Mapping Cone- and Rod-Induced Retinal Responsiveness in Macaque Retina by Optical Imaging

Kazushige Tsunoda,[1,2] *Yoshihisa Oguchi,*[3] *Gen Hanazono,*[3] *and Manabu Tanifuji*[1]

Purpose. To map the distribution of cone- or rod-induced retinal responsiveness by optical imaging from macaque retina.

Methods. The light reflectance changes in the posterior retina after a flash stimulus in anesthetized rhesus monkeys were measured by a modified fundus camera system equipped with a charge-coupled device (CCD) camera. The response topography of the optical signals was obtained in either light- or dark-adapted conditions.

Results. With infrared observation light, the whole posterior pole became darkened after the stimulus. The response topography in light-adapted conditions demonstrated a steep peak of darkening at the fovea, together with the gradual decrease of signal intensity away from the fovea toward the periphery. In dark-adapted conditions, the optical signal showed additional peaks along the circular region surrounding the macula at the eccentricity of the optic disc, together with the central peak at the fovea. A statistically significant positive correlation was obtained between the light reflectance changes in infrared observation light and the focal responses in multifocal electroretinogram (mfERG) at the corresponding retinal locations.

Conclusions. The response topography in the retina, obtained by optical imaging, was consistent with psychophysical cone or rod sensitivity in humans and anatomic cone or rod distribution in humans and macaques. The cone- or rod-induced retinal responsiveness within the posterior pole region was noninvasively recorded within a short recording time. (*Invest Ophthalmol Vis Sci.* 2004;45:3820–3826) DOI:10.1167/iovs.04-0394

Since the early pioneering work by Osterberg,[1] there have been numerous reports about the detailed anatomic distribution of photoreceptors in monkey or human retina,[2–4] and recently, the mosaic arrangement of three types of cones has been precisely analyzed, even in living subjects.[5] Because visual acuity or size of visual field depends not only on the anatomic density but more on the neural function of photoreceptors, mapping retinal responsiveness is important from both physiological and clinical points of view. However, detailed response topography of cone and rod photoreceptors have not been revealed with the current capabilities of the conventional objective measurements, such as the multifocal electroretinogram (mfERG),[6–8] flicker-evoked flowmetry,[9] and functional MRI (fMRI).[10]

Optical imaging based on intrinsic signals is a well-established imaging technique for visualizing neural activity. Since Blasdel and Salama[11] and Grinvald et al.[12] successfully visualized in living animals the functional organization of the primary visual cortex, such as the orientation column and ocular dominance column, this technique has been widely used in the past decade to map the functional organization of the various areas of the visual cortex,[13] such as V1,[14,15] V2,[16] V4,[17] MT,[18] and TE.[19] In optical imaging, the decrease in light reflectance elicited by neural activation is measured from the cortical surface by a charge-coupled device (CCD) camera, without using extrinsic probes. The reflectance changes are due to multiple metabolic changes after neural activation. For example, the optical signal measured at ~540 nm is dominated by blood volume changes in capillaries, that, at 600 to 650 nm, is dominated by the change in deoxygenation level of hemoglobin and that, in infrared light, is dominated by the change in tissue light-scattering.[20] Although different metabolic changes are emphasized at different wavelengths, optical responses obtained at these wavelengths give nearly the same spatial pattern that corresponds to the spatial pattern of activated neurons.[15,21]

The optical imaging technique with intrinsic signals has advantages over other functional neuroimaging techniques, such as positron emission tomography,[22,23] fMRI,[24–26] and near-infrared optical topography,[27,28] in that the spatial resolution is as good as 50 μm and the time resolution is less than 1 second. Although the reflectance change is not a direct measure of neural activation, the intrinsic signals coincide well with the activity of neurons examined by conventional extracellular recordings,[19,29,30] and the mass responses of clusters of neurons can be easily mapped simultaneously from large area of the cortex. Measuring intrinsic signals, however, requires surgical exposure of the cortical surface, and an optical chamber should be mounted on the skull to clear the cortical view and reduce the cortical movements induced by heartbeat and respiration. Thus, its application to clinical examination is severely limited.[31,32] The eyeball with transparent ocular media, on the contrary, works as an ideal optical chamber through which retinal reflectance changes can be clearly and stably monitored without any surgical invasion, and the optical imaging is likely to be of considerable value in ocular examination.

Previous studies, measuring light reflection from in vivo retina, mainly focused on the anatomic distribution of cone photopigment,[33,34] macular pigment,[35,36] and cone mosaic,[5] and few of them directly dealt with light reflectance changes related to neural activities.

