In Vivo Confocal Intrinsic Optical Signal Identification of Localized Retinal Dysfunction

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PURPOSE. The purposes of this study were to investigate the physiological mechanism of stimulus-evoked fast intrinsic optical signals (IOSs) recorded in dynamic confocal imaging of the retina, and to demonstrate the feasibility of in vivo confocal IOS mapping of localized retinal dysfunctions.

METHODS. A rapid line-scan confocal ophthalmoscope was constructed to achieve in vivo confocal IOS imaging of frog (*Rana pipiens*) retinas at cellular resolution. In order to investigate the physiological mechanism of confocal IOS, comparative IOS and electroretinography (ERG) measurements were made using normal frog eyes activated by variable-intensity stimuli. A dynamic spatiotemporal filtering algorithm was developed to reject the contamination of hemodynamic changes on fast IOS recording. Laser-injured frog eyes were employed to test the potential of confocal IOS mapping of localized retinal dysfunctions.

RESULTS. Comparative IOS and ERG experiments revealed a close correlation between the confocal IOS and retinal ERG, particularly the ERG a-wave, which has been widely used to evaluate photoreceptor function. IOS imaging of laser-injured frog eyes indicated that the confocal IOS could unambiguously detect localized (30 μ m) functional lesions in the retina before a morphological abnormality is detectable.

CONCLUSIONS. The confocal IOS predominantly results from retinal photoreceptors, and can be used to map localized photoreceptor lesion in laser-injured frog eyes. We anticipate that confocal IOS imaging can provide applications in early detection of age-related macular degeneration, retinitis pigmentosa, and other retinal diseases that can cause pathological changes in the photoreceptors. (*Invest Ophthalmol Vis Sci*. 2012;53:8139-8145) DOI:10.1167/iovs.12-10732

It is well established that many eye diseases involve pathological changes of photoreceptors and/or their support system, including different forms of retinitis pigmentosa (RP)¹ and age-related macular degeneration (AMD),²⁻⁵ a highly prevalent outer retinal disease. To prevent or slow the progress of vision loss associated with outer retinal disease, early detection and reliable assessment of medical interventions, including morphological examinations, are key elements. The

application of adaptive optics (AO) and optical coherence tomography (OCT) has enabled retinal fundus imaging with cellular resolution. However, disease-associated morphological and functional changes, if independently measured, are not always correlated directly in time course and spatial location.^{6,7} Therefore, a combined assessment of retinal function and structure is essential.

Psychophysical methods that access outer retinal function, such as visual acuity (VA) testing, are practical in clinical applications, but the VA test involves extensive higher-order cortical processing. Therefore, VA testing does not provide information on retinal function exclusively and lacks sensitivity for early detection of outer retinal diseases such as AMD.^{8,9} Electroretinography (ERG) methods, including full-field ERG,¹⁰ focal ERG,¹¹⁻¹⁵ and multifocal ERG,¹⁶⁻¹⁸ have been established for objective examination of retinal function. However, the spatial resolution of ERG may not be high enough to provide direct comparison of localized morphological and functional changes in the retina.

Stimulus-evoked fast intrinsic optical signals (IOSs) provide a promising alternative to ERG for objective measurement of retinal function with improved spatial resolution.^{19,20} We have recently demonstrated ex vivo IOS identification of localized retinal dysfunction in an inherited photoreceptor degeneration model.²¹ Because functional IOS images are constructed through spatiotemporal processing of pre- and poststimulus images, concurrent structural and functional measurements can be naturally achieved using a single optical instrument. Conventional fundus cameras have been used to detect IOSs from anesthetized cats and monkeys²²⁻²⁵ and awake humans.²⁶ Given limited axial resolution, fundus IOS imaging does not exclusively reflect retinal neural function due to complex contaminations of other ocular tissues. In principle, AO²⁷⁻²⁹ and OCT imagers³⁰⁻³² can provide cellular resolution. However, the signal source and mechanism of these imaging modalities are not well established, and functional mapping of fast IOSs that have time courses comparable to retinal electrophysiological kinetics is still challenging.

We recently developed a line-scan confocal microscope to achieve fast IOS imaging at high-spatial (μ m) and high-temporal (ms) resolution.³³ Rapid in vivo confocal IOS imaging has revealed a transient optical response with a time course comparable to that with ERG. In this paper, we report comparative confocal IOS imaging and retinal ERG recording to investigate the physiological mechanism of confocal IOS,³³⁻³⁵ and demonstrate confocal IOS identification of localized acute retinal lesions in an animal model, laser-injured frog eyes.

