High spatiotemporal resolution imaging of fast intrinsic optical signals activated by retinal flicker stimulation

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Abstract: High resolution monitoring of stimulus-evoked retinal neural activities is important for understanding retinal neural mechanisms, and can be a powerful tool for retinal disease diagnosis and treatment outcome evaluation. Fast intrinsic optical signals (IOSs), which have the time courses comparable to that of electrophysiological activities in the retina, hold the promise for high resolution imaging of retinal neural activities. However, application of fast IOS imaging has been hindered by the contamination of slow, high magnitude optical responses associated with transient hemodynamic and metabolic changes. In this paper we demonstrate the feasibility of separating fast retinal IOSs from slow optical responses by combining flicker stimulation and dynamic (temporal) differential image processing. A near infrared flood-illumination microscope equipped with a high-speed (1000 Hz) digital camera was used to conduct concurrent optical imaging and ERG measurement of isolated frog retinas. High spatiotemporal resolution imaging revealed that fast IOSs could follow flicker frequency up to at least 6 Hz. Comparable time courses of fast IOSs and ERG kinetics provide evidence that fast IOSs are originated from stimulus activated retinal neurons.

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References and links

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1. Introduction

High resolution imaging of stimulus-evoked retinal neural activities is important for understanding of visual information processing mechanisms in the retina. High resolution evaluation of localized retinal dysfunction can also be used in disease diagnosis and treatment outcome evaluation. It is well established that many eye diseases can cause pathological changes of photoreceptors and/or inner retinal neurons that ultimately lead to vision losses and even complete blindness. Different eye diseases, such as glaucoma [1, 2], diabetic retinopathy [3, 4], and macular degeneration [5], are known to target at different types of retinal neurons, causing localized lesions. Electroretinogram (ERG) [6] and multifocal ERG [7, 8] are frequently used to measure retinal neural activities, but the spatial resolution of ERG may not be high enough to capture the subtle pathological changes in the early stage of these diseases because ERG signals are pooled from an extended retinal area. Given the delicate structures and complicated functional interactions of the retina, detection of localized dysfunction of different cell populations requires a method that can examine stimulus-evoked retinal neural activities at high spatial and temporal resolutions.

It is possible to optically record stimulus-evoked neural activities in 3 dimensions with high spatial (~µm) resolution. A variety of voltage-sensitive dyes and ion selective indicators have been used to facilitate optical imaging of dynamic neural activity [9, 10]. However, potential phototoxicity of the dyes and the difficult loading procedures limit the applications of these techniques, especially in human subjects. Optical imaging of transient changes of intrinsic optical parameters in activated neural tissues avoids exogenous dyes or indicators. Fast intrinsic optical signals (IOSs) that are closely associated with action potentials and postsynaptic potentials have been observed in dissected neural tissue [11, 12]. Transient IOSs tightly correlated with phototransduction procedures have been also detected in isolated outer segments of photoreceptors [13–15] and isolated retinas [16, 17]. Both time-domain [18, 19] and frequency-domain [20, 21] optical coherence tomography (OCT) imagers have been used for depth-resolved recording of IOSs from isolated retinas [18, 19] and in intact eyes [20, 21]. Recent development of adaptive optics imagers [22, 23] has made in vivo IOS imaging of phototransduction changes of individual photoreceptors possible. However, clinical applications of IOSs for retinal diseases diagnosis have been hindered by their low sensitivity and limited specificity.

Transient IOSs recorded in the retina usually consist of stimulus-evoked retinal neural activities and corresponding metabolic changes of neural- and non-neural (such as glial) cells and hemodynamic activities. IOSs associated with hemodynamic and metabolic changes [24, 25] can be used to infer the physiological well-being of the retina, but they are not direct measure of neural activities. On the other hand, it has been demonstrated that fast IOSs have the time courses that are comparable to that of the retinal electrophysiological kinetics, i.e., electroretinogram (ERG) responses [26, 27]. Imaging fast IOSs may thus provide a new method to directly evaluate the functional integrity of photoreceptors and inner retina neurons. However, clinical applications of fast IOSs imaging require effective control of the signal contamination from the slow optical responses of hemodynamic and metabolic changes. Electrophysiological studies have demonstrated that only neural cells can track fast temporal changes (e.g., > 2 Hz) in light stimulus, and it is well established that flicker ERG can be used to separate electrophysiological responses of different retinal cells, such as rod- and cone-systems [28]. We demonstrate in this paper the feasibility of separating fast IOSs from slow optical responses by combining dynamic differential imaging and high frequency flicker stimulation. High-speed (1000 Hz) differential imaging revealed that fast IOSs could follow flicker stimuli up to at least 6 Hz, similar to the simultaneously recorded ERG signals.
2. Methods

