Intrinsic Signal Imaging and Data Analysis

A modified digital fundus camera system (NM-1000; Nidek, Aichi, Japan) was used to observe and measure the light reflectance changes from the ocular fundus. The fundus images were recorded with a charge-coupled device (CCD) camera (PX-30BC; Primetech Engineering, Tokyo, Japan), and the images were digitized with an IBM-compatible computer equipped with a video frame grabber board (gray-level resolution, 10 bits; spatial resolution, 640 × 480; temporal resolution, one-thirtieth of a second; Corona II; Matrox, Quebec, Canada). The camera was focused on the macular vessels, and the area recorded covered 45°, which included the macula, the superior and inferior vascular arcades, and the optic disc. We mainly investigated three retinal sites: the fovea, the posterior retina between the macula and inferior temporal artery, and the optic disc (Fig. 1A).

The fundus was continuously monitored with light from a halogen lamp filtered through an infrared interference filter (840–900 nm). Visible light could not be used for fundus monitoring because the light reflectance changes induced by bleaching of the photopigments have a polarity opposite to that of the intrinsic signals,[3,12–16] leading to incorrect mapping of the stimulus-evoked responses topographically.

Each recording trial consisted of 300 video frames collected at 30 frames per second for a total recording time of 10 seconds. To determine the time course of the flash-induced reflectance changes, we averaged the gray-scale values of 15 video frames collected in 0.5 second for each of the data points (Figs. 1, 6, and 7).

An unfiltered Xenon flash (duration: 1 ms) was given to the whole posterior pole of the ocular fundus or to a focal region of the posterior retina, 500 ms after the initiation of data acquisition. The maximum flash intensity (0-log-unit intensity) measured at the cornea was 308.0 cd · s/m^2 (measured at 50.2 mm from the object lens, by a photoradiometer: model IL-1700; International Light Technologies Inc., Peabody, MA). The timing of the data acquisition and stimulus delivery was under computer control.

Changes in light reflectance from the ocular fundus after the stimulus, such as a darkening (a decrease in light reflectance), and a brightening (an increase in light reflectance), of the retina, were measured. Under infrared observation, the light reflectance of the whole posterior retina decreased (the fundus image became darker) after a flash stimulus (Fig. 1B). The optical signal was calculated as follows: (1) the gray-scale values of the image obtained after the