In the current study, we applied the technique of optical imaging to macaque retina and, for the first time, successfully demonstrated the stimulus-evoked response topography that reflects cone- and rod-induced photoreceptor function. The purpose of this study was to investigate the basic properties of intrinsic signals in macaque retina, for the future establishment of a new diagnostic system by which early detection of functional disorders in retina, such as age-related macular degener-

From the [1]Laboratory for Integrative Neural Systems, Riken Brain Science Institute, Saitama, Japan; the [2]Laboratory of Visual Physiology, National Institute of Sensory Organs, Tokyo, Japan; and the [3]Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan.

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Corresponding author: Kazushige Tsunoda, Laboratory for Integrative Neural Systems, Riken Brain Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan; tsunoda@postman.riken.jp.

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ation (ARMD), retinitis pigmentosa, and various retinal diseases in infants, can be made noninvasively.

METHODS

The experiments were performed on two Rhesus monkeys (*Macaca mulatta*) (denoted M1 and M2) under anesthesia. After intramuscular injection of atropine sulfate (0.08 mg/kg), droperidol (0.25 mg/kg), and ketamine (5.0 mg/kg), the monkeys were paralyzed with vecuronium bromide (0.2 mg/kg per hour) and artificially ventilated with a mixture of N_2O, O_2, and isoflurane (70% N_2O, 30% O_2, isoflurane up to 2.0%). Electroencephalogram and electrocardiogram, expired CO_2 concentration, and rectal temperature were monitored throughout the experiments. Before recordings were made, the pupil was fully dilated with topical tropicamide (0.5%) and phenylephrine hydrochloride (0.5%), and eye movements were completely blocked with an additional dose of vecuronium bromide. The experimental protocol was approved by the Experimental Animal Committee of the Riken Institute. All experimental procedures were performed in accordance with the guidelines of the Riken Institute and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Optical Imaging and Data Analysis

We modified a digital fundus camera system (NM-1000; Nidek, Aichi, Japan) to measure the light reflectance changes from the ocular fundus. Fundus images were obtained using a CCD camera (PX-30BC; Primetech Engineering, Tokyo, Japan) and digitized with an IBM compatible computer, equipped with a video frame-grabber board (Pulsar; Matrox, Quebec, Canada: gray-level resolution, 10 bit; spatial resolution, 640 × 480; temporal resolution, 1/30 second; Fig. 1A). The recording area covered 45°, including the macula, superior and inferior vascular arcades, and optic disc, and the image was focused at the surface of macular vessels. Throughout the recording of a trial, the posterior fundus was continuously illuminated with light from a halogen lamp (observation light), which passed through one of the bandpass interference filters: 650 and 900 nm, with 30 and 120-nm bandwidths, respectively. Each recording trial took 8 seconds and consisted of 16 consecutive averaged images, each of which consisted of 15 gray-scale video frames (Fig. 1B). A white Xenon flash stimulus (duration: 1 ms) was given to the whole posterior region of the ocular fundus, 1250 ms after the initiation of data acquisition. The flash intensity measured in front of the eye was 41.9 cd-s/m^2. The timing of data acquisition and stimulus delivery was under computer control.

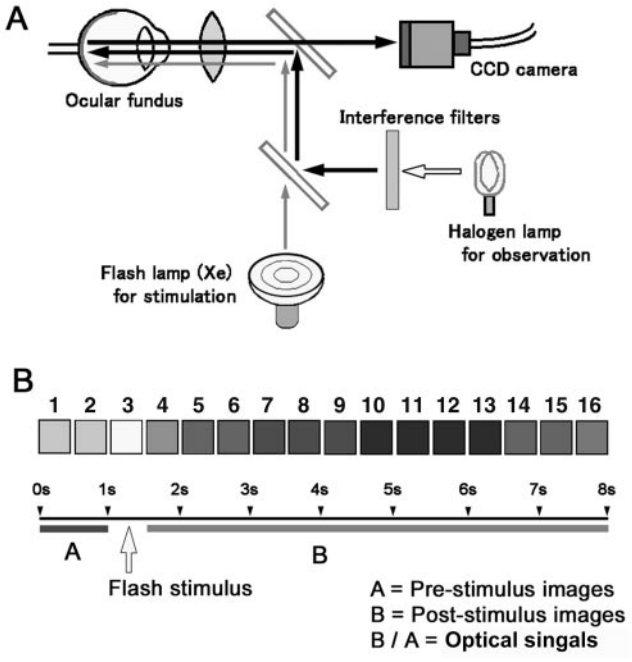

FIGURE 1. **(A)** Schematic drawing of the experimental setup. Throughout the recording trial, the fundus was continuously illuminated with observation light through one of the bandpass filters. **(B)** The recording protocol of one trial lasting 8 seconds (16 images). Before stimulus delivery, prestimulus images were taken to make a reference image of baseline light reflectance. Flash stimulus was administered in the middle of the third image. Each of the 16 consecutive boxes shows an averaged fundus image, containing 15 video frames taken across 500 ms.