2.1. Preparation of isolated retinas

Isolated leopard frog (*Rana Pipiens*) retinas were used for the experiments. Without the complications of hemodynamic changes and eye movements, isolated retina is a simple preparation to investigate the sources and biophysical mechanisms of fast IOSs, and for optimizing the design of imaging instruments. The experiments were performed following the protocols approved by the Institutional Animal Care and Use Committee of University of Alabama at Birmingham. Details of retinal preparation, the imaging device, and the ERG system have been reported in previous publications [18, 27]. Briefly, retinal dissection was conducted in a dark room with dim red illumination. After a long dark adaptation, the frog was euthanized by rapid decapitation and double pithing before the eyes were enucleated. The eyeball was hemisected below the equator with fine scissors to remove the lens and anterior structures before separating the retina from the retinal pigment epithelium. The dissection of the retina was performed in Ringer’s solution containing (in mM) [29]: 110 NaCl, 2.5 KCl, 1.6 MgCl$_2$, 1.0 CaCl$_2$, 22 NaHCO$_3$, and 10 D-glucose. The isolated retina was sliced into small wedges, allowing them to lie flat in the recording chamber. Typically, we selected a retina patch (~3 x 3 mm) which was at least 1 mm away from the edge of the optic nerve head. Optical imaging and electrophysiological recording were conducted simultaneously on the same retina patch.

2.2. Experimental setup

A flood-illumination microscope was modified for near infrared (NIR) light imaging of dynamic IOSs in stimulus activated isolated retinas. As shown in Fig. 1, the imaging system consisted of two (i.e., NIR and visible) light sources. The NIR light was used for IOS recording, and the visible light was used for retinal stimulation. The NIR light was produced by a 12-V 100-W halogen lamp (PHILIPS7724) with a band-pass filter (wavelength band: 800-1000 nm) in front, and the visible light stimulator was a fiber-coupled green light emitting diode (LED) with the central wavelength at 505-nm. The overall power of the NIR light delivered at the retina was ~1mW. Visible flicker stimuli between 1 and 6 Hz were used in this study. These were square pulses from a dark background. Each stimulus pulse had a 10-ms duration, with an intensity of ~$10^4$ 505-nm photons·µm$^{-2}$·ms$^{-1}$. Each recording session consisted of a 0.5-s pre-stimulus baseline image recording phase in total darkness, followed by a 3-s flicker stimulus phase, and then followed by a 1.5-s post-stimulus images recording phase, again in total darkness. It was estimated that <1% of the total photopigment was bleached by each stimulus pulse.

The images shown in this paper were recorded using a 10 bit CMOS camera (PCO1200, PCO AG, Kelheim, Germany), running at a frame rate of 1000 frames/s and spatial size of 400 x 400 pixels. The CMOS camera has 2 GB built-in RAM for fast image recording with a transfer speed of 820 MB/s. The ultrafast transfer speed made it possible to collect optical images at a high frame rate while allowing sufficient exposure time to ensure image quality. This imager allowed us to characterize fast IOSs with ms temporal resolution and µm spatial resolution. During the recording session, the frog retina was immersed in Ringer solution and pressed to a multi-electrode array. The photoreceptor layer was upward, closest to the visible light stimulus. The ganglion cell layer was in contact with the multi-electrode array for ERG measurement [18].
Fig. 1. Schematic diagram of the flood-illumination imager for NIR light imaging of fast IOSs. During the measurement, the isolated frog retina was illuminated continuously by the NIR light for recording of stimulus-evoked IOSs; while the visible light stimulator was used for retinal stimulation. Concurrent ERG measurement was conducted to record electrophysiological responses associated with retinal activation. At the dichroic mirror (DM), visible stimulus light was reflected and NIR recording light was passed through. The NIR filter was used to block visible stimulus light, and allow the NIR probe light to reach the CMOS camera.