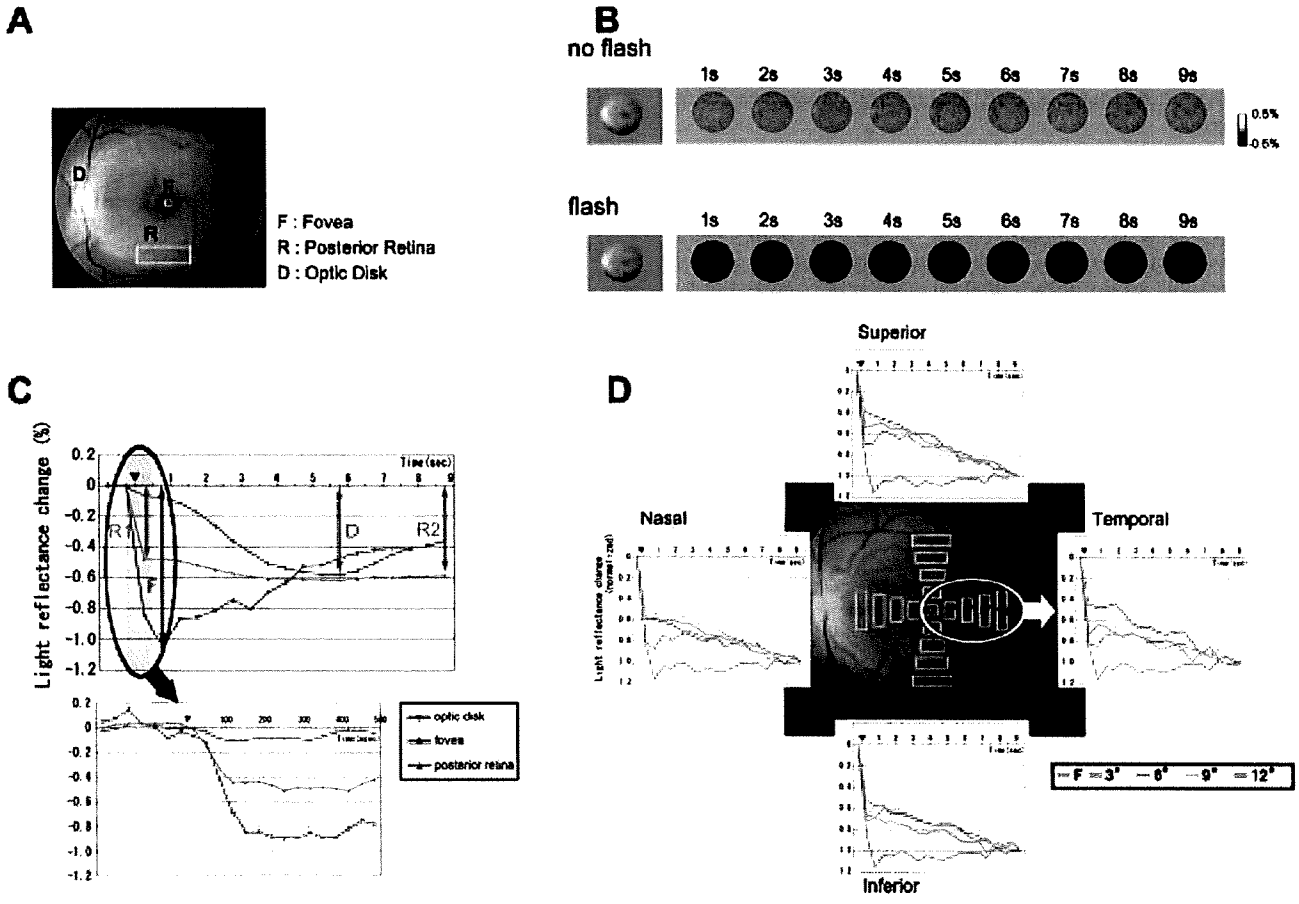

FIGURE 1. Fundus photograph and time courses of intrinsic signals after a flash stimulus. (A) Fundus photograph of normal retina showing the regions analyzed. (B) Time courses of two-dimensional images of ocular fundus showing the light reflectance changes during a 10-second trial without (*top*) and with (*bottom*) a flash stimulus. *Left*: fundus images taken at the beginning of a trial; *right*: light reflectance changes after a flash. Thirty consecutive video frames collected during 1 second were averaged for one poststimulus fundus image. Darkened regions indicate a decrease of light reflectance after the flash stimulus. (C) Plot of the time courses of light reflectance changes in a single trial after a diffuse flash stimulus in the three locations shown in (A). The time after the flash is shown on the abscissa. *Arrowhead*: point of delivery of the flash. Each point is the average of 15 video frames collected during 0.5 second of the light reflectance changes. *Colored arrows*: **F** signal at fovea, **D** signal at the optic disc and **R1** and **R2** signals in the nonfoveal posterior retina. The time course of the reflectance changes during the first 500 ms after a flash is shown in the *bottom* graph where each point is the average of two video frames during one-fifteenth of a second. (D) Time courses of light reflectance changes in a single trial after a diffuse flash, measured at the fovea and four different regions within 12° from the fovea in each quadrant. Amplitudes are indicated as values relative to the light reflectance changes at the end of each trial (1.0). The four regions tested in each quadrant are indicated as distances from fovea (3°, 6°, 9°, and 12°).

stimulus were divided, pixel by pixel, by those obtained during a 0.5-second period before the stimulus, and (2) this ratio was rescaled to 256 levels of gray-scale resolution to show the stimulus-induced reflectance changes.