Changes in light reflectance from the ocular fundus (optical signals), such as darkening (decrease in light reflectance) and brightening (increase in light reflectance) of the retina, were observed after flash stimulus. The optical signal was calculated as follows[19]: (1) Each image size was reduced to 320 × 240 pixels by 2 × 2 binning, and (2) the gray-scale values of the images obtained after stimulus were divided, pixel by pixel, by those obtained during a 1-second period before the stimulus. This ratio was rescaled to 256 levels of gray-scale resolution to show the stimulus-driven reflectance changes. Recording trials with severe motion artifacts or large physiological noise were excluded from the analysis.

Two-dimensional images of the optical signals were obtained from a single trial without averaging data from different trials (Fig. 2A). In the recording sessions to measure the signal time course, 8-second trials were consecutively run 20 times in monkey M1, with 10-minute intervals between flashes under dark condition. Among them, five trials with a high noise level were excluded. The result of 15 trials is shown in Figure 2B. In the recording sessions to map the response topography, each trial was consecutively performed after 15 (light-adapted condition) or 40 (dark-adapted condition) minutes' adaptation (Figs. 3A, 4A). The eye was light adapted by a Ganzfeld bowl with a background luminance of 30 cd/m^2. Basically, the result of a single trial could show a typical response topography. However, to obtain the response topography shown in Figures 3 and 4, three consecutive trials were averaged to reduce the "shot noise" derived from the observation light itself. To make a three-dimensional topographic map of signal intensity, a median filter (6 × 6 pixels) was applied (Figs. 3B, 4B).

In retinal optical imaging, there are two important issues in choosing the wavelength of observation light: (1) During the whole period of recording, the retina is continuously illuminated with the observation light to measure the reflectance changes. If visible light is used for observation, it evokes neural activities, and the reflectance can be changed without giving flash stimulus. The baseline retinal reflectance thus becomes unstable and unsuitable for evaluating the retinal response. For example, visible observation light at a wavelength of 650 nm could elicit changes in light reflectance without flash stimulus, and even after 10 minutes of adaptation to the observation light, the reflectance change of −0.08% was observed during the recording period of 8 seconds (Fig. 2B, left panel, no flash). Under infrared light (900 nm), however, the fluctuation of light reflectance was as little as −0.02% (Fig. 2B, right panel, no flash). (2) It is well known that after bleaching of photopigments, the macular reflectance for visible light illumination is dramatically increased (retinal image looks brighter; Fig. 2A, 650 nm) and this phenomenon could be very useful for functional evaluation of the macula. The problem is that the bleaching-related reflectance change has a polarity opposite to the reflectance change that is caused by tissue light-scattering and hemoglobin concentration changes, which are commonly observed as decreases in light reflectance (the retinal image looks darker; Fig. 2A, infrared). Considering that imaging with visible light is composed of multiple signal components with different polarities, the data of optical imaging under visible observation light is not useful for mapping retinal responses. Therefore, the observation light used in our study should have negligible absorption by the photopigments,[37] and this would allow a correct mapping of the stimulus-evoked response topography. In the present study, data with 650 nm light were also presented, representing re-

