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Functional optical coherence tomography reveals transient phototropic change of photoreceptor outer segments

Benquan Wang^{1,2}, Qiuxiang Zhang¹, Rongwen Lu¹, Yanan Zhi^{1,2}, and Xincheng Yao^{1,2,3,*}

¹Department of Biomedical Engineering, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

²Department of Bioengineering, University of Illinois at Chicago, Chicago, Illinois 60607, USA

³Department of Ophthalmology and Vision Sciences, University of Illinois at Chicago, Chicago, Illinois 60612, USA

Abstract

Dynamic near infrared microscopy has revealed transient retinal phototropism (TRP) correlated with oblique light stimulation. Here, by developing a hybrid confocal microscopy and optical coherence tomography (OCT), we tested sub-cellular source of the TRP in living frog retina. Dynamic confocal microscopy and OCT consistently revealed photoreceptor outer segments as the anatomic source of the TRP. Further investigation of the TRP can provide insights in better understanding of Stiles–Crawford effect (SCE) on rod and cone systems, and may also promise an intrinsic biomarker for early detection of eye diseases that can produce photoreceptor dysfunction.

The Stiles–Crawford effect (SCE) refers to directional sensitivity of the retina [1]. It is known that the retina is more sensitive to the light entering the center of the pupil, i.e., parallel light relative to eye axis, than the light passing through the periphery, i.e., oblique light illumination. The SCE is predominantly observed in cone photoreceptors, which is believed to suppress intraocular stray light to benefit image quality of photopic vision [2]. In contrast, psychophysical methods did not reveal significant SCE in rod photoreceptors that dominate scotopic vision [2]. The biophysical mechanism underlying the SCE difference between rod and cone photoreceptors is not well established. Recently, we observed transient retinal phototropism (TRP) correlated with oblique light stimulation, which might provide one possible explanation of the lack of rod SCE. Using a near infrared (NIR) transmission microscope equipped with a high-speed camera, we have consistently observed the TRP in both amphibian (frog) and mammalian (mouse) retinas [3]. High spatial and temporal resolution imaging revealed that the TRP was rod-dominant. It has been widely accepted that the waveguide property of retinal photoreceptors can affect the directionality of light acceptance [4–6]. Therefore, such rod-dominant TRP might affect the waveguide

acceptance and thus partially compensate for the loss of luminance efficiency in the rod system under oblique stimulation.

The purpose of this study is twofold: (1) to identify axial location (e.g., inner or outer photoreceptor segment) of the TRP at micrometer resolution; and (2) to validate the feasibility of mapping TRP through reflected light imaging modality. The transmitted light microscopy in our preliminary study provided excellent transverse resolution to differentiate rod and cone photoreceptors [3]. However, limited axial resolution hampered its application to identify sub-cellular source and mechanism of the TRP. Moreover, transmitted light imaging modality limited its potential for in vivo application. In this study, we combined line-scan confocal microscopy and spectral-domain optical coherence tomography (SD-OCT) to demonstrate reflected light recording and characterize sub-cellular source of the TRP in living frog retinas.

Figure 1 shows the schematic diagram of the confocal-OCT imaging system. In the line-scan confocal subsystem, a 780-nm superluminescent diode (QSDM-780-15D, Qphotonics) was used as the light source. A cylindrical lens was used to condense the light in one direction to reduce a focused line illumination. Back reflection and scattering light from the sample was detected by a high-speed (70,000 lines/s) linear charge-coupled device (CCD) camera (AViiVA EM2, e2v). The linear CCD (1×1014 pixels, $10 \mu\text{m} \times 10 \mu\text{m}$ each pixel) naturally acted as a slit aperture, which was conjugated with the focused line illumination, to achieve confocal configuration to reject out-of-focus light [7–9]. In the SD-OCT subsystem, a 850-nm SLD (D840, Superlum) was used as the light source. The bandwidth of the near infrared SLD was 100 nm, corresponding to axial resolution $\sim 3 \mu\text{m}$ in air. A $10\times$ water immersion objective with 0.3 numeric aperture (NA) was employed to provide $1.7 \mu\text{m}$ lateral resolution. The SD-OCT and line-scan confocal subsystems shared the same sample arm. A short-pass dichroic mirror DM1 (DMSP805, Thorlabs) was used to combine 780 and 850 nm light beams to achieve simultaneous OCT and confocal imaging. A long-pass dichroic mirror DM2 (cut-on wavelength 700 nm, Edmund Optics) was used to introduce green light (505 nm) for retinal stimulation. The stimulation orientation and TRP direction are defined in Figs. 2(A) and 2(B).