A deterioration of the signal can be caused by movement artifacts: small involuntary eye movements, blood pressure pulsations, and respiration-associated movements. Eye movements are the most serious artifacts because a different fundus position would be analyzed during the pre- and poststimulus periods. However, these artifacts can be minimized by giving sufficient amounts of muscle relaxants to block eye movements. Pulsations cause small movements of the retinal arteries and optic disc. However, the pulsation-derived reflectance changes are less than one tenth of the stimulus-induced reflectance changes and are almost negligible if 15 video frames are averaged during the 0.5 seconds (Fig. 1C).

Artifacts from respiratory movements produced large light reflectance changes, which were 20% to 40% of the stimulus-induced reflectance changes. This artifact is due to changes in blood flow or volume and periodic back-and-forth movement of the eye, synchronized with the respiration. In our recordings, the respiration-induced artifacts were significant, and the respirator had to be stopped during a recording period of 10 seconds. With the respirator stopped, we could record stable intrinsic signals whose quality was sufficient to map the retinal reflective changes in a single trial without either averaging or offline analysis with realignment of the images.[6]

To measure the time course of the signal changes, we recorded two trials under the same conditions and averaged the results. We found that each trial had to be recorded with at least a 20-minute interval to allow a recovery of the signal production from the previous stimulus. Thus, in experiments where the 11 stimulus intensities were recorded, up to 7 to 8 hours were necessary to record two trials under each condition. Because only two recordings were obtained under each recording condition, the type of statistics that could be used was limited. Unlike ERG recordings, the amplitudes of intrinsic signals are vulnerable to changes in the heart rate, blood pressure, and corneal reflectance. Because it is critical for quantitative comparisons to measure the responses under the same physiological conditions, averaging many trials was impractical for our experimental protocol.

Electroretinograms

A bipolar contact lens electrode (Mayo, Aichi, Japan) was used to record the ERGs. The ERGs were amplified ×10,000 and the band-pass filters were set at 0.3 to 500 Hz (Power Lab; AD Instruments, Colorado Springs, CO). A 45° brief white xenon flash stimulus was delivered through the same observation optical system to stimulate the retina while the fundus was monitored with the infrared observation light. As in intrinsic signal imaging, two ERGs were recorded for each recording condition and were averaged.

Recording Conditions

Initially, we compared the responses of the intrinsic signal images and the ERGs evoked by the same diffuse flash stimulus under different recording conditions: (1) flash intensities (Supplementary Fig. S1; all supplementary figures are online at http://www.iovs.org/cgi/content/full/48/6/2903/DC1), (2) flash intervals (Supplementary Fig. S2), and (3) background luminance (Supplementary Fig. S3). Second, we stimulated the retina focally and measured the intrinsic signals in the stimulated and nonstimulated regions.

Flash Intensity. The maximum intensity of the xenon flash was 308.0 cd · s/m^2, and neutral density filters were used to attenuate the intensity. The intrinsic signals and ERGs were recorded over an 8.8-log-unit range in 11 steps (−8.8, −7.8, −6.7, −6.0, −4.8, −3.7, −3.0, −1.8, −0.7, −0.3, and 0.0). The recordings were performed consecutively with 20 minutes between changes in the intensity under both the dark- and light-adapted conditions. In the light-adapted condition, each recording was followed by 10 minutes of light adaptation. For light adaptation, an 80-mm diameter white polyethylene ball, similar to a ping-pong ball, was cut in half and placed between the fundus camera and the eye. The ball was illuminated by two halogen lamps through fiber optics so that the luminance in the center was 30 cd/m^2. Although the luminance of the ball was not spatially uniform, we believe, that this did not affect the results of the experiments significantly because the luminance at 10° from the center varied by only 94.4% to 103.0% relative to the center (100%). The illuminated ball was removed a few seconds before each recording trial.

Flash Intervals. The intrinsic signals and ERGs were recorded after 0.5, 1.0, 3.0, 5.0, 10, 30, and 60 minutes of flash-to-flash intervals. After a 30-minute recovery period, the posterior retina was first bleached by a strong white flash stimulus (bleaching flash: −0.3 log units: 1.54×10^2 cd · s/m^2), followed by the various interval times listed. The intrinsic signals and ERGs were then measured with a flash (recording flash) of the same intensity as the bleaching flash.

