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In Vivo Functional Imaging of Retinal Neurons Using Red and Green Fluorescent Calcium Indicators

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Abstract

Adaptive optics retinal imaging of fluorescent calcium indicators is a minimally invasive method used to study retinal physiology over extended periods of time. It has potential for discovering novel retinal circuits, tracking retinal function in animal models of retinal disease, and assessing vision restoration therapy. We previously demonstrated functional adaptive optics imaging of retinal neurons in the living eye using green fluorescent calcium indicators; however, the use of green fluorescent indicators presents challenges that stem from the fact that they are excited by short-wavelength light. Using red fluorescent calcium indicators such as jRGECO1a, which is excited with longer-wavelength light (~560 nm), makes imaging approximately five times safer than using short-wavelength light (~500 nm) used to excite green fluorescent calcium indicators such as GCaMP6s. Red fluorescent indicators also provide alternative wavelength imaging regimes to overcome cross talk with the sensitivities of intrinsic photoreceptors and blue light-activated channelrhodopsins. Here we evaluate jRGECO1a for in vivo functional adaptive optics

imaging of retinal neurons using single-photon excitation in mice. We find that jRGECO1a provides similar fidelity as the established green indicator GCaMP6s.

Keywords

Adaptive optics; jRGECO1a; Calcium indicator; Retinal imaging; Ganglion cells

17.1 Introduction

We previously demonstrated functional imaging of retinal neurons in the living eye by combining adaptive optics (AO) and green fluorescent genetically encoded calcium indicators (Yin et al. 2013, 2014). In vivo functional imaging enables long-term study of the same retinal neurons over weeks to months making it ideal to the study of the time course of retinal function at the cellular scale during retinal disease and after vision restoration therapy. Because in vivo retinal imaging uses light to probe retinal function, the challenges of using green fluorescent indicators such as those of the GCaMP family are particularly relevant and stem from the fact that they are excited by short-wavelength light around 500 nm. Short-wavelength light is more phototoxic than long-wavelength light (Ham et al. 1976); the maximum permissible exposure of the longer 561 nm wavelength light used to excite the red fluorescent genetically encoded calcium indicator jRGECO1a (Dana et al. 2016) is approximately five times greater compared to 488 nm light used to excite GCaMP6s (Zhang et al. 2016). The excitation spectra of GCaMP indicators overlap with the spectral sensitivities of mouse medium-wavelength-sensitive (M-)opsin, rhodopsin, and melanopsin (Fig. 17.1a, Lyubarsky et al. 1999; Walker et al. 2008; Wang et al. 2011). The excitation spectra of GCaMP indicators also overlap with blue light-sensitive ion channels such as channelrhodopsin-2 (Nagel et al. 2003) making it challenging to use with optogenetics. To advance the utility of functional AO retinal imaging, we imaged jRGECO1a with single-photon excitation in mice. We found that jRGECO1a provides similar fidelity as GCaMP6s (Chen et al. 2013), which provides an alternative wavelength regime for functional AO retinal imaging along with improved light safety.

17.2 Materials and Methods

All animal procedures were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and the Guidelines of the Office of Laboratory Animal Care at the University of Rochester. All protocols were approved by the University Committee on Animal Resources at the University of Rochester.

17.2.1 Animal Preparation

Adult C57BL/6 J mice (The Jackson Laboratory, USA) were used in this study. For short (<30 min) procedures including intravitreal injections and fundus imaging, mice were anaesthetised using ketamine (0.1 mg g⁻¹, JHP Pharmaceuticals, USA) and xylazine (0.01 mg g⁻¹, Akorn Inc., USA). For AO imaging (~ 2 h), mice were anaesthetised using a cocktail containing fentanyl (0.05 µg g⁻¹, West-Ward Pharmaceuticals Corp., USA), dexmedetomidine (Dexdomitor, 0.5 µg g⁻¹, Orion Corp., Finland), and midazolam (5 µg g⁻¹,

West-Ward Pharmaceuticals Corp., USA). At the conclusion of the AO imaging session, mice were given a reversal cocktail containing naloxone ($1.2 \mu\text{g g}^{-1}$, Hospira Inc., USA), atipamezole (Antisedan, $2.5 \mu\text{g g}^{-1}$, Orion Corp., Finland), and flumazenil ($0.5 \mu\text{g g}^{-1}$, Hikma Pharmaceuticals, UK). Drugs were administered intraperitoneally. Adequate anaesthesia was evaluated by checking the toe-pinch and corneal reflexes. During AO imaging, additional heating was provided to maintain the internal body temperature at 37°C , and animals were ventilated on 100% oxygen.

