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***In vivo* confocal imaging of fast intrinsic optical signals correlated with frog retinal activation**

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Abstract

Using freshly isolated animal retinas, we have conducted a series of experiments to test fast intrinsic optical signals (IOSs) that have time courses comparable to electrophysiological kinetics. In this Letter, we demonstrate the feasibility of *in vivo* imaging of fast IOSs in intact frogs. A rapid line-scan confocal ophthalmoscope was constructed to achieve high-speed IOS recording. By rejecting out-of-focus background light, the line-scan confocal imager provided the resolution to differentiate individual photoreceptors *in vivo*. Rapid confocal imaging disclosed robust IOSs with time courses comparable to retinal electroretinogram kinetics. High-resolution IOS images revealed both positive (increasing) and negative (decreasing) light responses, with subcellular complexity.

Functional evaluation is important for retinal disease detection and treatment evaluation. It is well known that many eye diseases can cause pathological changes of photoreceptors or inner retinal neurons that ultimately lead to vision losses and even complete blindness. Different eye diseases, such as age-related macular degeneration [1], retinitis pigmentosa [2], and glaucoma [3], are known to target different types of retinal neurons, causing localized lesions or cell losses. The electroretinogram (ERG) [4], focal ERG, multifocal ERG [5], and perimetry [6] have been established for functional examination of the retina. However, spatial resolution and signal selectivity of the ERG and perimetry may not be high enough to provide precise identification of localized retinal dysfunctions. While it is possible to combine morphological (such as high-resolution OCT) with functional (such as ERG) evaluation [7] to improve retinal disease study and diagnosis, conducting these separate measurements is time consuming and cost inefficient. Moreover, morphological and functional changes of the retina are not always correlated [8]. Given the delicate structure and complicated functional interaction of the retina, detection of localized dysfunction requires a method that can examine stimulus-evoked retinal functional activities at high-spatial and temporal resolutions.

Intrinsic optical signal (IOS) imaging may provide a noninvasive method for concurrent morphological and functional evaluation of the retina. Several imaging techniques, such as fundus cameras [9,10], adaptive optics ophthalmoscopes [11–13], and optical coherence

tomography (OCT) imagers [14–16], have been explored to detect transient IOSs associated with retinal stimulation. In principle, both stimulus-evoked retinal neural activity and corresponding hemodynamic and metabolic changes can produce transient IOSs associated with retinal stimulation. While hemodynamic and metabolic changes associated slow IOSs can provide important information in functional assessment of the visual system, they are relatively slow and cannot directly track fast neural activities in the retina. Fast IOSs, which have time courses comparable to electrophysiological kinetics, are desirable for direct evaluation of the physiological health of photoreceptors and inner neurons. Using freshly isolated frog retinas, we have conducted a series of experiments to validate high-spatial (subcellular) and high-temporal (millisecond) resolution imaging of stimulus-evoked fast IOSs in the retina [17–20]. In this study, we demonstrate the feasibility of *in vivo* imaging of fast IOSs in the retina of intact frogs.

The schematic diagram of the line-scan confocal ophthalmoscope is shown in Fig. 1. In this system, a fast linear CCD camera (SG-11-01k80-00R, DALSA), with pixel size of $14\ \mu\text{m} \times 14\ \mu\text{m}$ and pixel sampling rate up to 80 MHz, was employed to achieve high-speed and high-resolution imaging. Our recently reported line-scan confocal microscope [21] was modified to an animal ophthalmoscope for *in vivo* imaging of the retina. In this system, an NIR (center wavelength, 830 nm; bandwidth, 60 nm) superluminescent laser diode (SLD) (SLD-35-HP, Superlum) was used for IOS imaging, and a green LED was used for retinal stimulation. Moreover, an NIR LED was placed beside the eye to provide oblique illumination of the pupil, and a full-field CCD camera was used to monitor the pupil to allow easy alignment of the NIR SLD light for IOS recording. The cylindrical lens (CL) condensed the NIR recording light into one dimension to produce a focused line illumination, which was conjugated with the linear CCD sensor. Lateral and axial resolutions of the system are theoretically estimated at $\sim 1\ \mu\text{m}$ and $\sim 10\ \mu\text{m}$, respectively.