Background Luminance. The background luminance was changed from 0 to 200 cd/m^2 in five steps, to examine the effects of background luminance on the intrinsic signal images. For the intrinsic signal imaging, a strong white flash (−0.3 log units: 1.54×10^2 cd · s/m^2) was used as a stimulus. For the ERGs, the flash intensity, that evoked the maximum b-wave (−3.0 log: 3.08×10^{-1} cd · s/m^2) was used.[17] Each recording trial was recorded with a 20-minute interval.

Finally, a focal stimulus was projected onto the retina by inserting a transparent film in the optical pathway of the Xenon strobe. The film was placed at a point that was conjugate to the retina. The shape and size of the stimulus on the retina was determined by the pattern on the film.

Results

The time course of the intrinsic signals evoked by a brief flash stimulus was different for different regions of the ocular fundus. A representative flash-evoked response from a single trial under dark-adapted condition is shown in Figure 1C. The reflectance changes at the fovea were rapid and reached a negative peak (darkening) within 100 to 200 ms after the flash. The darkening then gradually returned to the prestimulus baseline. The changes in the signals at the optic disc were much slower and reached a peak 5 to 6 seconds after the flash. The signals in the nonfoveal posterior retina were composed of both fast and slow components. The light reflectance decreased rapidly within 100 ms (flexural point) and then gradually decreased, reaching a trough 5 to 6 seconds after the flash. As shown in Figure 1D, the time course of the intrinsic signals of the posterior retina was approximately the same over the whole field except for the small central region within the avascular foveal area (i.e., 300 μm in diameter).[18] The light reflectance at the fovea did not decrease after the initial negative peak.

There is some evidence that the signal in the nonfoveal posterior retina is composed of at least two components. First, the threshold of the fast and slow signals were different by 4 log units of flash intensity in the dark-adapted condition and 1 or 2 log units in the light-adapted condition, as shown by the following results. Only the later phase of the slow reflectance change was observed after a dim flash stimulus. Second, only the amplitude of the late phase of the signal is vulnerable to changes in the heart rate, whereas that of the early phase is not changed (see the Discussion section).

To analyze the flash-induced intrinsic signals and electrophysiological responses, we used the value of the initial peak of light reflectance change at the fovea (**F**: 15 × 15 pixels), the value at the flexural point of light reflectance change (**R1**), or the value at the end of the recording trial (**R2**) in the inferior retina (60 × 40 pixels), and the lowest value of light reflectance at the optic disc (**D**: ~70 × 50 pixels; Fig. 1C).

Stimulus Intensity

The intrinsic signal images and ERGs recorded after a diffuse flash are shown in Figures 2A, 3A, 4A, and 5A, and the intensities of the intrinsic signals and ERG amplitudes are shown in Figures 2B, 3B, 4B, and 5B.

Under dark-adapted conditions (Fig. 2), the amplitudes of a- and b-waves of the ERGs increased as the stimulus intensity increased.[19] The intrinsic signals of **D** and **R2** had the same threshold as that of the b-wave, and their amplitudes also increased as the intensity increased. The amplitudes of **D** and **R2** reached a plateau at −6.0 log units and did not change significantly with higher intensities. **R2** increased again with higher flash intensities over −0.3 log unit in monkey (M)1 and −0.7 log unit in M2.

The threshold of **R1** was higher than that of **D**, **R2**, and the ERG a-wave. The amplitude of **R1** increased gradually with increasing flash intensities. The amplitude of **F** also increased with increasing flash intensities, but its threshold was higher than that of any of the other intrinsic signals.

Under light-adapted conditions (30 cd/m^2), the amplitudes of the a- and b-waves increased progressively with increasing flash intensities, but that of the b-wave decreased with intensities higher than −3.0 log units, due to the photopic hill phenomenon (Fig. 3).[17] The thresholds of **D** and **R2** of the intrinsic signal images were higher than those in the dark-adapted condition by 2.1 log units in M1 and 2.8 log units in M2. The thresholds of the **D** and **R2** signals and ERG a- and b-waves were the same in M1.

