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***In vivo* characterization of genetic expression of virus-transduced calcium indicators in retinal ganglion cells using a low-cost funduscope**

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Abstract

Virus-transduced calcium indicators are effective reporters of neural activity, offering the advantage of cell-specific labeling. To track the level of *in vivo* expression of genetically encoded calcium indicators (GECIs) in rodent retina, we developed a noninvasive imaging approach based on a custom-modified low-cost and simple fundus system that enabled us to monitor and characterize *in vivo* bright-field and fluorescence retinal image. The system clearly resolves individual retinal ganglion cells (RGCs) and axons. RGC fluorescence intensity and number of observable fluorescent cells show a consistent rising trend from week 1 to week 3 after viral injection, indicating a uniform increase of GCaMP6f expression. At defined time points, we prepared wholemount retina mounted on a transparent multielectrode array (MEA) and used calcium imaging to identify the optimal time for studying the responsiveness of RGCs to external electrical stimulation. The results show that the fluorescence-endoscopy fundus system is a powerful and widely accessible tool for evaluating *in vivo* fluorescence reporter expression.

Introduction

Genetically encoded calcium indicators (GECIs) have been widely used for observing neural activity, especially in vision research, because they enable repeated measurement of many cells in parallel at single-cell resolution. For example, some genetically encoded activity reporters were developed for optimizing the stimulation parameter of retinal prosthesis study [1], or monitoring responses in targeted retinal cell populations during visual information processing activity with relatively high spatial resolution [2]. Technically, the reporters can be delivered to cells via electroporation, biolistics, viral vector transduction, or generation of transgenic animal engineering [3]. Of these methods, viral transduction provides the greatest cellular specificity in the retina [1][2]. Therefore, a suitable tool for tracking GECI genetic expression *in vivo* following transduction is highly desirable.

Generally, several systems are currently used for microscopic fundus imaging in rodents, including scanning laser ophthalmoscopes incorporating adaptive optics [4], commercial fundus systems [5], and two-photon microscopy [6]. Compared with these relatively

complex and expensive imaging techniques, fundus imaging in fluorescence mode, is cost-effective for screening purposes or characterization of the gene expression in transduced neurons [7][8]. In addition, the user-friendly examination procedure facilitates the training and execution time needed to perform the examination.

We developed a custom endoscope-based fundus system for monitoring and characterizing *in vivo* bright-field and fluorescence retinal images, by adapting a low-cost and simple fundus system based on the concepts proposed by Paques et al. [7] and Schejter et al [8]. To monitor the genetic expression of GECI GCaMP6f [9] transduced in mouse RGCs by adeno-associated viral vector (AAV), we performed long-term tracking following intravitreal injection. The result was further validated by *in vitro* calcium imaging in retinal wholemount preparation.

Methods

A. Overview

Two groups of adult mice (C57BL6/J) receiving an intravitreal injection of an AAV vector encoding a GECI (AAV2-CAG-GCaMP6f) were used to perform the fundus imaging and calcium imaging experiment. For *in vivo* fundus imaging, the mice were anesthetized using a mixture of ketamine (80mg/kg) and xylazine (10mg/kg). The pupil was dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. Topical tetracaine hydrochloride was applied for local corneal anesthesia. The animals were positioned such that the eye barely touched the endoscope tip, and a drop of saline (NACL 0.9%) was used to keep the eye hydrated and coupled with the endoscope. To make images comparable at different time points, the optic disc was aligned at the center of camera, and the edge of dilated pupil was arranged perpendicular to the endoscope tip. For each subject, the fundus images were recorded from week 1 to week 3 post injection at 4-day intervals. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC) at the University of Southern California.

B. Fundus Imaging System

The custom endoscope-based fundus imaging system is illustrated in Fig. 1A, with divided excitation and emission pathways to facilitate fluorescence imaging of the retina at single-cell resolution. For the emission pathway (Fig. 1A, C), an endoscope with a 5 cm otoscope and 3 mm outer diameter (1218 AA, Karl Storz, Tuttlingen, Germany) was positioned in front of camera. Assembled a step down ring, the manufactured adaptor was used to connect the selected digital camera (D5100 with AF-S VR Micro-Nikkor 105mm f/2.8G lens, Nikon, Tokyo, Japan) with the endoscope, with the ability to replace optical emission filters. For the Excitation pathway (Fig. 1A, B), a xenon lamp house (LH-M100CB-1, Nikon, Tokyo, Japan) was used as the light source to generate collimated light with illumination power from 1.5 to 6mW at the tip of endoscope. The light can be projected through the excitation filter to a custom made optical fiber connector that transmits the light source to the endoscope by means of the commercial optic fiber cable (495NA, Karl Storz, Tuttlingen, Germany).