17.2.2 AAV-Mediated Gene Transfection of Mouse Retinal Neurons

Adeno-associated viral (AAV) vectors were used to transfect mouse retinal neurons with genes of interest. Constructs used were AAV2.CAG.jRGECO1a.WPRE.SV40, titre 5×10^{12} GC mL^{-1} , produced in the laboratory of Dr. Cepko, Harvard Medical School; AAV9.Syn.GCaMP6s.WPRE.SV40, titre 4.04×10^{13} GC mL^{-1} ; and AAV2.Syn.GCaMP6s.WPRE.SV40, titre 2.38×10^{13} GC mL^{-1} purchased from the University of Pennsylvania Vector Core. Intravitreal injections of $2 \mu\text{l}$ per eye were made in 3-week-old animals. One male and one female mouse received AAV2.jRGECO1a, two males received AAV9.GCaMP6s, and four females received AAV2.GCaMP6s. A fundus camera (Micron III, Phoenix Research Laboratories, USA) with custom filters (GCaMP6s, excitation FF01–498 SP [Semrock Inc., USA], emission ET525/50 [Chroma Technology Corporation, USA]; jRGECO1a, excitation FF02–525/40 [Semrock Inc., USA], emission band-pass 590/47) was used to assess and map gene expression.

17.2.3 In Vivo Functional AO Calcium Imaging

In vivo retinal imaging was done with a custom-built mouse adaptive optics scanning light ophthalmoscope (see Geng et al. 2012 for system details). Reflectance imaging of blood vessels was performed using an infrared 790 nm laser diode (S790-G-I-15, Superlum Diodes Ltd., Ireland), and simultaneous fluorescence imaging of either GCaMP6s or jRGECO1a was performed using a multichannel laser diode (iChrome MLE-L, Toptica Photonics Inc., USA); GCaMP6s (excitation, 488 nm; emission band-pass filter, FF01–520/35) (Semrock Inc., USA); and jRGECO1a (excitation, 561 nm; emission band-pass filter, FF01–630/92) (Semrock Inc., USA). Wavefront sensing was performed using a 905 nm laser diode (QFLD-905–10S, QPhotonics LLC, USA). All imaging lights were scanned over a $5 \times 6.7^\circ$ field on the retina. Light intensity at the pupil was $185 \mu\text{W}$ for 796 nm, $100 \mu\text{W}$ for both 488 and 561 nm, and $7 \mu\text{W}$ for 904 nm. A $50 \mu\text{m}$ confocal pinhole was used for infrared reflectance and $75 \mu\text{m}$ pinhole for fluorescence.

Mice were positioned in a custom-built holder with bite bar, and contact lenses were placed on the eyes to prevent the corneas from drying. A 365 nm LED (M365 L2-UV; Thorlabs, USA) was presented in Maxwellian view over an 8° diameter patch of the retina to drive short-wavelength-sensitive (S-)opsin. Stimuli were temporally modulated, uniform field, square waves. Figure 17.1a shows the measured spectra of the stimulus and imaging lights, spectral sensitivities of mouse opsins, and action spectra of GCaMP6s and jRGECO1a.

Eye motion was computed using the reflectance images of retinal vasculature (Dubra and Harvey 2010), and motion correction was applied to both reflectance and fluorescence data.

Cell segmentation was performed manually. Response time course was obtained by calculating the mean pixel intensity of each region of interest as a function of time. A fast Fourier transform algorithm was used to compute the signal amplitude and phase at the stimulus frequency (F1) and the DC component (F0). Normalised response was quantified as the ratio F1/F0. All data analysis used MATLAB (MathWorks, USA).

17.3 Results

Widespread expression of jRGECO1a and GCaMP6s was achieved in C57BL/6 J mice retina. Figure 17.1b, c shows fluorescent fundus images of jRGECO1a and GCaMP6s expression, respectively. Both AAV9.Syn.GCaMP6s and AAV2.Syn. GCaMP6s resulted in expression of GCaMP6s; however, AAV2.Syn.GCaMP6s produced more consistent expression across eyes injected as well as more widespread retinal coverage in each eye (data not shown). Figure 17.1d, e shows fluorescent neurons expressing jRGECO1a and GCaMP6s imaged in vivo with AO. Note the visibility of axon bundles in Fig.17.1d indicating expression in ganglion cells. Axon bundles were also observed in retina expressing GCaMP6s but are not visible in Fig.17.1e.

Robust measurements of neuronal responses to a flashing 365 nm LED stimulus were made using jRGECO1a. Figure 17.2a–f shows response time courses for six example neurons; ON cells are shown in a, c, and e and OFF cells in b, d, and f. Response amplitude was quantified (see methods), and histograms of responses to a 365 nm stimulus and “no stimulus” are shown in Fig.17.2g, h, respectively. No response amplitudes above 0.04 were observed in the “no stimulus” condition so we considered cells with response amplitudes above 0.04 in the 365 nm stimulus condition as “responsive” and further analysed their response phase. Figure 17.2i shows a frequency response phase histogram of “responsive” cells. Note that the histogram appears bimodal; the two separate distributions correspond to ON and OFF cells.