The threshold of **R1** was the same in both dark- and light-adapted conditions. In both conditions, the threshold of **F** was the same in M1 but was 0.7 log unit lower than that in M2 in the dark-adapted condition. What was striking was that the amplitude of **R2** was smaller with brighter flashes in M2 under light-adapted conditions and the light reflectance change became approximately zero at −0.3-log-unit intensity. There was a tendency for the amplitude of the **R2** signal to decrease with intensities that were 1.0 to 2.0 log units higher than the threshold of the **R1** signal.

Effect of Changes in Flash Intervals

After a bleaching with a bright flash, the amplitudes of the a- and b-waves were reduced and the amplitudes increased with increasing time in the dark (Fig. 4).[20,21] The recovery of the ERG amplitudes appeared slower than that in other studies because our flash intensity was 1.7 log units more intense than that of the ISCEV (International Society for Clinical Electrophysiology of Vision) standard flash.

For the intrinsic signals, only the **F** signal had a pattern similar to that of the ERGs (i.e., the amplitude increased gradually with longer intervals in the dark after the preceding flash). **D**, **R1**, and **R2** had peaks at 3 to 5 minutes after the preceding flash, and the amplitudes decreased at 10-minute intervals. These findings indicate that the source of the intrinsic signals of the optic disc and the nonfoveal posterior retina are different from that of the fovea.

Effect of Background Luminance

The amplitudes of the a- and b-waves decreased progressively as the background illumination increased (Fig. 5).[22,23] The amplitudes of **D** and **R1** of the intrinsic signals under light-adapted conditions (10–200 cd/m^2) were 45% to 65% as large as that in the dark-adapted condition in M1 and 60% to 80% in M2. The amplitude of **F** in the light-adapted condition was approximately 90% as large as that in dark-adapted condition in M1 and 80% to 100% in M2.

In contrast, the **R2** signal, was markedly decreased under light-adapted conditions. Even with a weak background of 10 cd/m^2, **R2** was decreased by approximately 90% in M2, and an increase of 20% in light reflectance was observed in M1. This

