 mm distance from the ONH (Figs. 4(A3) and 4(B3)). Thus, we chose 2 mm from the ONH as the imaging area for the light stimulation experiment.

OCT B-scan sequence images (Fig. 5(A)) provided structural information about the retina without blood flow information in individual functional layers before and during the light stimulation. The blood flow information was clearly observed in OCT angiographic images at individual functional layers such as the GL, IPL, OPL and CH (Fig. 5(B)). Differential OCT angiographic images (Fig. 5(C)) demonstrated dynamic angiographic changes in individual layers over time, with a peak time at ~7.5 s after the stimulation. The enlarged B-scan image (Fig. 5(D)) provided a clear retinal structure of individual functional layers. Figure 5(E) shows more detailed angiographic information, such as blood flow, vessel size and location, with the relative blood flow changes indicated by different colors from red (fast blood flow) to blue (slow blood flow). Also, various sizes of blood vessels were observed. The enlarged dynamic angiographic images (Fig. 5(F)) at different time points provided a clear visualization of blood flow changes, with the changing locations corresponding to blood vessel locations in the angiographic images (Fig. 5(E)).
Fig. 5. (A) B-scan OCT sequence; (B) B-scan angiography sequence; (C) Dynamic angiography change with the pre-stimulus baseline subtracted; (D) Enlarged picture of image A0; (E) Enlarged picture of image B5; (F) Enlarged pictures of images C2 (F1), C5(F2). Scale bar indicates 200 µm.

Flattened images (Figs. 6(A), 6(B) and 6(C)), which provided straightforward segmentations of retinal layers compared to the original images (Fig. 5), showed better visualization of each retinal layer, blood vessel and dynamic angiographic changes. A differential M-scan tomogram (Fig. 6(D)) provided additional information about dynamic angiographic changes in individual functional layers over time. A noticeable increase in dynamic angiographic changes was induced several seconds after the light onset, especially in the IPL and OPL (see Fig. 6(E) for the averaged dynamic angiographic changes of individual functional layers). The dynamic angiographic changes occurred at 5 s and 6.2 s and reached their maximum at 10.3 s and 12.8 s after the stimulus onset in the IPL and OPL, respectively. The increased dynamic changes were sustained even during the post-stimulation phase in which the light was switched off. Dynamic changes occurred immediately after stimulation of the GL, although the magnitude of the dynamic changes was not as apparent as those in IPL and OPL. In the CH, we did not detect significant changes.

Fig. 6. Flattened images of Fig. 5(A7) (A), Fig. 5(B7) (B), and Fig. 5(C7) (C). (D) Differential M-scan tomogram of dynamic angiographic changes, corresponding to Fig. 5(C). (E) Averaged angiographic changes of individual functional layers. Red and blue arrowheads indicate the onset and time-to-peak, respectively.
Figure 7 shows three representative experimental results, which had similar patterns except the response times were slightly different in the experiments. The differential M-scan tomogram (Figs. 7(A2), 7(B2) and 7(C2)) showed large dynamic angiographic changes in the IPL and OPL, small changes in the GL and almost no significant changes in the CH. On average, the response time was 6.3 (mean) ± 1.2 s (sd) in the IPL and 7.4 ± 1.7 s in the OPL, and the time to peak was 12.54 ± 1.59 s in the IPL and 14.31 ± 1.97 s in the OPL. In the GL, the response was increased first and then decreased (Figs. 7(A3), 7(B3) and 7(C3)) but the response changes were small compared to those in the IPL and OPL (the peak change in the GL was only 0.7% and those in the IPL or OPL were 9% and 12%, respectively).

4. Discussion

In this study, we developed and tested a spectral domain OCT angiography system. The resolution and DOF were sufficient for imaging the mouse retinal vascular system, as the OCT images of mouse retina (Fig. 4) properly showed retinal structure and vasculature information. We further demonstrated dynamic angiographic changes induced by flickering light stimulation, confirming neurovascular coupling in the retina. Several studies have assessed the relation of flickering light stimulation and retinal hemodynamic responses using various techniques [47–52]. Recently, OCT angiography has been used to image hemodynamic responses in response to flickering light stimulation. Radhakrishnan et al. and Wei et al. investigated retinal blood flow changes induced by light stimulation [51, 52]. In their studies, OCT en-face images were used to show blood flow changes and to provide geometrical information about the blood flow changes from the entire retina, without depth-resolved information at individual retinal layers. Moreover, their studies did not provide time-resolved hemodynamic changes caused by flickering light stimulation. To the best of our knowledge, this is the first study to demonstrate time-resolved dynamic OCT angiography at individual functional layers with flickering light stimulation. The SV method was used to identify blood flow changes in OCT images. The SV calculates speckle changes in OCT intensity signals between the frames and these speckle changes depend on the object’s movement. This SV calculation in OCT images can detect the speckle changes that are
relatively similar to laser speckle imaging. Several studies already showed quantitative flow in OCT images using speckle changes [53–55].

