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***In vivo* observation of transient photoreceptor movement correlated with oblique light stimulation**

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Abstract

Rod-dominated transient retinal phototropism (TRP) has been observed in freshly isolated retinas, promising a noninvasive biomarker for high resolution assessment of retinal physiology. However, *in vivo* mapping of TRP is challenging due to its fast time course and sub-cellular signal magnitude. By developing a line-scanning and virtually structured detection based super-resolution ophthalmoscope, we report here *in vivo* observation of TRP in frog retina. *In vivo* characterization of TRP time course and magnitude were implemented by using variable light stimulus intensities.

Keywords

Retina; photoreceptor; functional imaging; super-resolution microscopy; ophthalmology

1. INTRODUCTION

Age-related macular degeneration (AMD) is a leading cause of impaired vision and legal blindness. In the U.S. alone, more than 10 million people have early AMD, and 1.75 million patients suffer from visual impairment due to late AMD [1]. Early detection is one essential step to prevent the sight-threatening damages of late AMD. Optical imaging techniques, such as fundus photography, fluorescein angiography, and optical coherence tomography (OCT) [2,3], can be used to reveal AMD associated morphological distortions, such as drusen in the retinal pigment epithelium (RPE) and Bruch's membrane complex. However, physiological abnormalities may occur before detectable morphological distortion. It is well known that retinal photoreceptor dysfunction is the key in AMD associated vision loss [4]. Both psychophysical methods, such as the Amsler grid test [5], visual acuity test [6] and dark adaptometry [7,8], and electrophysiological methods, such as focal and multifocal electroretinography (ERG) [9–12], have been explored to provide functional examination of the visual system. However, these methods essentially rely on subjective test, which involves complex neural processing and therefore hampers the signal specificity, without necessary spatial resolution to identify localized retinal dysfunctions. Moreover, rod photoreceptors are known to be affected first, compared to cone photoreceptors, in early AMD [13,14]. A high-resolution method for objective examination of retinal physiology is desirable to advance early diagnosis of AMD.

Transient retinal phototropism (TRP) was recently observed in freshly isolated amphibian and mammal retinas stimulated by oblique visible light illumination [15]. Functional OCT of living eye-cups and time-lapse microscopy of retinal slices revealed that TRP has an anatomic origin within the outer segment (OS) layer [16], presumably caused by an unbalanced shrinkage of rod OSs [17]. Comparative electrophysiological investigation of isolated retina further identified the physiological source of TRP to the phototransduction processes before hyperpolarization of the rod cell membrane [18,19]. Therefore, the rod-dominated TRP promises a noninvasive biomarker for objective assessment of rod function, promising a high-resolution method for early detection of AMD. However, *in vivo* mapping of the rod-dominated TRP is challenging due to its rapid time course and sub-cellular movement magnitude. In both isolated mouse and frog retinas, the TRP peak magnitude is less than 1 μm , with the onset time within 10 ms after stimulus delivery [15,17,18].

Here, we demonstrated for the first time *in vivo* observation of TRP using a custom-designed super-resolution ophthalmoscope [20]. The custom-built digital ophthalmoscope employed virtually structured detection (VSD) to achieve a sub-cellular level of spatial resolution, and it combined a rapid line-scanning strategy to realize a millisecond level of temporal resolution. *In vivo* imaging of frog retinas stimulated by variable light intensities was conducted to characterize the properties of the TRP time course.

2. METHODS AND MATERIALS

2.1 Animal preparation

Adult northern leopard frogs (*Rana Pipiens*) were used in this study. The frogs were first anesthetized through the skin by immersion in 800 mg/liter tricaine methanesulfonate (TMS, MS-222; MP Biomedicals, Inc.) solution. After confirmation of anesthesia, the frog was fixed in a custom-built holder and the pupils were fully dilated with topical atropine (1%) and phenylephrine (2.5%). The holder provided five degrees of freedom to facilitate adjustment of body orientation and retinal areas for *in vivo* imaging. All experiments in this research were performed following the protocols approved by the Animal Care Committee (ACC) at the University of Illinois at Chicago, and conformed to the statement on the use of animals in ophthalmic and vision research, established by the Association for Research in Vision and Ophthalmology (ARVO).