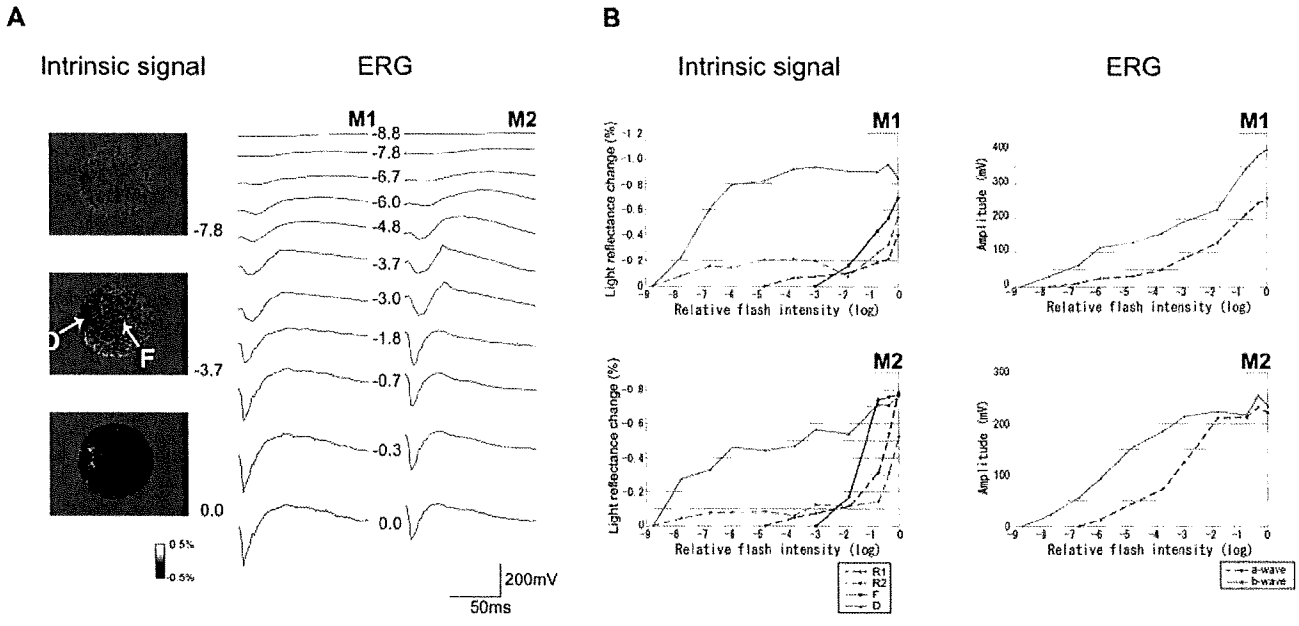

FIGURE 2. Intrinsic signal images and ERGs after a diffuse stimulus in dark-adapted conditions. (**A**) Fundus images of the intrinsic signals (*left*) and ERGs (*right*) after a diffuse flash in the dark-adapted condition with stimulus intensities from −8.8 to 0 log units. *Left*: intrinsic signal images from a single trial averaged from 5.0 to 8.0 seconds after the flash. The darkened region in fundus images indicates light reflectance decrease after the flash. The ERGs recorded from monkeys M1 and M2 are shown. The relative log flash intensity responses to the maximum flash are indicated. D: optic disc, F: fovea. (**B**) *Left*: amplitudes of **R1**, **R2**, **F**, and **D** of the intrinsic signals in response to increasing flash intensities are shown as light reflectance changes for M1 and M2. *Right*: the amplitudes of the ERG a- and b-waves in response to the same stimulus series. Note that negative values of light reflectance changes are plotted to indicate the strength of intrinsic signals.