Anesthetized northern leopard frogs (*Rana pipiens*) were used for this study. The frog was selected for taking advantage of high (~ 0.4) NA [22] and large size photoreceptor (cone, $\sim 3\ \mu\text{m}$; rod, $\sim 6\ \mu\text{m}$ [23]), which made it possible to resolve the individual photoreceptor *in vivo* without the requirement of adaptive optics. The experimental procedure was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. After 2 to 4 h dark adaptation, the frog was anesthetized by immersing it into tricaine methanesulfonate (TMS, MS-222) solution (500 mg/liter) [24]. After confirmation of the anesthesia, the frog was placed in a home-built animal holder for IOS imaging. The animal holder provided five degrees of freedom to allow easy adjustment of the animal orientation and retinal area for IOS measurement.

During IOS recording, the frog eye was continuously illuminated by the NIR light. With the line-scan confocal system, high-resolution *in vivo* images revealed individual blood vessels [arrowheads in Fig. 2(a)] and photoreceptors [arrowheads in Fig. 2(b)]. In order to ensure high-temporal resolution for IOS recording (Fig. 3), one subimage (250×50 pixels) area was selected to achieve high-speed (200 frames/s) measurement. During the IOS imaging, the retinal ERG response was recorded simultaneously. Figure 3 represents retinal IOS responses recorded in intact frogs. In Fig. 3(a), high-spatial resolution images revealed both positive (increasing) and negative (decreasing) IOS response, with subcellular complexities. Figure 3(b) shows IOS dynamics of individual pixels selected randomly from the image area. It was observed that the peak IOS magnitude of subcellular locations was up to 20% $\Delta I/I$, where ΔI was the light intensity change and I was the background light intensity. As shown in Fig. 3(b), the positive and negative IOSs had comparable time courses, in terms of time delay and time to peak (relative to stimulus onset). By ignoring the signal polarity, Fig. 3(c) shows averaged IOS magnitude (i.e., absolute value of the IOS) and the corresponding retinal ERG. As shown in Fig. 3(c), fast IOSs occurred almost immediately (< 10 ms) and

reached peak magnitude within ~300 ms after the stimulus onset. Comparable retinal ERG was observed.

In summary, the feasibility of *in vivo* imaging of retinal activation was demonstrated with intact frogs. A rapid line-scan confocal ophthalmoscope was constructed to achieve high spatiotemporal resolution imaging of fast IOSs. By rejecting out-of-focus background light, the system resolution was significantly improved in comparison with our previous flood-illumination imager [25]. High-resolution confocal images revealed individual frog photoreceptors *in vivo*. Robust IOSs were clearly imaged from the stimulus activated retina, with subcellular resolution. High-resolution images revealed fast IOSs that had time courses comparable to retinal ERG kinetics. Our experiment indicates that rapid line-scan IOS imaging of intact frogs provides a simple platform for *in vivo* investigation of fast IOSs correlated with retinal activation. Previous studies suggested that both binding and release of G-proteins to photoexcited rhodopsin may produce transient IOSs in the photoreceptor [26,27]; IOS sources and mechanisms in other neural cells have also been investigated [28]. We anticipate that better study of the fast IOSs can provide insight for developing advanced instruments to achieve concurrent morphological and functional evaluation of human retinas, with high-spatial resolution to differentiate individual retinal cells.

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Fig. 1. Schematic diagram of line-scan confocal ophthalmoscope for reflected light IOS imaging: CO, collimator; CL, cylindrical lens; BS, beam splitter; SM, scanning mirror; DM, dichroic mirror; Lx, optical lenses. The NIR filter, in front of the linear CCD, was a long-pass filter that blocked visible stimulus light and transmitted NIR light reflected from the retina. The optical magnification of the system was estimated at ~ 14 , corresponding to $1\text{ }\mu\text{m}=\text{pixel}$ in retinal image.

Fig. 2.
(a) Representative *in vivo* image (500×400 pixels) of frog retina. (b) Enlarged picture of the subimage (100×100 pixels) marked by the white square in (a). Individual blood vessels [white arrowheads in (a)] and photoreceptors [white arrowheads in (b)] were clearly observed.

Fig. 3.

(Color online) (a) Representative spatial IOS image sequence. Each illustrated frame is an average over 100 ms interval (20 frames). The black arrowhead indicates the onset of the 10 ms green flash stimulus. 200 ms prestimulus baseline and 900 ms post-stimulus IOS recordings are shown. (b) Representative IOS responses of individual pixels randomly selected from the image area. The gray bar indicates the stimulus onset and duration. (c) The top black trace shows the IOS magnitude (i.e., absolute value of the IOS) averaged over the whole image area, corresponding to the image sequence shown in (a). The gray trace shows one control experiment without stimulation. The black trace below shows concurrent frog ERG. The gray bar indicates the stimulus delivery.