Fluorescence images were obtained using a 469 nm excitation filter (MF469-35, Thorlabs, Newton, NJ) placed in the removable filter holder on the light source breadboard (Fig. 1B), and a 535 nm emission filter (MF525-39, Thorlabs, Newton, NJ) positioned in the adaptor in front of the camera lens. Bright-field and green-field images were obtained with and without emission filter respectively, but both required replacing the excitation filter with a neutral density filter (NE06B-A, Thorlabs, Newton, NJ). The camera settings typically used were: 1) image resolution: fine (4928×3264); 2) manual operating mode and focus: 1; 3) aperture: F3.3; 4) ISO: 3200; and 5) focal length: 105 mm. For bright-field or green field imaging, the shutter speed was set to 1/8 seconds, and for fluorescence imaging the shutter speed is set between 8 and 25 second empirically.

C. Imaging Processing

The raw images were processed using different image processing tools. For the fluorescence intensity analysis, we used MATLAB (The MathWorks, Natick, MA) to analyze the fluorescence intensity change tendency. To eliminate the temporal and spatial variation caused by the change of properties of biological lens or the alignment between the eye and the tip of endoscope, the green field intensity was used for normalization. The green channel was first split up from the raw RGB fluorescence image, and 10 consistently observable cells expressing GCaMP6f fluorescence across different time points were randomly selected from the fundus images. The normalized fluorescence intensity was calculated by the average of fluorescence intensity within each cell divided by the baseline green field reflection taken from identical region of interest (1). For each time points, 6 to 8 trails of image pair were taken to compute the final value.

$$I_{\text{normalized}} = \frac{I_{\text{fluorescent}}}{I_{\text{baseline}}} \quad (1)$$

For cell counting, we used ImageJ (National Institutes of Health, Bethesda, MD) to process the data. The green channel was first split up from the raw fluorescent data, and the background subtraction tool was applied with rolling ball radius of 40 pixels. After removing the background, the inverted image was taken and the threshold was adjusted so that distinct cell contour could be clearly observed. To eliminate the noise generated by the CCD camera, the noise correction function, including outlier removal and despeckle, were used for correcting the hot and dead pixels. Moreover the images were further processed by binary function fill holes and watershed functions to fill in the nucleus and partition the overlapping cells. Finally, we used the analyze particles function to count the number of observable cells, with the size set to 500-infinity.

D. Calcium Imaging and Electrical Stimulation

The other group of virus-injected mice were euthanized corresponding to fundus imaging time points for identifying the neurophysiological properties of RGCs expressing GCaMP6f. To simulate external stimulation as retinal prosthesis, we fabricated MEAs in a class 100 cleanroom and patterned the arrays from indium tin oxide (ITO) on no. 1 cover glass

substrates (Vaculayer, Mississauga, Ontario, Canada). A dual-insulation layer, including SU-8 epoxy photoresist and silicon nitride, was formed atop the ITO. The insulation layer was removed to create 75 μm diameter disk electrodes.

The retina was placed on the transparent MEA chamber with ganglion cell side face down and imaged with an inverted epifluorescence microscope. Fluorescence excitation was provided by a super bright cool white light-emitting diode (LED). Excitation and emission light were filtered through a commercial filter set (GFP-4050A, Semrock, Rochester, NY) for GCaMP6f. Images were viewed through a Nikon (Tokyo, Japan) Plan Apo 0.75-numerical aperture (NA) 20 objective and captured by an electron-multiplied charge-coupled device (EMCCD) camera. (iXon, Andor Technology, Belfast, Northern Ireland). The retina was continuously superfused by bicarbonate-buffered Ames' Medium (Sigma-Aldrich, St. Louis, MO) during the course of each experiment at a flow rate of 4–5 ml/min and a temperature of 33°C.

Electrical stimuli consisted of a 50 sec train of charge-balanced, biphasic current pulses at 20 Hz with 5ms duration to maximizing the calcium transient. Voltage stimuli were generated from a computer-controlled stimulus generator (STG-2008, Multi Channel Systems, Reutlingen, Germany) and fed through a custom voltage-to-current converter. The amplitude was determined by 1.2 times the minimum threshold of RGCs in the region of interest.

Results

A. System Performance

To evaluate the system performance, bright-field, green-field and fluorescence fundus images were acquired from virus-transduced GCaMP6f mice. Representative images are in Fig. 3. In the fluorescence images, axons and individual RGCs could be visualized (Fig. 3C, D) and appeared to be relatively well resolved when compared to the previous fluorescence endoscope-based fundus imaging work [8]. Tests of different exposure time settings suggest that the effective resolution is primarily limited by optical point spread function, instead of movement related blurring (not shown).

B. Long-term Expression Tracking

Representative fluorescent images for different stages of virus-induced GCaMP6f expression are shown in Fig. 4. At an early stage of about 1 to 1.5 week post injection, only a few RGCs can be distinguished from the relatively weak background. The number of observable cells as well as the fluorescence intensity within each cell both demonstrate a consistent rising trend with time. In addition, the gradually intensified background for late stage possibly can be attributed to increased scattering with enhanced fluorescence intensity in RGC dendrites.