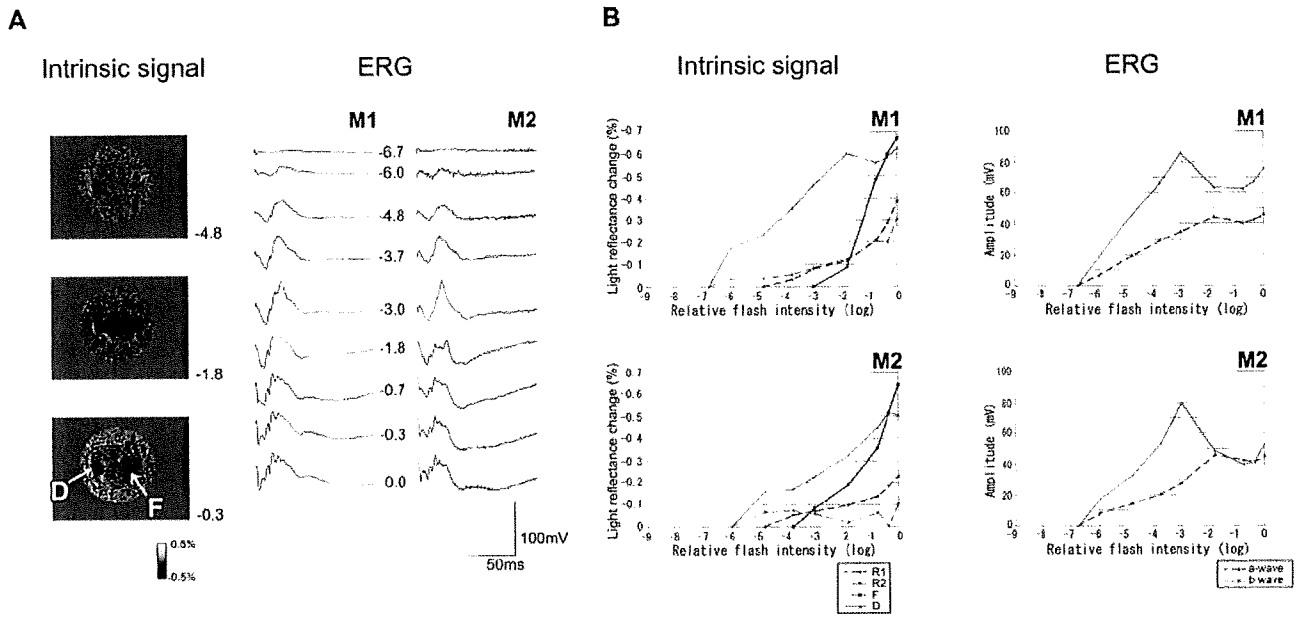

FIGURE 3. Intrinsic signals and ERGs after a diffuse stimulation under light-adapted conditions. (**A**) Fundus images of the intrinsic signals (*left*) and ERGs (*right*) after different stimulus intensities (−6.7–0-log-unit intensity). Representative signal images for a single trial averaged from 5.0 to 8.0 seconds after a flash are shown. (**B**) Amplitudes of **R1**, **R2**, **F**, and **D** of the intrinsic signals and the a- and b-waves of the ERGs in response to various flash intensities are as described in Figure 2.

means that the posterior retina appeared brighter after a flash at the later phase of a recording trial with bright background illumination.

Responses at Optic Disc

We have shown that the thresholds of the intrinsic signals at the optic disc were comparable to the threshold of the ERG b-waves and that even a dim stimulus can evoke a strong signal at the optic disc (Fig. 2). To determine the contribution of blood-related changes to the intrinsic signals at the optic disc, we measured the responses from different regions within the optic disc (Fig. 6A): (1) the central region where the central retinal artery and vein run perpendicular to the imaging plane (Center), (2) over the superior branch of the central retinal artery (Artery), (3) over the superior branch of the central retinal vein (Vein), (4) temporal and nasal regions where large