METHODS

Experimental Setup

Figure 1 shows the schematic diagram of our line-scan confocal ophthalmoscope for confocal IOS imaging. The optical rationale of the line-scan confocal IOS imaging was reported in our previous

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publication.³⁴ In this study, the imaging system was upgraded with a new fast linear CCD camera (EV71YEM2CL1014-BA0; E2V, Tarrytown, NY). The new camera was equipped with camera link interface that greatly facilitated system control and data synchronization. The line-scan confocal imaging system consisted of two light sources: near infrared (NIR) for IOS recording and visible green for retinal stimulation. The NIR light was produced by a superluminescent laser diode (SLD) (SLD-35-HP; Superlum, County Cork, Ireland) with center wavelength of 830 nm. A single-mode fiber-coupled 532 nm DPSS laser module (FC-532-020-SM-APC-1-1-ST; RGBLase LLC, Fremont, CA) was used to produce the visible light for stimulating or injuring the retina locally. It provided adjustable output power from 0 to 20 mW at the fiber end. A mechanical slit (VA100; Thorlabs, Newton, NJ) was placed behind the collimated green stimulus light to produce a rectangle pattern and provide precise adjustment of stimulus width.

A custom program (Labview 2011; National Instruments Corp., Austin, TX) was developed for system synchronization, high-speed image acquisition, and real-time image display. Before each IOS recording, stimulus timing and location in the field of view were tested for repeatability and accuracy. During each experiment, the retina was continuously illuminated by the NIR light at $\sim 600 \mu\text{W}$. For each IOS recording trial, 400 ms pre-stimulus and 800 ms after-stimulus images were recorded at the speed of 100 frames/s, with frame size of 350×100 pixels ($\sim 300 \mu\text{m} \times 85 \mu\text{m}$ at the retina). Exposure time of the line-scan CCD camera was $71.4286 \mu\text{s}$. Scanning speed of the mirror was 100 Hz.

The ERG was recorded via placement of differential electrodes on two eyes. The ERG signal was amplified with a physiological amplifier (DAM 50; World Precision Instruments, Sarasota, FL) equipped with a band-pass (0.1 Hz to 10 kHz) filter. The preamplified ERG was digitized using a 16-bit DAQ card (NI PCIe-6351; National Instruments) with a resolution of 1.6 mV and was sent to the computer for averaging, display, and storage.

Animal Preparation

The northern leopard frog (*Rana pipiens*) was selected to take advantage of the high-quality optics of the ocular lens³⁶ and the large size of the retinal photoreceptors (cone, $3 \mu\text{m}$; rod, $6 \mu\text{m}$).³⁷ Together, these characteristics made it possible to resolve individual photoreceptors (blue arrowheads in Fig. 2A) and blood vessels (yellow arrowheads in Fig. 2A and see Supplementary Material and Supplementary Movie 1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10732/-DCSupplemental>) in vivo. The experimental procedure was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and carried out in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Frogs were dark adapted for at least 2 hours prior to functional IOS imaging. Then, the frog was anesthetized by immersion in tricaine methanesulfonate solution (TMS; MS-222; 500 mg/L).³⁸ Pupils were fully dilated with topical atropine (0.5%) and phenylephrine (2.5%). After confirmation of the anesthesia, the frog was placed in a custom-built holder for IOS imaging. The holder provided five degrees of freedom to facilitate adjustment of body orientation and retinal area for IOS imaging.

Experimental Design

A rectangular stimulus bar with $30 \mu\text{m}$ width (Fig. 3A) and 20 ms duration was used for localized retinal stimulation. Estimated maximum stimulus flash intensity was 3.5×10^5 photons/ $\mu\text{m}^2/\text{ms}$ (7×10^6 photons/ μm^2 for 20 ms) at the retina. Neutral density filters were used to adjust light intensity for retinal stimulation. The IOS and ERG were recorded over a 5.0 log unit range in nine steps, namely, -5.0 , -4.0 , -3.0 , -2.5 , -2.0 , -1.5 , -1.0 , -0.5 , and 0.0 . Stimulus flashes were presented at 2-minute intervals (Fig. 4). IOS and ERG recordings were performed consecutively in the same frog eye, as explained in the Results section.