Robust measurements of neuronal responses were also made using GCaMP6s. No differences in function between AAV9.GCaMP6s and AAV2.GCaMP6s were observed so data were pooled. Figure 17.3 shows responses to 365 nm LED stimuli measured with GCaMP6s. Data are presented as in Fig.17.2; example response time courses of ON and OFF cells are shown in Fig.17.2a–f, histogram of response amplitudes to 365 nm LED stimulus in Fig. 17.2g, histogram of response amplitudes to “no stimulus” in Fig. 17.2h, and response phase histogram for all cells with response amplitude greater than 0.04 in Fig.17.2i.

17.4 Discussion

The purpose of this study was to explore the use of a red-shifted genetically encoded calcium indicator for in vivo functional AO imaging of the retina. We show that AO ophthalmoscopy of both red-shifted and green fluorescent calcium indicators, jRGECO1a and GCaMP6s, provide robust measurements of neuronal activity in the retina. Our virus constructs resulted in widespread and bright expression of jRGECO1a and GCaMP6s; however, the mean intensity and signal-to-noise ratio of jRGECO1a were lower than GCaMP6s (Figs. 17.2a–f and 17.3a–f). Response amplitudes measured with GCaMP6s also

tended to be larger than with jRGECO1a (Figs. 17.2g and 17.3g); however, these differences do not permit a precise comparison of the two because many factors affect measured intensity. For example, measured resting fluorescence and signal-to-noise ratio are dependent on the ocular optics as well as level of expression, which often differs from eye to eye despite consistent injection parameters. In the original report on jRGECO1a, the authors show that jRGECO1a exhibits similar performance to the GCaMP6 indicators; however, jRGECO1a responses in vivo were still smaller than GCaMP6s likely because of signal saturation at high firing rates (Dana et al. 2016).

One of the benefits of using red fluorescent calcium indicators for functional AO retinal imaging is improved light safety from imaging with longer-wavelength light. It is well established that high-intensity blue light induces photochemical damage in the retina and retinal pigment epithelium (Ham et al. 1976; Wu et al. 2006; Hunter et al. 2012). The longer-wavelength 561 nm light used in this study to excite jRGECO1a provides approximately a fivefold improvement in light safety compared to the 488 nm light used to excite GCaMP6s (Zhang et al. 2016). The greater safety of red fluorescent calcium indicators is particularly important for in vivo imaging of the retina because a major benefit of this technique is the ability to perform repeated imaging of the retina over weeks to months, which carries the risk of phototoxicity.

Recently discovered red-shifted channelrhodopsins (Lin et al. 2013; Klapoetke et al. 2014; Tomita et al. 2014) may be used with green fluorescent calcium indicators to enable all optical recording and activation of neuronal responses. However, the most sensitive channelrhodopsin found to date is activated by blue light (Klapoetke et al. 2014). Thus, a red fluorescent calcium indicator would be necessary in a combined preparation.

Red calcium indicators derived from mRuby such as jRCaMP1a and jRCaMP1b may be more ideal for combined use with optogenetics because they do not exhibit photoswitching in response to blue light, unlike jRGECO1a (Dana et al. 2014).

A remaining serious challenge for optical recording of neuronal responses in the eye is the activation and bleaching of photoreceptors with the intense light needed to excite the fluorescent calcium indicator. Shifting the excitation spectra to longer wavelengths reduces photoreceptor activation with single-photon excitation. Future implementation of two-photon imaging of red fluorescent calcium indicator will greatly reduce photoreceptor activation and bleaching.

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Fig. 17.1.

(a) Action spectra of mouse opsins, 365 nm LED stimulus, excitation and emission spectra of GCaMP6s and jRGECO1a, and fluorescence excitation imaging lasers. (b, c) Fluorescent fundus images of jRGECO1a (b) and GCaMP6s (c) expression. Scale bar: 500 µm. (d, e) In vivo adaptive optics images of jRGECO1a (d) and GCaMP6s (e) fluorescent neurons. Scale bar: 10 µm

Fig. 17.2. Responses measured with jREGECO1a. (a–f) Response time courses of cells to a 365 nm LED stimulus. Shaded bars indicate stimulus presentation. Three trials for each cell are shown. ON responses are shown in a, c, and e and OFF responses in b, d, and f. (g) Frequency histogram of cell responses to 365 nm stimulus ($n = 323$). Dashed vertical line indicates a response threshold of 0.04. (h) Frequency histogram of cell responses to “no stimulus” ($n = 238$). (i) Frequency histogram of response phase for cells with response greater than 0.04 in the 365 nm stimulus condition ($n = 185$). ON and OFF labels indicate cells with respective responses

Fig. 17.3.

Responses measured with GCaMP6s. (a–f) Response time courses of cells to a 365 nm LED stimulus. Shaded bars indicate stimulus presentation. Three trials for each cell are shown. ON responses are shown in a, c, and e and OFF responses in b, d, and f. (g) Frequency histogram of cell responses to 365 nm stimulus ($n = 987$). Dashed vertical line indicates a response threshold of 0.04. (h) Frequency histogram of cell responses to “no stimulus” ($n = 681$). (i) Frequency histogram of response phase for cells with response greater than 0.04 in the 365 nm stimulus condition ($n = 752$). ON and OFF labels indicate cells with respective responses