2.2 Experimental setup

Figure 1 shows a schematic diagram of the line-scanning super-resolution ophthalmoscope. The light source is a near-infrared superluminescent diode (SLD-35-HP; Superlum Ireland, Inc.) with a center wavelength at 830 nm and a bandwidth of 60 nm. A focused line, produced by a cylindrical lens, scanned across the retina under the control of a scanning galvanometer mirror (GVS001; Thorlabs, Inc.). The line profile reflected from the retina was recorded by a high-speed two-dimensional CMOS camera (FastCam Mini AX50; Photron, Inc.). The imaging system provided an optical magnification of 43.55 (the focal length of the frog eye was assumed as 2.87 mm). To achieve fast imaging speed for an *in vivo* imaging, the line scanning was performed in one dimension for super-resolution imaging. A total of 255 line-profiles were acquired to reconstruct one super-resolution image. In this

experiment, the imaging speed of the camera was set at 30,000 frames/s (fps), corresponding to a 100-fps speed for VSD-based super-resolution imaging. The light that entered the frog pupil had a beam diameter of ~2 mm and a power of ~2.5 mW.

The visible light used for retinal stimulation was produced by a fiber-coupled light-emitting diode (LED) with a central wavelength at 505 nm (M505F1; Thorlabs, Inc.), and its incident angle was adjusted by a kinetic mount (KC1; Thorlabs, Inc.) that held both the fiber tip and the collimator. The stimulating power was first measured by a powermeter (PM200; Thorlabs, Inc.) placed at the rear focal plane of the lens before the eye (L4 in Fig. 1) and was then converted to stimulation intensity on retina considering cornea reflection (~4%), ocular media absorption (<10%) and photon loss due to passing through the inner layers of the retina (~20%) [21]. A slit was placed at the conjugate plane of the retina to provide a localized stimulation pattern with sharp boundaries on the retina.

2.3 Image processing and data analysis

The *in vivo* retinal images acquired by the line-scanning ophthalmoscope were first registered with custom-programmed software to remove the eye movement caused by respiration. Then a rod OS movement magnitude map was generated for each registered image using the optical flow method [22] with the first image as the reference. Time course of the movement magnitude was then obtained with the following procedures. Each point in a time course is an average of all the pixel values that are believed to reflect stimulus-evoked movement in the magnitude map that corresponds to that time point. The pixel value was regarded as a signal when it satisfied the following two requirements. First,

$$I_k(x_p, y_p) > \bar{I}_{pre}(x_p, y_p) + 3\delta_{pre}(x_p, y_p)$$

where $I_k(x_p, y_p)$ is the intensity of the pixel (p) with a coordinate index (x_p, y_p) on a magnitude map with frame index k , $\bar{I}_{pre}(x_p, y_p)$ is the mean intensity of the pixels with the same index (x_p, y_p) in all pre-stimulus magnitude maps and $\delta_{pre}(x_p, y_p)$ represents the standard deviation. Second, the intensities of the pixels with the same index (x_p, y_p) in the following n magnitude maps should also be larger than $\bar{I}_{pre}(x_p, y_p) + 3\delta_{pre}(x_p, y_p)$, i.e.,

$$I_{k+1}(x_p, y_p), \dots, I_{k+n}(x_p, y_p) > \bar{I}_{pre}(x_p, y_p) + 3\delta_{pre}(x_p, y_p)$$

These two procedures efficiently eliminate both the background noise and the random movements that are not elicited robustly by stimulation.

3. RESULTS

3.1 *In vivo* super-resolution imaging of retinal photoreceptors

The VSD based super-resolution ophthalmoscope was used for this study. In this custom-built instrument, a rapid line-scanning modality was integrated to achieve an imaging speed of 100 frames/s, with a field of view of $100 \mu\text{m} \times 200 \mu\text{m}$ on the retina. The imaging plane

was focused on the photoreceptor layer during the experiment. Individual photoreceptors were clearly imaged with subcellular spatial resolution and millisecond temporal resolution (Fig. 2a), which allowed quantitative measurement and dynamic monitoring of photoreceptor movements.