FIGURE 1. Schematic diagram of the line-scan confocal ophthalmoscope for in vivo IOS imaging. CO, collimator; CL, cylindrical lens; BS, beam splitter; SM, scanning mirror; DM, dichroic mirror; MS, mechanical slits; Lx, optical lenses.

Both normal and laser-injured frogs were used in this study. To produce a localized retina laser injury, a $30 \mu\text{m}$ width green laser light bar with output power of 1 mW at the retina surface was continuously delivered into the retina for 30 seconds. Thirty minutes after local damage was induced, a full-field stimulus (Fig. 5) was applied to injured and noninjured areas to obtain the retinal response pattern.

Data Analyses

The raw images were processed using a custom program with a user-friendly GUI interface in MATLAB R2011a (MathWorks, Natick, MA). Raw images were registered to compensate for eye movements before IOS calculation (Fig. 3A). Basic procedures of IOS data processing have been previously reported.¹⁹

As shown in Figure 2A, ocular blood vessels can superimpose on photoreceptor cells, and hemodynamic changes inherent to rapid blood flow may contribute to fast IOS recording. Retinal blood vessels can be mapped based on dynamic optical changes correlated with blood flow.³⁹ We adapted the strategy to separate stimulus-evoked fast IOS in retinal photoreceptors from blood flow-induced optical change. Key procedures of the dynamic spatiotemporal filtering are summarized as follows:

(1) To calculate the mean $\bar{I}(x, y)$ of each pixel in the pre-stimulus baseline recording (n frames):

$$\bar{I}(x, y) = \frac{1}{n} \sum_{j=1}^{j=n} I_j(x, y) \quad (1)$$

(2) To calculate the standard deviation $\sigma(x, y)$ of each pixel in the pre-stimulus baseline recording (n frames):

$$\sigma(x, y) = \sqrt{\frac{1}{n} \sum_{j=1}^{j=n} [I_j(x, y) - \bar{I}(x, y)]^2} \quad (2)$$

(3) To conduct spatiotemporal filtering of potential noises: Because blood flow changes dynamically, the variability of light intensity at the blood vessels is much larger than that at the blood-free area; that is, before the stimulus, the temporal $\sigma(x, y)$ of blood flow is much larger than that of photoreceptors. Upon stimulation, blood flow may increase, but within the short recording time (1 second), hemodynamic change is slow. Therefore, the temporal $\sigma(x, y)$ change of blood flow is insignificant compared with the fast IOSs from the photoreceptors. To reject noise attributable to blood flow, values three standard deviations above or below the mean at each pixel were used

FIGURE 2. (A) Representative in vivo confocal image of frog retina. Individual blood vessels (*yellow arrowheads* and see Supplementary Material and Supplementary Movie 1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10732/-/DCSupplemental>) and photoreceptors (*blue arrowheads*) were clearly observed. (B) $3\text{-}\sigma$ map of the pre-stimulus images to show blood vessel pattern. Scale bars (in *white*) represent $50\text{ }\mu\text{m}$.

as a filtering criterion. This filter ($3\text{-}\sigma$) allowed us to plot the vasculature profile as shown in Figure 2B. In other words, the pixel change will be assumed to reflect noise, if

$$\bar{I}(x, y) - 3\sigma(x, y) < I_t(x, y) < \bar{I}(x, y) + 3\sigma(x, y) \quad (3)$$

Therefore, a high threshold is used to define stimulus-evoked IOS in the retinal area superimposed by blood vessels. The signals at pixel (x, y) with light intensity greater than the mean above three standard deviations are positive and those with light intensity less than the mean below three standard deviations are negative. IOS images with pixels that fall into the noise range are forced to be zero, and only positive or negative IOSs are left. Therefore, after dynamic spatiotemporal filtering (Fig. 3C), most hemodynamic-driven optical signals (Fig. 3B) can be rejected.

RESULTS

Confocal IOS Imaging of Localized Retinal Response

Experiments were designed to validate the feasibility of line-scan confocal IOS imaging of localized retinal response in intact frog eye. The stimulus light intensity was at -1 log unit. Each illustrated frame in Figure 3 is the average of two raw IOS images obtained during a 20 ms epoch. Additionally, 40 ms pre-stimulus and 80 ms post-stimulus recordings are shown.