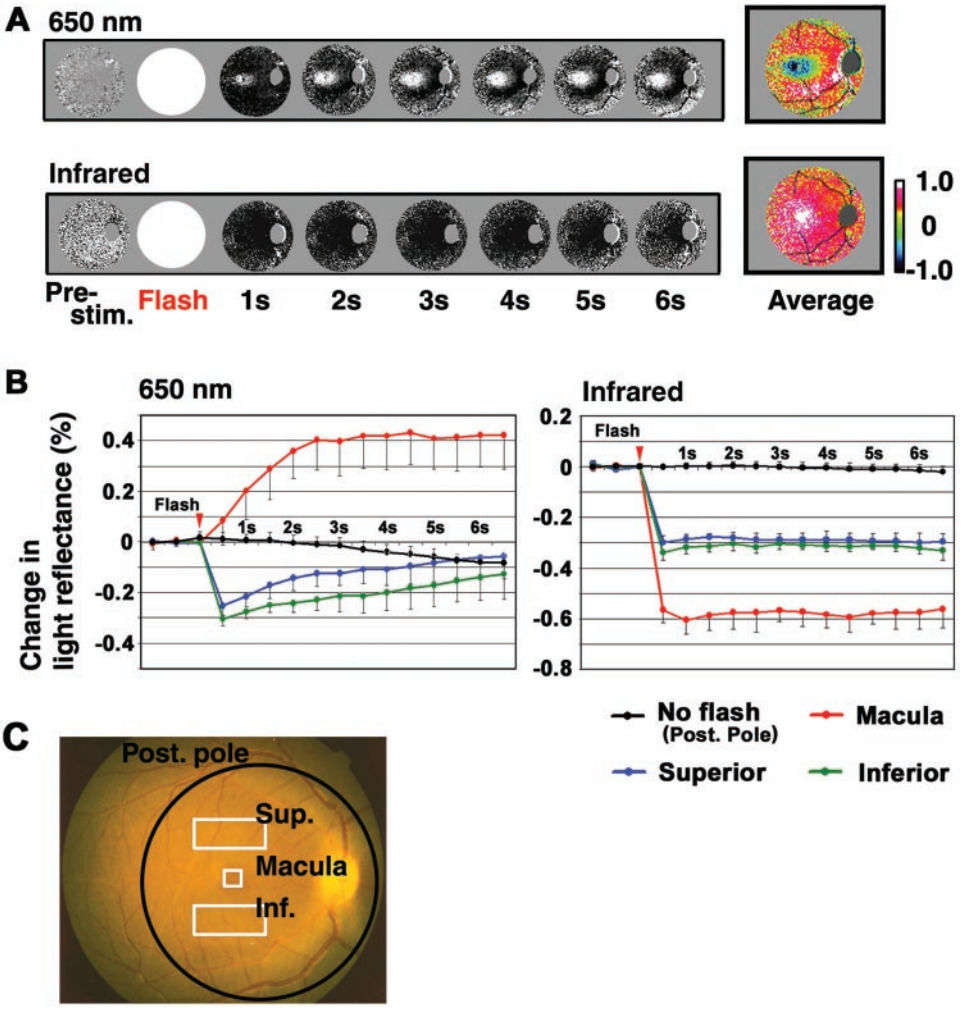

FIGURE 2. (**A**) Time courses of two-dimensional images of retina showing light reflectance changes after a flash stimulus observed with 650 nm and infrared light (900 nm), measured in the posterior pole region of normal retina in (**C**). The images in the second column were saturated by the strong reflection of the flash stimulus. The averaged reflectance changes during initial 3 seconds after stimulus are shown in pseudocolor maps on the *right*, with landmarks such as retinal vessels and the optic disc indicated in *black*. Color indicates relative light reflectance decrease (darkening) from prestimulus level. (**B**) Time courses of light reflectance changes, with or without stimulus under observation light at 650 and 900 nm (infrared) at various regions in a normal eye. The data were averaged among 15 trials. Spontaneous reflectance changes (no flash) in the posterior pole region, and stimulus evoked reflectance changes in superior, inferior, and macular regions were plotted as averaged reflectances ± SD within the corresponding local regions in (**C**). (**C**) Fundus photograph of a normal retina, indicating the recording areas. Sup., superior posterior pole; Inf., inferior posterior pole.

cording with visible light, because red observation light (600–700 nm) is most commonly used in brain optical imaging.

Multifocal Electroretinogram

mfERG measures the local retinal activity in multiple small regions in the posterior retina by presenting on a television monitor an achromatic flicker that alternates independently between black and white in individual hexagonal segments.[7,8,38] Multifocal ERGs were recorded and analyzed with a visual evoked response imaging system (VERIS; EDI, San Mateo, CA) with a Burian-Allen bipolar contact lens electrode. Foveal position was adjusted by projecting the retinal image onto the monitor through a modified retinoscope. The luminance of each hexagon was 200 cd/m^2 (white) or 10 cd/m^2 (black), with the surround at 100 cd/m^2. Thirty-seven equally sized hexagonal elements (each 3.6° wide) were displayed at a 75-Hz frame rate, and recordings were collected in either the normal or artificially damaged eye of monkey M1, with 4 minutes' total duration. Amplitudes of the focal first-order responses were estimated by a scalar product method.[7] The correlation between the amplitude of the local mfERGs and the magnitude of the local reflectance changes was calculated. For this, the fundus was divided into 15 separate regions. Three optical measurements taken on separate days were compared with one mfERG (Figs. 3C, 3D, 5C, 5D).