Fig. 5 shows the statistical analysis for the tendency change of the fundus imaging pattern across different subjects ($n=5$). In the intensity analysis, each curve represents the averaging normalized intensity from the selected RGCs at different sampling points. The results indicate that all subjects share a similar rising trend from week 1 to week 2 post injection.

However, for most of the cases, the fluorescence intensity reaches a plateau or decreases slightly after week 2.

The only dramatic declining case was later found to be attributed to the deterioration of the biological lens. For cell counting analysis, a prolonged increasing trend can also be identified in terms of the number of observable RGCs on the fundus view. Both findings support a uniform increase of GCaMP6f expression on average. In addition, this long-term tracking study further demonstrates that the proposed endoscope-based system can reproducibly acquire images from the selected regions of interest.

C. In vitro Calcium Imaging Validation

To test whether the images obtained by fundus imaging could be used to establish experimental time windows for neurophysiological experiments, the other group of virus-injected mice was used for *in vitro* calcium imaging at defined time points. As shown in Fig. 6, burst stimulation with an ITO electrode was applied to elicit strong fluorescence responses in GCaMP6f-expressing RGCs. Through subtraction between the post-stimulus and baseline images, the responsive cells can be located and correctly labeled in the field of view.

Since the initiation of expression is highly non-uniform across the retina, we selected 3 to 4 regions for each retina where expression is relatively higher. The statistical results shown in Table 1 indicate that the ratio for the responsive RGC versus the total observable cells is highest at week 3 post injection. For earlier time points, there were few observable cells and the responsiveness rate of those cells was also extremely low. If the incubation time was longer than optimal, GCaMP-induced cytotoxicity might occur in some of the RGCs, thus leading to the reduction of ratio [2][10]. The excitation light will cause bleaching of photoreceptors, which limits the applicability of calcium imaging for studies involving light stimulation. However, even in the presence of strong excitation light, RGCs remain responsive to electrical stimulation, because the photoreceptor response is transient even if the excitation light is continuously on.

The *in vitro* calcium imaging results correspond to the *in vivo* fundus imaging shown in Fig. 5. In most cases, the fluorescence intensity within RGCs began to express around 1 to 1.5 week and started plateau or decrease slightly around 3 week, which corresponds to the trend of *in vitro* neurophysiological responsiveness.

Conclusion

We have presented an *in vivo* fundus system that allows high-resolution digital fundus imaging in the mouse eye. Additionally, the long-term tracking experiment demonstrates the system can serve as a powerful tool for stably obtaining cellular-resolved fluorescent images and this information can be effectively used for characterizing the change of expression of virus-induced calcium indicator, thus greatly reducing the time to identify transduction outcomes. Moreover, the *in vitro* calcium imaging shows that the fluorescence fundus imaging can be used to determine the time window for neurophysiological studies of transduced RGCs.

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Figure 1.

(A) System schematic diagram. 1: light source, 2: convex lens, 3: excitation filter, 4: objective lens, 5: optic fiber, 6: endoscope, 7: *in vivo* eye, 8: adaptor, 9: emission filter, 10: lens of camera, 11: camera. (B) Excitation part of the system, including compartments 1 through 5 in (A). (C) Emission part of the system, including compartments 6 through 11 in (A).

Figure 2.

Calculation of normalized fluorescence intensity of individual cell. Locally selective fluorescence intensity was normalized by the green field intensity from identical region of interest. (A) Group of cells selected from fluorescence fundus image. (B) Baseline captured from identical location of relevant green field image.

Figure 3. Representative fundus images in a C57 mouse. (A) Bright field image. (B) Green field image. (C) Fluorescent image. (D) Magnification of (C).

Figure 4. Representative fundus images demonstrating the long-term expression of GCaMP6f in mouse RGCs. From (A) to (E) are 11, 15, 19, 23, 27 days post injection respectively.

Figure 5. Normalized intensity in selective RGCs and number of observable RGCs in the fundus field of view across 5 subjects. For each time point, 6-8 trials of data were averaged to demonstrate the tendency of fluorescent expression.

Figure 6.

Electrical stimulation activates RGCs as revealed through large changes in GCaMP6f fluorescence intensity in well expressed region *in vitro*. (A) Before Stimulation, cells are at baseline fluorescence. (B) Original calcium imaging in response to external stimuli from the ITO electrode in the middle, the electrically activated cells became visibly brighter. (C) Image subtraction of (B) from (A) baseline highlights the responding cells in the field of view.

Table 1**Responsive cell (*in vitro*)**

Statistical results for the ratio of responsive RGC versus total cell expressing GCaMP6f in the region of interest. The data was acquired from 3-4 well-expressed regions of each retina.

Time Post injection	Ratio (mean)	Ratio (std)	# of RGC w/ GCaMP6f
2 weeks	29.60%	$\pm 6.38\%$	43.3
3 weeks	86.97%	$\pm 3.83\%$	86.3
4 weeks	69.24%	$\pm 5.00\%$	94.0