3.2 *In vivo* mapping of TRP evoked by oblique retinal stimulation

To elicit TRP, a collimated green light beam was delivered to the retina at $\sim 12^\circ$ with respect to the axial direction of the photoreceptors (Fig. 2b). Ocular differences among individual samples were neglected. Only a portion of the retina that was $\sim 50\ \mu\text{m}$ in width at the center of the field of view was exposed to the oblique stimulation for TRP recording (red-dashed rectangle in Fig. 2a).

The magnitudes of photoreceptor movements were employed to quantify TRP. Figure 2c shows a representative magnitude map of photoreceptor movement acquired when the retina was exposed to localized stimulation. As Fig. 2c matches Fig. 2a, the red-dashed rectangle shown in Fig. 2c also represents the stimulated retinal area. Fig. 2c reveals the oblique stimulation elicited robust photoreceptor movements were primarily confined within the stimulated retinal region. Representative magnitude maps acquired from different phases of the experiment are presented in Fig. 2d to illustrate the spatiotemporal dynamics of TRP. As the stimulation onset was set as 0 s and the stimulation period lasted for 0.5 s, Fig. 2d covers the photoreceptor movements before, during, and after the stimulation. Robust photoreceptor movements were observed after the onset of stimulation.

3.3 Temporal dynamics of photoreceptor correlated with stimulation intensity

Having demonstrated the capability of recording photoreceptor movement using the super-resolution ophthalmoscope, we further employed stimulations with different intensities to investigate their effects on movement magnitudes. The line-scanning modality of the super-resolution ophthalmoscope made it possible to monitor the dynamic changes in the movement magnitude map (Fig. 2d) with a temporal resolution of 10 ms. A time-magnitude course was then used to reflect the dynamics of photoreceptors. Three stimulation intensities, i.e., 1.97×10^5 , 0.67×10^5 , and $0.197 \times 10^5\ \text{photons} \cdot \mu\text{m}^{-2} \cdot \text{ms}^{-1}$, were employed in this study. The flash duration was set to 500 ms with the purpose of eliciting robust photoreceptor movement. For each stimulation intensity, 12 time-magnitude courses were acquired from 6 samples. Each trace in Fig. 3a is the mean of the time-magnitude courses and is accompanied by the standard deviations of data about the mean (colored area). The relationship of the standard deviation amplitudes to the waveform in each trace indicates a general similarity between the temporal dynamics of photoreceptors obtained from different samples. The waveforms of all three traces exhibit a stable and flat stage prior to the flash presentation, a rapid rise upon the onset of the stimulation, and a recovery phase after the peak. Two parameters of the waveform, peak amplitude (the maximum value of photoreceptor movement magnitude) and time-to-peak (time taken to reach the peak amplitude), were compared to assess the difference caused by increasing the stimulation intensity. The results show that the difference between peak amplitudes of the three traces was statistically insignificant, suggesting the peak amplitude is irrelevant to the variation of stimulation intensities within a certain range (Fig. 3b). However, the time-to-peak values

show a remarkable correlation with stimulus intensities, indicating brighter intensity results in earlier saturation of the photoreceptor movement (Fig. 3c). These results together further demonstrated that higher intensities initiated a much more rapid photoreceptor movement. Moreover, brighter stimulation also initiated a faster falling phase of the waveform. The movement magnitude started to decrease even during the stimulation period when the brightest stimulation was applied (orange trace in Fig. 3a).

4. DISCUSSION

In this study, we demonstrated for the first time *in vivo* observation of TRP. The VSD-based super-resolution imager allowed direct observation of individual photoreceptors with subcellular resolution, which enabled quantitative measurement of photoreceptor changes evoked by oblique light stimulation. Robust photoreceptor movements were observed within the localized stimulation pattern. The line-scanning super-resolution imager provided 10 ms temporal resolution to investigate the time course of photoreceptor dynamics. The photoreceptors presented a rapid response after the onset of the stimulation and persisted during and after the stimulation period for at least 1.5 seconds (Figs. 2d and 3a), which is consistent with our previous observation with isolated retinas [18] and retinal slices [17].