Argon Laser Photocoagulation

To reduce the function of outer retina artificially, the left fundus in M1 was locally coagulated with argon laser photocoagulation (ALC; wavelength: 514 nm, spot size: 500 μm, power: 160 mW).[39] As many as 1500 spots were densely scattered in the top half of the retina within the equator, so that the distance between each spot was as close as 100 μm (Fig. 5). The optical imaging and mfERG were conducted 1 month after ALC, when the ALC spots had formed complete scars, and no macular edema was observed, either by fluorescein angiography or funduscopic examination.

RESULTS

With observation light having a wavelength of 650 nm, the light reflectance from the macula increased (retinal image became brighter), whereas the light reflectance from the posterior pole region apart from the macula decreased (retinal image became darkener) after a flash stimulus (Fig. 2A). The time course of light reflectance changes in the macular region was quite different from that in perimacular regions (Fig. 2B). The increase in light reflectance after a flash in the macular region is attributable to the bleaching of photopigments by visible light.[33,34,37,40] In contrast, the decrease in light reflectance in the perimacular region is attributable to the hemoglobin-related reflectance changes or tissue light-scattering changes that are observed in the cerebral cortex.[20]

With infrared observation light, the whole posterior region became darker for at least 6 seconds after the stimulus, and the pseudocolor map shows that there was a peak of light reflectance decrease at the center (Fig. 2A). The signal time course shows that the light reflectance from the retina decreased after the flash stimulus and reached its peak no later than 1 second after stimulus onset (Fig. 2B). The response topography in the

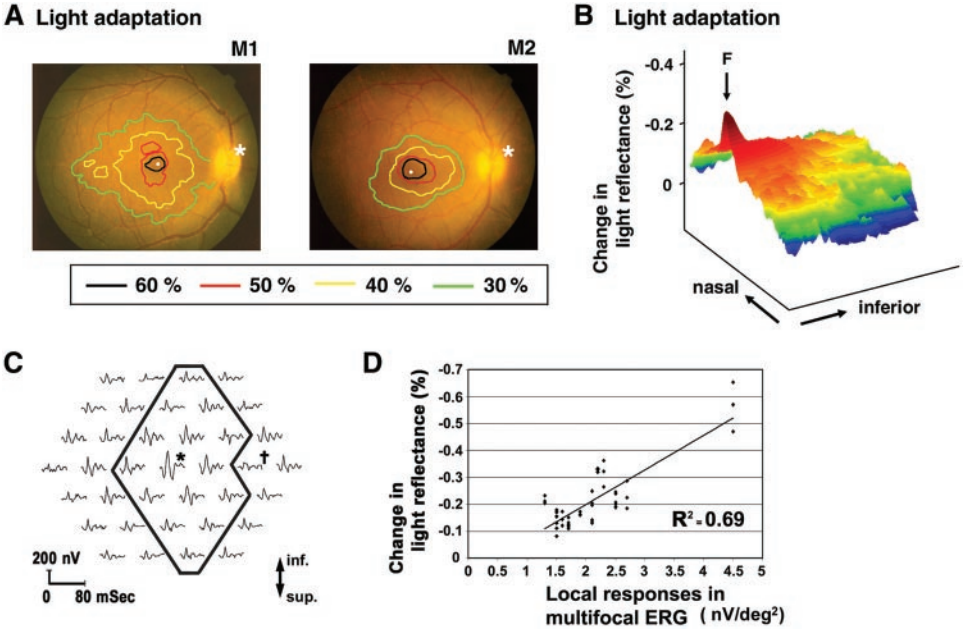

FIGURE 3. (**A**) Response topography of normal retina in light-adapted conditions in two monkeys (M1 and M2). *White dot*: foveal center; *asterisk*: optic disc. Regions with 60%, 50%, 40%, and 30% of the peak signal intensity at the fovea are outlined by different colors. (**B**) Pseudocolor topographic maps of light reflectance changes in the inferior retina, profiled along the horizontal meridian, in the light-adapted condition. Three consecutive trials were averaged to make up the topographic map in M1 (same in Fig. 4). The location of the fovea is indicated by F (*arrow*). (**C**) Array of 37 local mfERG responses in normal retina. The responses of 15 elements (inside the frame) were taken for correlation analysis. ERGs with * and † indicate the location of macula and optic disc, respectively. (**D**) Correlation between the optical signal (decrease in light reflectance) obtained with 900 nm illumination and the scalar product of the first-order kernel of the mfERG from the corresponding retinal locations ($R^2 = 0.69$, $P < 0.001$, $n = 45$). To emphasize the negative peak, we used graphic scaling opposite to convention.