2.3. Data processing

IOS images shown in Fig. 2a are stimulus-evoked optical responses represented in the unit of $\Delta I/I$, where $\Delta I$ is the dynamic optical changes and $I$ is the background light intensity. The IOS images were constructed using the following procedure [27]: 1) The images from the 0.5-s pre-stimulus baseline recording phase were averaged, pixel by pixel, and the averaged intensity of each pixel was taken as the background intensity $I$ of each pixel; 2) The background intensity $I$ was subtracted from each subsequent recorded frame, pixel by pixel, to get the $\Delta I$ of each pixel. 3) The $\Delta I/I$ image sequence was constructed to show the dynamic IOS patterns of the retina.

In order to reduce the contamination of optical signal due to metabolic changes of activated retinal cells, a dynamic differential processing procedure was used to separate fast IOSs from slow optical changes (Fig. 4a). Dynamic differential IOS images were constructed using the following equation [30]:

$$IOS_{t(x,y)} = \frac{I_{t(x,y)} - I_{ref(x,y)}}{I_{ref(x,y)}}$$  \hspace{1cm} (1)

where $I_{t(x,y)}$ was the intensity value of a pixel $(x, y)$ at a time point $t$; $I_{ref(x,y)}$ was the dynamic reference baseline of $m$ consecutive frames, which could be quantified by:

$$I_{ref(x,y)} = \frac{\sum_{i=t-m}^{t-1} I_{t(x,y)}}{m}$$  \hspace{1cm} (2)
In other words, the averaged pixel value of \( m \) consecutive frames recorded before the time point \( t \) was used as a reference baseline to calculate the differential IOS. For the dynamic differential IOSs shown in this article, we selected \( m = 100 \) (i.e., images recorded over 100 ms) for the dynamic reference baseline.

3. Results

Figure 2 shows the optical responses in the frog retina activated by a 1 Hz visible light flicker stimulus. Figure 2a shows slow, high magnitude IOSs component, which lasted much longer and did not go back to baseline before the delivery of the next stimulus flash (2d). Dynamic differential processing revealed a separate, fast IOSs component that closely followed the temporal changes of the flicker stimulus (Fig. 2b). High spatial resolution IOS images also revealed localized optical responses that correlated with cellular structures, and both positive (increasing) and negative (decreasing) IOSs were observed in the stimulus activated retinal area (Fig. 2c). Overall, the first stimulus flash evoked IOSs that were dominantly positive-going, and IOSs elicited by following flashes were dominantly negative-going (Fig. 2b, 2d-f). While the negative-going response was primarily confined to the center of the stimulus activated retinal area, the positive-going response appeared to spread into a relatively larger area (Fig. 2a and Fig. 3b).