Both amphibian (frog) and mammalian (mouse) retinas have previously been used to demonstrate the TRP [3]. Freshly isolated leopard frog (*Rana pipiens*) retina was selected for this study. Relative large photoreceptors (rods $\sim 5\text{--}8 \mu\text{m}$, cones $\sim 1\text{--}3 \mu\text{m}$) [10, 11] allowed unambiguous observation of photoreceptor mosaic, and thus to readily enable quantitative analysis of the TRP at sub-cellular resolution. Animal handling was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. During the experiment, the frog was euthanized by decapitation followed by double pithing. The eyeball was then dissected and transferred to Ringer's solution (containing in mMol/L: 110 NaCl, 2.5 KCl, 1.6 MgCl_2 , 1.0 CaCl_2 , 22 NaHCO_3 , and 10 D-glucose) [12]. After hemisecting the eyeball, retina was isolated and moved to a chamber filled with Ringer's solution for imaging. The whole process was conducted in a dark room with dim red light illumination.

In our preliminary study of TRP, we observed clear photoreceptor mosaic pattern in NIR transmission microscopy image, and rods and cones could be directly differentiated [Fig. 2(C), white cells: rods, black cells: cones]. In this study, photoreceptor mosaic pattern was also unambiguously observed in enface confocal image [Fig. 2(D)], although it was difficult to differentiate rod and cone cell types in the reflected light images. The integrated OCT provided cross-sectional observation of retinal structure at micrometer resolution [Fig. 2(E)]. The combined confocal and OCT enabled simultaneous enface [Fig. 2(D)] and cross-sectional [Fig. 2(E)] measurements. For each experiment, 100-ms pre-stimulus and 400-ms post-stimulus images were recorded at the speed of 200 frames per second. A 10-ms oblique stimulus [Fig. 2(A), 20° or -20° , 2×10^5 photons/ $\mu\text{m}^2/\text{ms}$] was introduced to stimulate the retina. Transient photoreceptor displacement could be observed after the delivery of the retinal stimulation [see Media 1, Fig. 2(E)]. In order to quantify the photoreceptor displacement, we adopted the optical flow code developed by Sun *et al.* [13]. Optical flow is a well-established method for calculating target movements between two images. It can accurately identify movements of fine details in sequential images, which makes the method suitable for tracking small movements of retinal cells. Stimulus-evoked transient photoreceptor displacements could be quantified in magnitude and direction maps. In order to automatically segment active area and inactive area in displacement magnitude map, we used the classic automatic thresholding method for bi-modal images: Otsu's method [14]. Segmented active area was color coded in the direction map according to predefined scale [Fig. 2(B)]. Inactive area in direction map was coded as black.

Figure 3(A) illustrates confocal and OCT results of the retina stimulated with 20° and -20° oblique light flashes. The results were calculated from the average of 10 images acquired between 100 ms and 150 ms after the onset of the stimulus light. As shown in Figs. 3(A3) and 3(A4), dynamic confocal imaging revealed that retinal photoreceptors moved toward the 0° direction, i.e., right direction, corresponding to the 20° oblique stimulation. In other words, the photoreceptor moved toward the incident light, which was consistent to our early observation using a digital NIR microscope [3]. Cross-sectional retinal OCT images revealed that the TRP primarily localized at the photoreceptor outer segment [Figs. 3(A6) and 3(A7)]. The OCT images in Figs. 3(A7) and 3(A8) confirmed that the 20° stimulation led the photoreceptor to shift right, i.e., toward the direction of stimulus light.

In order to further verify the orientation dependence of the TRP, we switched stimulus light direction from 20° to -20° . As shown in Figs. 3(B1)–3(B8), the TRP was consistently observed in the stimulated area (Fig. 3B2). Figure 3(B3) shows that displacement angle was $\sim 180^\circ$, which indicated the photoreceptors moved left due to -20° stimulation. The cross-sectional retinal OCT images [Fig. 3(B7)] also consistently revealed the orientation dependence of the TRP. It was observed that when the stimulus light changed from 20° to -20° , photoreceptor displacement direction switched from right to left. In other words, when stimulus light direction was reversed, photoreceptor displacement direction was also reversed. Figure 3(B6) confirmed that the photoreceptor displacement started from IS/OS junction and primarily occurred at the OS.