light-adapted condition demonstrated a steep peak of darkening at the fovea, together with the gradual decrease of signal intensity away from the fovea toward the periphery (Figs. 3A, 3B). This is consistent with the topography of psychophysical cone sensitivity in normal human subjects measured with bright background.[41,42] To make sure that the central peak of darkening reflected the cone-related retinal response, the local optical signal values in the light-adapted condition were compared with the local neuronal activities, measured electrophysiologically by mfERG, that are thought to reflect the cone-mediated retinal activity (Figs. 3C, 3D).[7,38] We found a statistically significant correlation between the light reflec-

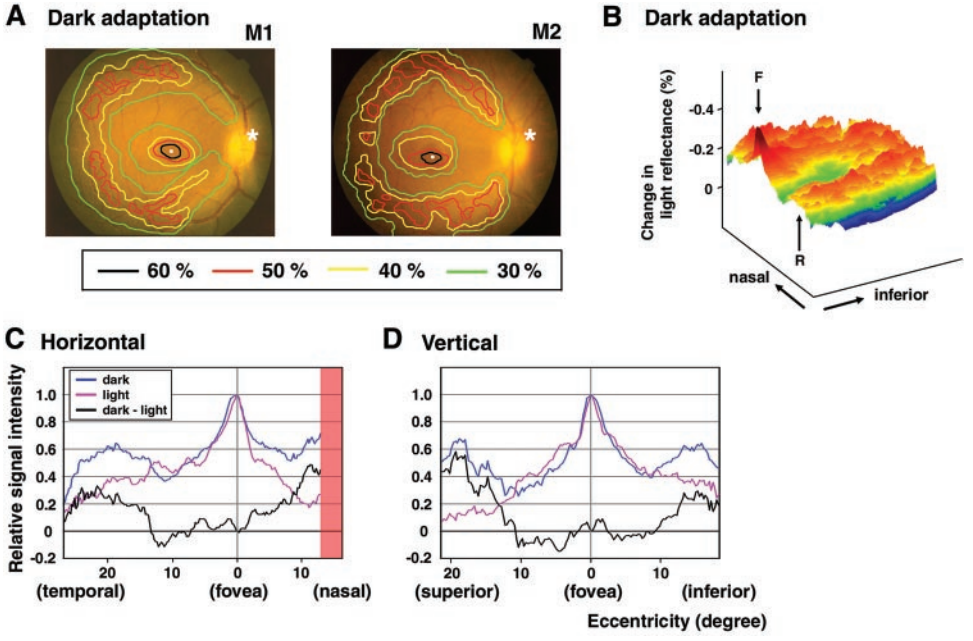

FIGURE 4. (**A**) Response topography of normal retina under dark-adapted conditions. (**B**) Pseudocolor topographic maps of light reflectance changes in the inferior retina, profiled along the horizontal meridian, under dark-adapted conditions. F: the fovea; R: crest of the rod ring. (**C**, **D**) Signal-intensity profiles along the horizontal (**C**) and vertical (**D**) meridians as a function of eccentricity from the foveal center, under dark- and light-adapted conditions. The signal intensity was averaged among three consecutive trials in monkey M1. The peak intensities at the fovea were normalized to 1.0 in both conditions. *Black traces*: estimated rod-induced relative response profiles. The *red* region on the *x*-axis corresponds to the optic disc in the nasal retina.