Different stimulus intensities were used to characterize TRP magnitudes and time courses (Fig. 3). The time-magnitude profiles suggest an intimate correlation between *in vivo* TRP and phototransduction. As shown in Fig. 3a, strong stimulation produced accelerated TRP time course, which might reflect more rhodopsin and additional amounts of cascaded reactions activated [23]. Similar effects were also observed in our previous intrinsic optical signal (IOS) and rod OS shrinkage studies, supporting that photoreceptor movement is a major component of IOS and the unbalanced rod OS shrinkage is the mechanical source of TRP [17,24,25]. It is also interesting to note that increased stimulation intensity corresponds to a shortened saturation stage and an accelerated recovery process in the time-magnitude courses of *in vivo* TRP. Moreover, for the stimulation with the photon flux of 1.97×10^5 photons· μm^{-2} ·ms $^{-1}$, the photoreceptor movement magnitude started to decrease before stimulus offset (orange trace in Fig. 3a). The accelerated TRP recovery process might reflect extra deactivation processes of phototransduction, such as the quenching of activated rhodopsin, the hydrolysis of transducin, and/or the synthesis of cytoplasmic cyclic guanosine monophosphate [26–28], triggered by strong light stimulation. Time-lapse microscopy of living retinal tissues has revealed light stimulus-evoked rod OS shrinkage [17]. Comparative study of isolated retinas with low sodium treatment showed that TRP begins before hyperpolarization of the rod in the phototransduction cascade [18]. These observations suggest that physical source of the movement/shrinkage of the rod OS might be located on or adjacent to the discs [23,29–31]. Further TRP studies with mutant mouse models may provide insights about biophysical source and mechanism of TRP [32,33].

In summary, *in vivo* imaging of TRP demonstrates its potential to work as a biomarker of rod functionality. We anticipate that further development of *in vivo* imaging of TRP may provide a high spatial resolution method for functional evaluation of rod physiology, allowing early diagnosis of rod photoreceptor dysfunction due to AMD and other eye diseases.

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Figure 1.

Schematics of the line-scanning ophthalmoscope for *in vivo* super-resolution imaging. The illumination for retinal imaging is produced by a superluminescent diode (SLD). The line profile of illumination scanned across the retina is produced by the cylindrical lens. L1–L4 are lenses with focal lengths of 80 mm, 400 mm, 80 mm, and 25 mm, respectively. The black-dashed line represents the virtual optical axis for a perpendicular stimulation on the retina. The oblique stimulation is then achieved by moving the green LED away from the axis with a certain angle, i.e., applying an “off-axis” setup in the stimulation path.

Figure 2.

Spatiotemporal characteristics of *in vivo* TRP evoked by a localized oblique stimulation. (a) Representative *in vivo* super-resolution image of the photoreceptor layer. Yellow arrowheads indicate retinal blood vessels. (b) Cartoon illustration of the retinal photoreceptors and oblique stimulation. A collimated green light beam obliquely illuminates retinal photoreceptors. The sub-window shows the sagittal plane of a stimulated photoreceptor and the definition of the stimulation angle. IS: inner segment; OS: outer segment. (c) Representative magnitude map of photoreceptor movement recorded at time 0.4 s after the onset of stimulation. Temporal dynamics of photoreceptors before (d1 – d2), during (d3) and after (d4 – d6) the stimulation. The stimulation started at time 0 s and lasted for 500 ms. Red-dashed rectangles in (a) and (c) represent the retinal area illuminated by the green stimulus. The color bar is applied to both (c) and (d).

Figure 3.

Temporal characteristics of photoreceptor movement correlated with stimulation intensities. (a) The time-magnitude courses of photoreceptor movement corresponded to three descending stimulation intensities as respectively indicated by the legend. Each trace is an average of 12 datasets recorded from 6 different retinal samples. No repeated or overlapped stimulation occurred at the same retinal location. The colored area that accompanies each trace illustrates the standard deviations. Shaded area in grey represents the 500-ms stimulation. PR: photoreceptor. (b) The mean and standard deviation of peak amplitudes of the traces in (a) are shown. (c) The mean and standard deviation of the time-to-peak of the traces in (a) are shown. Significance was determined by a one-way ANOVA with post-hoc Tukey honestly significant difference test for multiple comparisons, * $p < 0.05$, ** $p < 0.01$, NS, not significant, $n = 12$ retinal locations for each stimulus intensity.