As shown in Figure 3B, an IOS pattern was observed after a rectangular stimulus was delivered, whereas the blood vessels showed persistent optical changes. After setting the pixels falling within the range defined by equation (3) to zero, most of

FIGURE 3. (A) Confocal images of frog retina; each illustrated frame was the average over 20 ms. Epochs of 40 ms (pre-stimulus) and 80 ms (post-stimulus) are shown. The *green rectangle* in the third frame of column A indicates the size and location of the stimulus pattern. (B) The spatial IOS image sequence before filtering blood dynamics. (C) The spatial IOS image sequence after filtering blood dynamics. (D) The IOS strength distribution image sequence after filtering blood dynamics. Scale bars (in *white*) represent $50\text{ }\mu\text{m}$.

FIGURE 4. Comparative IOS and ERG analysis. IOS (A) and ERG (B) were recorded from the same groups of frogs. Each tracing represents an average of four responses evoked by light flashes of progressively brighter intensities over 5.0 log units ($\log I/I_{\max}$) as indicated by the legend. (C) Normalized magnitude and (D) time to peak of ERG a-wave, b-wave, and confocal IOS plotted as a function of stimulus strength.

the rapid blood flow activities were excluded from stimulus-evoked retinal responses. With a clean background, the stimulus-activated IOS pattern can be visualized clearly from Figure 3C. Both positive and negative signals were observed almost immediately after retinal stimulation. Figure 3D shows the IOS pattern by plotting absolute magnitude and ignoring the signal polarities.

Physiological Source of the Confocal IOS

Experiments were designed to determine the physiological source of confocal IOS by comparing IOS imaging and ERG recording. Figure 4A shows representative IOS magnitude dynamics elicited by nine different stimulus strengths over a 5 log unit range. Figure 4B illustrates ERG waveforms recorded under the same conditions. The amplitude of the a-wave was measured from baseline to trough. The amplitude of the b-wave was measured from the a-wave trough to the b-wave peak. IOS and ERG signals were not measured simultaneously. Rather, they were recorded with the same stimulus/illumination light and in the same eye. Both IOS and ERG signals were averaged based on four trials/eyes. For the first and third trial/eye, IOS was first recorded, then ERG. For the second and fourth trial/eye, the order was changed to ERG recording first, followed by IOSs. In this way, differences in experiment conditions between IOS and ERG recordings could be minimized. It was typically observed that the IOS occurred almost immediately after the stimulus delivery, reaching peak magnitude within 150 ms. To compare time courses of IOS and ERG dynamics, ERG a-wave, b-wave, and IOS magnitudes were normalized as shown in Figure 4C. The amplitude of the b-wave first increased almost linearly with the gradual increased

intensity of the stimulus, reached a maximum, and then decreased as light intensity became higher than -1.5 log units. The a-wave is widely accepted as a measure of photoreceptor function.⁴⁰ At low stimulus light intensities (below -3 log units), a-wave amplitude increased slowly with increased stimulus intensity, whereas it increased much faster when the light intensity was above -3 log units. Maximum a-wave amplitude was found at the light intensity of -0.5 log units, 10 times higher than the maximum of -1.5 log units for the b-wave. As can be seen from Figure 4C, the overall trend of IOS magnitude was quite consistent with that of a-wave amplitude, including the threshold and maximum response. This suggests that confocal IOSs predominantly originate from retinal photoreceptors.

Time-to-peak values of the IOS and ERG recordings also show similar dependency on stimulus intensity, decreasing as the light intensity increased (Fig. 4D).

IOS Detection of Localized Retinal Lesion

This experiment was designed to demonstrate IOS identification of localized retinal lesions in laser-injured frog eyes.

Figure 5A1 shows retinal structure before laser damage. A full-field stimulus with moderate intensity (at -1.5 log units) was applied to conduct confocal IOS imaging. The corresponding 3-D surface envelope of the IOS image recorded within 0.1 seconds after stimulus delivery is illustrated in Figure 5A2. For better visualization of the overall IOS distribution pattern, we smoothed the IOS image using a mean filter (kernel size $15 \mu\text{m} \times 15 \mu\text{m}$). A relatively homogeneous signal distribution pattern was displayed (Fig. 5A3).

FIGURE 5. Structural and IOS images before and after laser injury. Retinal structure of normal frog eye (**A1**) and the same retinal area after laser injury (**B1**); three-dimensional IOS image with full-field stimulus before (**A2**) and after (**B2**) laser injury; the corresponding overall IOS distribution images obtained by smoothing (**A3**, **B3**). Scale bars (in black) represent 50 μm .