Fig. 2. (a) IOS imaging of frog retina activated by 1.0 Hz flicker stimulation. Arrowheads represent the delivery of the flicker pulses. (b) Dynamic differential IOS imaging of the same retina shown in a. Raw images were acquired with a frame speed of 1000 frames/s. Each illustrated frame is an average of 250 frames over 250 ms interval. Each stimulus pulse lasted 10 ms (c) Enlargement of the 7th frame in b. (d) Gray and black traces 1-3 show IOSs and dynamic differential IOSs of retinal areas marked by the red squares 1-3 in c. Green trace depicts the flicker stimuli. (e) Statistics of IOSs in the activated retina shown in a. (f) Statistics of dynamic differential IOSs in the activated retina shown in b. A threshold (0.5\% \( \Delta I/I \)) was used to reduce the effect of background noises on the statistics. In e and f, the red and blue traces present the ratios of retinal areas with positive and negative IOSs, respectively. The black trace shows the difference (i.e. subtraction of the red and blue traces) of the retinal areas with positive and negative IOSs.
Figure 3 compares concurrent IOS and ERG recordings of the frog retina activated by flicker stimuli from 1.0 to 6.0 Hz. The time courses of both IOS and ERG responses were tightly correlated with the flicker stimulus. In order to ensure the repeatability of the IOSs, two experimental trials were conducted at each stimulus frequency. The results shown in Fig. 3c and 3d indicate that both IOS and ERG responses were very repeatable. The experiments demonstrated that the dynamic differential IOSs can follow flicker stimuli up to at least 6 Hz. For higher frequency flickers (4 and 6 Hz), an additional OFF response (arrowheads in Fig. 3c) was detected in both IOS and ERG signals. For the 6 Hz flicker stimulus, the IOS and ERG OFF response reached their magnitude peaks at ~350 ms and ~325 ms after the onset of the last flash, respectively. Dynamic differential IOS imaging consistently showed positive and negative optical responses. Figure 3c shows that the fast IOSs in response to the first visible light flash was positive-going, and the fast IOSs elicited by the following flashes were negative-going IOSs. The peak magnitude (4.1-4.5%) and the time-to-peak (~120 ms) of the fast IOS response evoked by the first stimulus flash are almost constant across the stimulus frequencies (1-6 Hz). However, the magnitude and time delay of the IOS responses evoked by following flashes were gradually reduced with increasing stimulus frequency (Fig. 4b and 4c).

Fig. 3. (a) Representative raw image of isolated frog retina. The retinal image (400 x 400 pixels) was split to 10 x 10 sub-images for statistics of localized dynamic differential IOSs shown in b. (b) Optical responses activated by the localized visible light spot (green spot). Each trace shows the fractional difference of positive and negative dynamic differential IOSs in each sub-image (40 x 40 pixels) shown in a. (c) Averaged dynamic differential IOSs (left) and concurrent ERG measurements (right) activated by 1-6 Hz flicker stimuli. Two experimental trials were conducted for each stimulus frequency. (b) Normalized cross-correlation ratio of two retinal recording trails for each stimulus frequency shown in Fig. 3c. Black bars show IOS responses, and gray bars show ERG measurements.

Similar changes were also observed in concurrent ERG recording (Fig. 3c). In each retinal flicker measurement, the first visible light flash typically evoked a rapid (time-to-peak: ~100 ms), but low magnitude, negative (decreasing) a-wave which was followed by a high magnitude positive (increasing) b-wave (Fig. 3c and Fig. 4d). The ERG signals elicited by the following flashes were mainly positive b-waves (Fig. 3c). The time-to-peak (~110 ms) of the ERG a-wave evoked by the first stimulus flash is constant across the stimulus frequencies (1-
6 Hz). The peak magnitude (108-125 mV) and time-to-peak (~200 ms) of the first b-wave is also quite repeated (Fig. 3c). However, the magnitude and time delay of the ERG responses evoked by the following flashes were reduced with increasing stimulus frequency (Fig. 4e and 4f).

![Figure 4](image)

**Fig. 4.** (a) P1 and T1 present the peak magnitude and time delay (relative to the stimulus onset) of the optical response evoked by the first stimulus flash, and P2 and T2 show averaged peak magnitude and time delay of optical responses elicited by the following stimuli. (b) Comparison of P1 and P2 at different stimulus frequencies shown in Fig. 3c. (c) Comparison of T1 and T2 at different stimulus frequencies shown in Fig. 3c. (d) Both a- and b-waves were observed in the ERG response evoked by the first stimulus flash. b1 and b2 show peak magnitude of ERG b-waves elicited by the first and subsequent stimulus flashes. Ta and Tb show the time delays, relative to the stimulus onset, of the a- and b-waves. (e) Comparison of b1 and b2 at different stimulus frequencies shown in Fig. 3c. (f) Comparison of Ta and Tb at different stimulus frequencies shown in Fig. 3c.