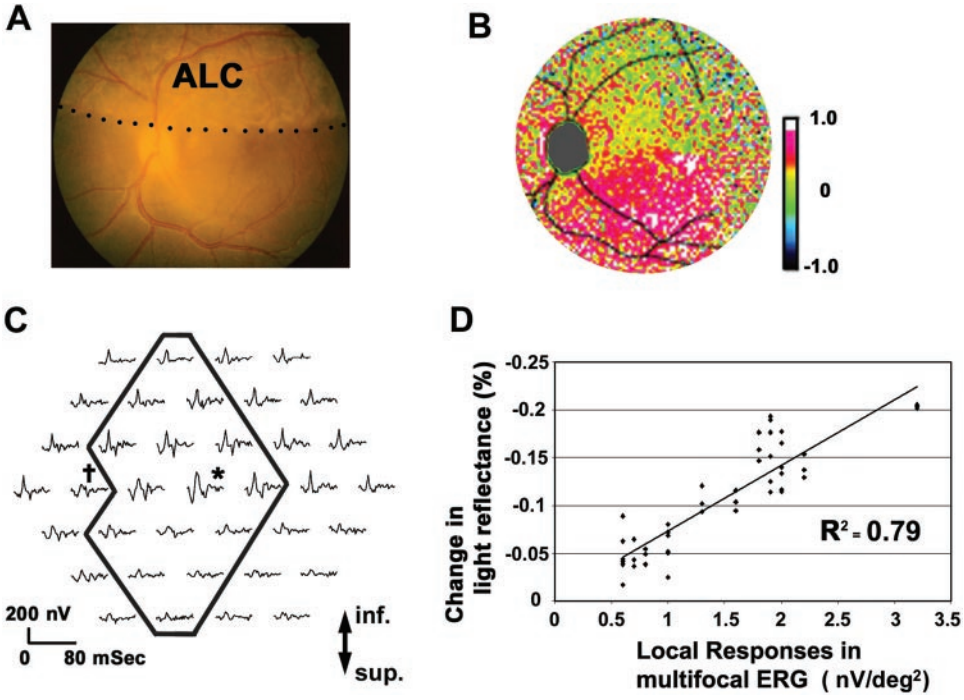

FIGURE 5. (**A**) Fundus photograph of the artificially damaged eye. The top half of the posterior retina was densely coagulated with ALC, sparing the macular area. (**B**) Pseudocolor map of optical signals averaged over the initial 3 seconds under infrared light. Color indicates relative light reflectance decrease (darkening) from prestimulus level. (**C**) Array of 37 local responses of mfERG, taken from the fundus in (**A**). ERGs with * and † indicate the location of macula and optic disc, respectively. (**D**) Correlation between the optical signal (decrease in light reflectance) obtained with 900 nm illumination and the scalar product of the first-order kernel of the mfERG from the corresponding retinal locations ($R^2 = 0.79$, $P < 0.001$, $n = 45$).

tance change under infrared observation light and the focal responses in mfERG at the corresponding sites in normal retina ($R^2 = 0.69$, $P < 0.001$, $n = 45$), indicating that the topography of optical signal response reflects cone-related retinal function.

If the optical signal reflects the central accumulation of photoreceptors in the light-adapted condition, the local retinal region, which is called rod ring with high rod density, should also be activated in the dark-adapted condition.[3,4] After 40 minutes of dark adaptation, the optical signal showed additional peaks along the circular region surrounding the macula at the eccentricity of the optic disc, together with a central peak at the fovea (Figs. 4A, 4B). By assuming that the optical signals measured under light-adapted condition reflect only the cone-induced response, we estimated the relative response profile of rods by subtracting the signal in the light-adapted condition from that in the dark-adapted condition. The optical signal amplitudes of the cone-induced central peak were almost the same in dark- and light-adapted conditions (data not shown). The averaged signal intensity along the horizontal and vertical meridian in three recording trials was plotted as a signal intensity profile relative to the peak value (Figs. 4C, 4D). The profiles along the horizontal and vertical meridians showed that the estimated rod response had peaks at 22.0° in temporal, 18.5° in superior, and 15.0° in inferior retina from the foveal center. These peak eccentricities were consistent with the location of peak in psychophysical rod sensitivity in normal human subjects.[43,44] The response peak at the nasal retina was not measurable because of the presence of the optic disc.

To see whether optical imaging could map the area with a functional lesion, we used ALC for local coagulation of the left fundus in M1 (Fig. 5A). One month after the procedure, the structure of the outer retina, including photoreceptors and pigment epithelial cells, was thought to be widely damaged,[39] and the electrophysiological responses in the damaged region were reduced accordingly (Fig. 5C). Similar to the data from the normal eye, the light reflectance decrease and the mfERG signal showed statistically significant correlation in the eye artificially damaged by ALC ($R^2 = 0.79$, $P < 0.001$, $n = 45$). The results of optical imaging and electrophysiological measurement were well correlated not only in their response amplitudes but also in the spatial location of reduced responses: the border between normal and reduced response regions in both measurements corresponded to the border between the normal site and the area of photocoagulation (Figs. 5A–C).

DISCUSSION

Local neural activity induces various metabolic changes, and some of them cause changes in light reflectance from tissue. For example, neural activation elicits increases in deoxygenated hemoglobin concentration and in tissue blood volume. Intrinsic signals in cerebral cortex, stimulus-induced light reflectance changes, partly originate from light absorption changes due to these hemoglobin concentration changes.[20,21] Neural activation also elicits microscopic morphologic changes, such as glial cell swelling associated with ion and water movement and changes in synaptic vesicle density associated with synaptic transmission. These morphologic changes alter tissue light-scattering, which is considered to be another source of intrinsic signals in the cerebral cortex.[20,45,46] In our recording protocol with infrared observation light, the most plausible source of the optical signals is the stimulus-induced light-scattering changes derived from photoreceptors and surrounding glial cells (e.g., Müller cells[47–49]). That is because (1) strong darkening was observed, even in the foveal center, which lies within a capillary-free zone and is therefore less susceptible to hemoglobin concentration changes,[50] and (2) under infrared light, the contribution of changes in deoxygenated hemoglobin concentration and local blood volume to the optical signal is thought to be much smaller than that of tissue light-scattering.[20]