Figures 5(C) and 5(F) provide more detailed information about the increased blood flow after flickering light stimulation. These hemodynamic responses can be explained by vascular coupling mechanisms. Major arterioles on the retinal surface could be dilated by a flickering light to produce increased blood flow in the retinal vessels and to meet increased oxygen and glucose needs of active neurons at the inner and middle retinal layers. Blood flow increases in the optic disc serve the needs of the active axons of the retinal ganglion cells [56]. This hemodynamic response of the retinal nerve system is strongly related to the cerebral circulation [56]. The cerebral blood flow increases are related to glucose consumption and this glucose metabolism is connected to synaptic activity. The neuron requires oxygen for hemodynamic responses, the increase in local blood flow caused by O\textsubscript{2} exhaustion and metabolic excess of CO\textsubscript{2}. Another hypothesis is that cytosolic free nicotinamide adenine dinucleotide - hydrogen (NADH) supplies a signaling pathway that increases nitric oxide (NO) production, which increases blood flow in stimulated areas. It needs more precise and controlled data about stimulation-induced changes in firing rate energy, glucose and O\textsubscript{2} consumption, as well as oxy- versus deoxy-hemoglobin content to confirm the mechanism of the neurovascular coupling in the retina and optic nerve [2, 56–58].

The differential M-scan tomograms (Figs. 6(D), 7(A2), 7(B2) and 7(C2)) provide intuitive visualization about the dynamic angiographic changes at individual functional layers over time. The increased dynamic angiographic changes were observed in the GL, IPL and OPL. The hemodynamic responses in the IPL and OPL were much higher than that in the GL. This may be caused by different metabolic demands after light stimulation. Using a confocal microscope, Kornfield et al. measured vessel diameters and red blood cell flux in the retinal trilaminar vascular network and found that blood flow in the three different retinal layers was differentially regulated after flickering light stimulation [59]. The differential regulation of blood flow in the different retinal layers may be due to the differences in neuronal metabolic demand. The oxygen in the superficial layer is supplied by both superficial layer vessels and the vitreous humor. The pO\textsubscript{2} near the retinal surface was high enough and thus it was not necessary to increase blood flow during light stimulation [60]. The oxygen and nutrients were supplied to synapses in the inner plexiform retina and outer plexiform layer by blood vessels in the intermediate layer and deep layer, respectively. Also, synapses in both layers can be activated by stimulation conditions [59]. When the stimulation is applied to these layers, the blood flow changes to meet the increased metabolic demand of the neurons. The changes in the CH were not significant, probably because of anatomical differences between choroidal and retinal circulations. The large arteries in the outer part of the choroid are fed by ciliary arteries. The central retinal artery in the optic nerve serves the retinal vasculature. The autoregulation in retinal circulation is well developed, whereas it is not in choroidal vessels. This may cause a different functional hyperemia response when flickering light stimulation is applied to the retina and CH [56].

Our results were basically consistent with previous studies of retinal neurovascular coupling, although there were minor discrepancies in terms of response times, response durations and magnitudes. These discrepancies may originate from different experimental conditions such as light stimulation characters (wavelength, frequency, illuminance, and duration, etc.), stimulation location and recording sensitivity. Riva et al. described different species showing similar changes but slightly different flow changes [2]. This may suggest that flickering light induced vasodilatation is caused by different mechanisms, which remain to be investigated.

In summary, we demonstrated an OCT angiography technique that can observe the time-resolved hemodynamic responses in different retinal layers induced by flickering light stimulation. We observed differential hemodynamic responses at different retinal layers, probably due to different metabolic demands, with significant increases in the IPL and OPL,
small increases in the GL but without significant changes in the CH. This study demonstrated the potential of using OCT angiography for investigating coupling mechanisms between retinal neural activities and hemodynamics. We anticipate that the functional OCT angiography can be readily adapted to human studies to explore sensitive angiographic markers of various eye diseases.

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