In summary, the TRP was consistently observed in both *en face* confocal and cross-sectional OCT images. Comparative analysis of the confocal and OCT images indicated that the TRP

was orientation dependent, i.e., the photoreceptor moved toward the direction of oblique stimulus light. Although both confocal and OCT measurements revealed convincing TRP changes, some slight difference was observed. For confocal data, it was observed that the distribution of the TRP direction was approximately normally distributed with a mean value of 0° (right movement) or 180° (left movement) [Figs. 3(A4) and 3(B4)]. However the distribution of the TRP direction in OCT data slightly deviated away from normal distribution [Figs. 3(A8) and 3(B8)]. Most likely, this is due to different signal-to-noise ratios (SNRs) of the confocal and OCT images. It is known that the OCT images were severely affected by speckle noise [15]. Moreover, the confocal image was recorded from a relatively large area, and average effect might enhance relative SNR. Depth-resolved OCT imaging unambiguously revealed that the TRP was predominantly occurred at photoreceptor OS. Although we do not totally understand the mechanism underlying this phenomenon, early study has reported disc-shape change of photoreceptor rod [16]. Orientation unbalanced disc-shape change might contribute to the observed TRP. We speculated that the TRP may compensate for some part of the light efficiency loss caused by oblique illumination. We are currently pursuing comparative TRP imaging and electrophysiological measurement for better investigation of anatomical source and biophysical mechanism of the TRP. This is not only important for better understanding of the SCE on rod and cone systems, but can also be essential for establishing an intrinsic biomarker for early detection of eye diseases that can produce photoreceptor dysfunctions. Many eye diseases, such as age-related macular degeneration (AMD), can produce photoreceptor damages associated with severe vision loss including legal blindness. Currently, there is no outright cure for the retinal degenerative. A key strategy for preventing vision loss due to late AMD is to preserve function and be vigilant for changes in early AMD signifying progression. It is well established that physiological integrity of rod photoreceptors is more vulnerable than that of cones in early AMD [17,18]. Therefore, the rod-dominant TRP promises a new strategy to allow objective assessment of localized rod dysfunctions at high resolution.

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Fig. 1.

Diagram of the hybrid confocal-OCT imaging system. (A) Top view: SLD1, superluminescent diode, $\lambda=850$ nm, $\lambda=100$ nm; SLD2, superluminescent diode, $\lambda=780$ nm, $\lambda=50$ nm; CO1–CO3, collimators; CL, cylindrical lens, f 150 mm; L1–L5, lenses, focal lengths are (in mm) 120, 60, 125, 160, and 30, respectively; BS, beam splitter; DM1, short-pass dichroic mirror; GALVO, galvo mirror; DM2, long-pass dichroic mirror; STI, stimulus light; OBJ, objective, 10 $\times$, NA 0.3; SAMP, sample; CAM, linear camera; GL, glass block; MIR, mirror; PC, polarization controller; SM, spectrometer. (B) Side view of blue and purple rectangle areas in (A). Only confocal part is illustrated.

Fig. 2. (A) Illustration of 20° (left) and -20° (right) oblique stimulations. (B) Color code of TRP direction. (C) Frog photoreceptor mosaic pattern acquired with a NIR transmission microscope. (D) Frog photoreceptor mosaic pattern acquired with the line-scan confocal microscope. (E) Representative frame of the OCT movie (see Media 1). Dynamic OCT imaging revealed TRP in the area (white window) activated by the oblique stimulation. NFL, nerve fiber layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; ELM, external limiting membrane; ISE, inner segment ellipsoid; OS, outer segment. Scale bars: 50 μm .

Fig. 3.

(A) Confocal and OCT observation of the TRP evoked by 20° oblique stimulation. (A1) Confocal image of retinal photoreceptors. Green rectangle represents the 20° oblique stimulus light. Red dashed line shows the location for OCT recording (A5). (A2) TRP magnitude map derived from confocal images. (A3) TRP direction map derived from confocal images. Inactive area is mapped by black background. Active/inactive areas are separated according to displacement magnitude map (A2) by using Otsu's thresholding method. Active area is the location with detectable photoreceptor displacement, while inactive area is the location without detectable displacement. (A4) Histogram of TRP directions in (A3). (A5) Cross-sectional retinal OCT image. The green area indicates the 20° oblique stimulus. (A6) TRP magnitude map derived from OCT images. OCT cross-sectional image is merged in the red rectangle for easy identification of axial location of detectable displacement. (A7) TRP direction map derived from OCT images. (A8) Histogram of TRP directions in (A7). (B) Confocal and OCT observation of the TRP evoked by -20° oblique stimulation. (B1) Confocal image of photoreceptors. Green rectangle represents -20° oblique stimulus light. Red dashed line shows the location for OCT recording (B5). (B2) TRP magnitude map derived from confocal images. (B3) TRP direction map derived from

confocal images. (B4) Histogram of TRP directions in (B3). (B5) Cross-sectional retinal OCT image. The green area indicates the -20° oblique stimulus. (B6) TRP magnitude map derived from OCT images. (B7) TRP direction map derived from OCT images. (B8) Histogram of TRP directions in (B7). Scale bars: 50 μm .