In this study, we have revealed characteristic response topographies of the retina both in light- and dark-adapted conditions, (1) there was a strong peak of signal intensity in a small area at the foveal center, with a steep decrease away from the fovea in both conditions; (2) the contours of the same signal intensities in the perimacular region were horizontally elongated; and (3) rod-induced signal increased toward the

periphery, forming a circular region with high signal intensity at the same eccentricity of the optic disc. These response patterns were consistent with the results in subjective psychophysical measurements of cone and rod sensitivities,[41–44] and seemed to reflect the current neural activity related to the photoreceptors. As a proof, the spatial property of the optical signals obeyed the several principal features of cone and rod density revealed by macaque and human anatomic studies.[1–4] There are, however, some discrepancies between the response topography and the anatomic distribution of photoreceptors: The dip of the rod ring was not apparent in the horizontal meridian in the response topography, and the crest of the rod ring in the response topography was more peripherally located in superior and temporal retina than that in the anatomic rod density in macaque. These discrepancies may be attributable to the nature of response topography of photoreceptors, which may not completely correspond to their anatomic density distribution; the spatial spread of optical signals, which may deteriorate the fine spatial information; and variability in photoreceptor density distribution among different species or different individuals.[3]

Intrinsic signals can be recorded from the retina noninvasively and more stably than from the cortex. However, there are some potential artifacts that may influence the results. Because of the optical property of the fundus camera system, the intensity of flash stimulus applied to the retina could not be spatially homogeneous, because of the luminance distribution, which has its peak at the center, and the stray light of the flash in the eyeball. In the present system, the light intensity in the central optical axis is theoretically 10% larger than that in the periphery (20° apart from the fovea). In the pilot experiments, we recorded optical signals by adjusting the optical axis of the fundus camera to the foveal center or the peripheral regions apart from the foveal center (10–20°). The difference in the signal intensities of the foveal response in both conditions was as small as the trial-to-trial deviation in each condition, indicating that this problem was not significant in mapping the topography of retinal responsiveness. Opacification in the ocular media, such as corneal edema and vitreous opacity, could also be a cause of the optical artifact.

Eye movement can be another major source of the artifact. Because very small reflectance changes were measured to extract the stimulus-evoked optical response, the location of retinal images before and after stimulus should be completely matched. Slight eye movement during a recording trial causes the quality of the data to deteriorate significantly, which can be corrected by off-line realignment of the retinal images. In the present study, each recording trial was conducted after ensuring that the muscle paralysis was effective enough to stop the eye movements. This artifact, however, could be a serious problem, when we apply it for the clinical examination of alert subjects, who may also produce additional physiological artifacts, such as head movement and change in blood pressure. We are now designing a new recording system to reduce such possible artifacts, and we believe that the trial-to-trial variability could be minimized to that of animal experiments with further mechanical development.

Although lipofuscin autofluoresces,[51] it would be impossible for this to affect our measurement because (1) the first measurements were taken 250 ms after the flash, whereas the excitation of lipofuscin occurs at a much shorter time ($<$0.1 ms), and (2) if there was continuous illumination, the constant autofluorescence would be deducted, because reflectance records after the flash were subtracted from those before the flash.

There have been many reports in which light reflectance from in vivo retina was measured for evaluating the macular function. Most of them focused on the anatomic distribution of cone photopigment,[33,34] macular pigments,[35,36] and cone mosaic,[5] and a few of them dealt directly with light reflectance changes related to neural activities.[52,53] DeLint et al.[53] pointed out that slower reflectance changes in the fovea during light and dark periods may be linked to cone photoreceptor activity. The results presented by them may be related to the stimulus-evoked light reflectance changes in our study; however, it is difficult to determine, because our technique is different and our measurements are much briefer than their measurements, in which the signal lasts for as long as 15 minutes.

The advantage of this functional measurement of retina is that cone- or rod-induced retinal responsiveness within the posterior pole region can be noninvasively monitored with fine spatial resolution, even from a single trial. There is an urgent need for the development of an objective method to measure retinal function, by which functional disorders can be detected before symptomatic or structural changes occur, especially in adult patients with ARMD or retinitis pigmentosa and in small infants in whom functional examination is always difficult to perform. Although the source of the optical signal is not fully understood, with further technical development, this functional measurement could have a strong potential as a new diagnostic tool for early detection of subtle (subclinical) retinal dysfunction in adults and infants.

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