In order to demonstrate the feasibility of detecting localized retinal damage, a 30 μm lesion was introduced after a control test (Figs. 5A1–A3). Thirty minutes after the laser exposure, the same full-field stimulus was applied to this retinal area. From the structural images of Figures 5A1 and 5B1, we could barely observe any visible changes. However, IOS images with full-field stimulus showed a signal-absent slit area located at the place where the laser damage was introduced (Fig. 5B2). With use of the smoothing method described above, the IOS magnitude image (Fig. 5B3) showed a clear 30 μm wide rectangle of markedly reduced signal. Therefore, our experiment indicated that rapid line-scan IOS imaging of intact frogs could be used for *in vivo* investigation of this localized retinal lesion.

DISCUSSION

In summary, a rapid line-scan confocal imager was employed to achieve cellular resolution IOS imaging of retinal photoreceptors *in vivo*. The confocal IOS patterns show tight correlation with localized retinal stimulation (Fig. 3). A spatiotemporal filtering algorithm was developed to separate stimulus-evoked fast IOS response from blood flow. Given the fact that blood flow could induce significant optical fluctuation without the requirement of retinal stimulation, the blood flow associated artifact could be readily excluded by dynamic threshold

rejection. This spatiotemporal filtering assumes that blood flow-associated optical changes at any one location were consistent before and after stimulus delivery. Although it is possible that retinal stimulation may produce hemodynamic changes in the blood vessel area, we did not detect significant changes in the short (0.8 seconds) after-stimulus recording epoch.

Comparative ERG measurements were conducted to investigate physiological sources of the confocal IOS. Our experiments revealed tight correlation between the IOS response and ERG a-wave. Both magnitudes and time courses of the IOS and a-wave showed similar responses to stimulus intensity changes. The time to peak of IOSs fell between the a-wave and b-wave. It is well established that the a-wave leading edge is dominated by retinal photoreceptors, and the later phase is truncated by electrophysiological response of inner retinal neurons, particularly ON ('on') bipolar cells. If we record a pure photoreceptor response, that is, if postphotoreceptor neurons are blocked, the a-wave should take more time to return to baseline, which results in longer time to reach peak compared with the a-wave of standard ERGs.^{41–43} From this perspective, if we assume that the fast IOSs originate from retinal photoreceptors, the measured time to peak of the IOS should be longer than that of the standard a-wave but shorter than the b-wave, which is consistent with our experimental results. Therefore, we speculate that the confocal IOSs originate mainly from retinal photoreceptors. In addition,

because of the frog eye's high numerical aperture (0.4), the axial resolution of confocal IOS imaging was estimated at $\sim 10\ \mu\text{m}$. This resolution was sufficient to distinguish the photoreceptors from other retinal layers. Previous studies with isolated photoreceptor outer segments and isolated retinas have demonstrated transient IOSs associated with phototransduction.^{44–46} Both binding and release of G-proteins to photoexcited rhodopsin might contribute to the positive (increased) and negative (decreased) IOSs.⁴⁵ Localized biochemical processes might produce nonhomogeneous light intensity changes, that is, positive and negative signals mixed together.

A laser-injured frog model was used to validate confocal IOS identification. By inducing localized retinal lesions through green laser exposure, we demonstrated that confocal IOS imaging can provide high transverse resolution, at least $30\ \mu\text{m}$. Based on early investigations of laser damage in other animal models,^{47–49} we estimated that our laser exposure could produce severe photoreceptor damage. Further investigation is required to quantify the laser thresholds that can produce detectable cone and rod photoreceptor damages.

Better development of high-resolution confocal IOS imaging can lead to reliable physiological assessment of individual retinal photoreceptors. This prospect is particularly important for rods, known to be more vulnerable than cones in aging and in early AMD.^{2,50} The most common cause of severe vision loss and legal blindness in adults over 50.^{2,51} Early detection and reliable assessment of medical interventions are key elements in preventing or slowing the progress of AMD-associated vision loss. Both morphological⁵² and functional^{50,53} tests are important for reliable detection of AMD. Currently, there is no established strategy to allow objective assessment of retinal dysfunction at high resolution to enable direct comparison between localized physiological and morphological abnormalities in early AMD or other eye diseases. Confocal IOS imaging will enable concurrent morphological and functional assessment of localized retinal dysfunctions in vivo. Further, it can be combined with technologies that assess structure and function of the photoreceptor support system that is affected even earlier in AMD. This combination could revolutionize the study, diagnosis, and therapy assessment of AMD.

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