4. **Discussion**

In summary, we demonstrated the feasibility of using dynamic differential imaging to record fast IOSs elicited by flicker stimuli. In this process, the visible light flicker activated retinal neurons and elicited fast IOSs, and dynamic differential processing served as a high-pass filter to separate the fast IOSs from the slow, high magnitude optical responses that might be related to the metabolic changes in the retina (Fig. 2). The fast IOSs had time courses (T1 and T2: 40-120 ms, Fig. 4c) that were comparable (not identical) to ERG kinetics (Ta and Tb: 100-240 ms, Fig. 4f). For higher frequency flickers (e.g., 4 and 6 Hz), the d-wave (i.e., OFF response) was observed after the termination of the last visible light flash (Fig. 3c). The OFF responses of the IOS and ERG signals peaked at ~350 ms and ~325 ms, respectively (Fig. 3c).

It is well established that ERG a-wave reflects electrophysiological response of activated photoreceptors, and ERG b- and d-wave are associated with activities of inner retinal neurons, i.e., ON- and OFF bipolar cells [6–8]. The similarity of IOS and ERG time courses provides the evidence that the fast IOSs are correlated with stimulus-evoked responses of retinal neurons. Both fast IOSs and ERG were tightly correlated with retinal flicker stimulation, and could follow stimulus frequency up to at least 6 Hz (Fig. 3). This provided additional evidence that fast IOSs might originate from neural cells, because it is well known that only retinal neural cells can track high frequency (e.g., > 2 Hz) flickers. Because IOS and ERG may record activities of different retinal neural populations, we did not expect to see identical signal polarities and time courses. However, the similarity between the outcomes of the two recording methods did indicate that ISO was related to visible light elicited neural activities in the retina.
High spatial resolution images consistently reveal the correlation between fast IOSs and retinal cellular structures (Fig. 2c). Previous experimental study and theoretical analysis suggested that the fast IOSs may originate from dynamic volume changes of activated neuron bodies when ion and water flow across the cell membrane [31, 32]. Water influx in response to ionic currents through gated channels during depolarization causes cellular swelling that can produce changes in tissue light scattering [32–35] and polarization changes [31]. There are also several other possible processes [36], such as neurotransmitter secretion [37], reorientation of membrane proteins and phospholipids [11, 12, 38], and refractive index change of neural tissues [39], to produce transient IOSs during neural activation.

Both positive and negative IOSs were observed associated with the retinal flicker stimulation (Figs. 2 and 3). We speculate that the positive-going IOSs elicited by first stimulus flash were originated primarily from the rod-system, and following negative-going IOSs were originated primarily from the cone-system. Previous studies have demonstrated that transient IOSs were related to phototransduction procedures [13–17]. Both the binding and releasing of the G-proteins to the photoexcited rhodopsin might have contribution to the observed IOSs [14]. Moreover, transient IOSs were also detected from the inner neurons of the retina [18, 19, 21, 27, 40]. It is well established that the rod-system has much higher sensitivity than cone-system under dim light conditions, but the time course of the rod-system is slower than the cone-system. This may reasonably explain the longer latency of the positive peak (T1 in Fig. 4c) than the negative responses (T2 in Fig. 4c).

Moreover, the different light sensitivities of rod- and cone-systems may also affect the spatial patterns of positive and negative IOSs. Because of the high light sensitivity of the rod-system, diffused visible light in the surrounding area may provide enough stimulation for the rod-system, but not for the cone-system. As shown in Fig. 2a and Fig. 3b, the negative IOSs were primarily confined in the center area of the stimulus, but positive IOSs could spread into a relatively larger area. We speculate that the first stimulus flash evoked IOSs in the dark-adapted retina, and acted as a light adaptation illumination for the following flashes. Therefore, the IOSs may be sensitive to the light adaptation condition. Further experiments with variable background light levels will allow better understanding of the IOS patterns evoked by flickers. It is well established that, in coordination with controlled light adaptation, flicker ERG can be used to dissect electrophysiological responses of different retinal cells, such as rod- and cone-dominated systems [28, 41, 42]. We anticipate that further development of IOS imaging of retinal flicker responses will lead to a new method for retinal diseases detection and treatment outcome evaluation.

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