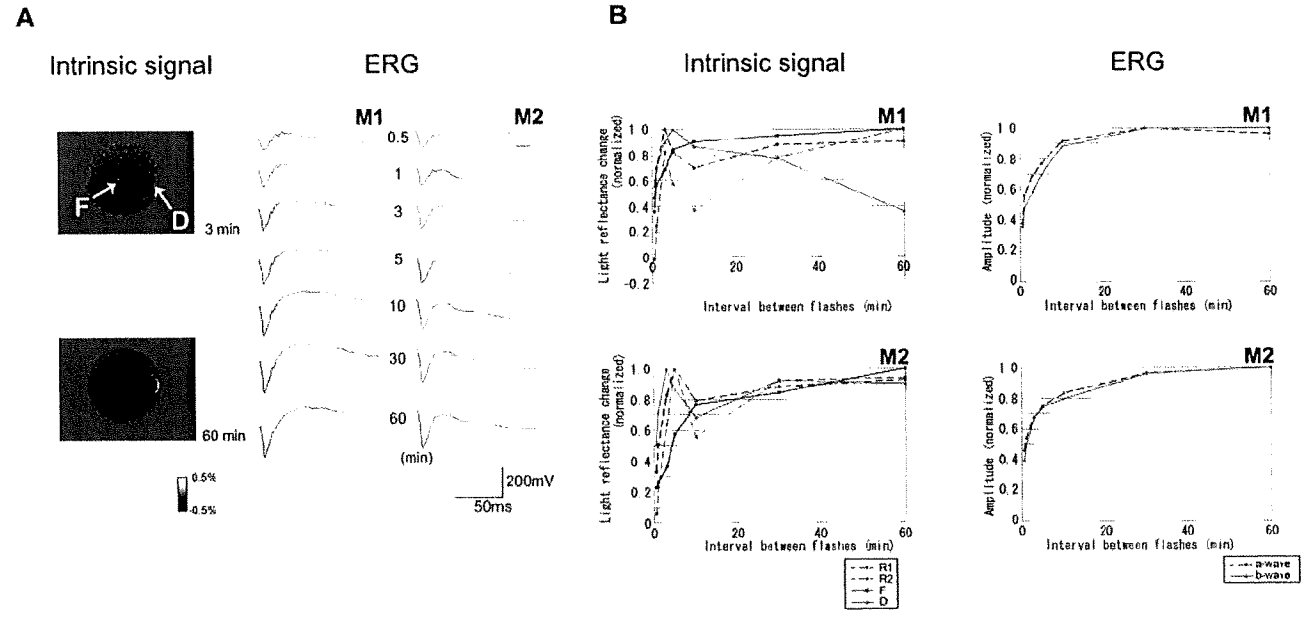

FIGURE 4. Intrinsic signals and ERGs after a diffuse stimulus recorded at different times after bleaching. (**A**) Fundus images of intrinsic signals (*left*) and ERGs (*right*) evoked by a diffuse flash (−0.3 log unit) at different intervals (0.5–60 minutes) after a bleaching flash at the same intensity. Representative images from a single trial averaged from 5.0 to 8.0 seconds after a flash are shown. (**B**) Amplitudes of **R1**, **R2**, **F**, and **D** of the intrinsic signals and a- and b-waves of the ERGs at different flash intervals. Amplitudes are relative to the maximum for each signal component.

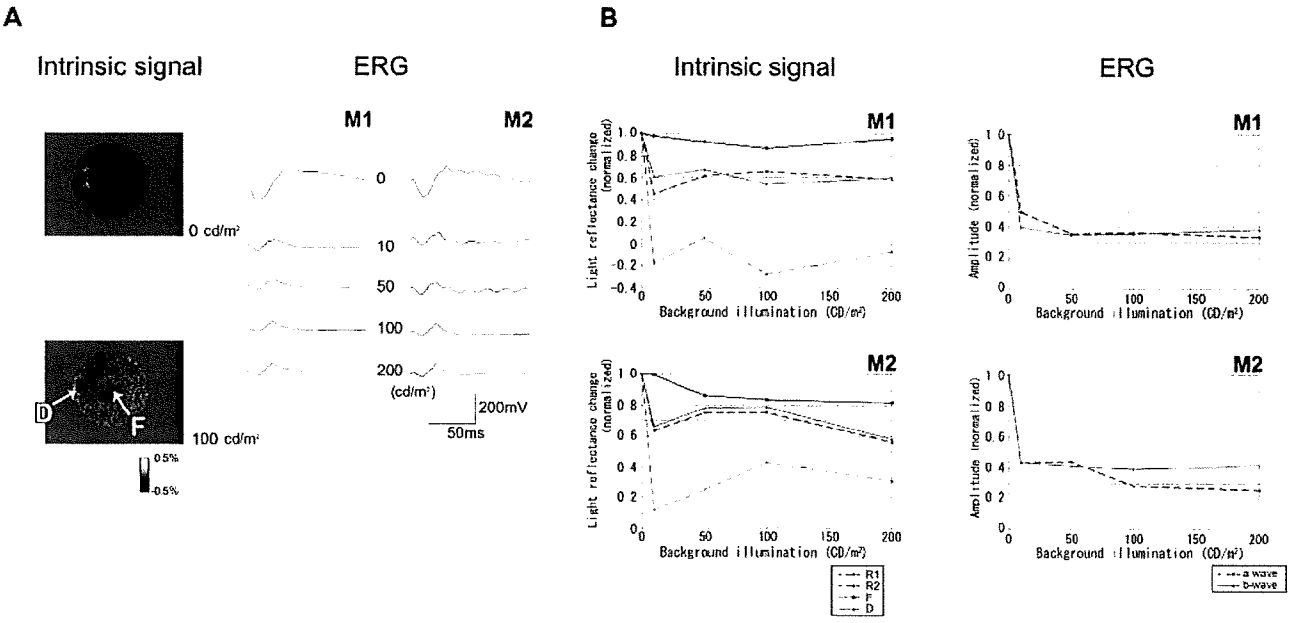

FIGURE 5. Intrinsic signal images and ERGs after a diffuse flash stimulus on different background luminances. (A) Fundus images of intrinsic signals (*left*) and ERGs (*right*) after a diffuse flash on different background luminances (0–200 cd/m^2). Representative signal images in a single trial averaged from 5.0 to 8.0 seconds after a flash (−0.3 log unit) are shown. For the ERGs, a flash with the maximum intensity which evoked a b-wave without photopic hill phenomenon was used (−3.0 log units). (B) Amplitudes of **R1**, **R2**, **F**, and **D** of the intrinsic signals and the ERG a- and b-waves with different background luminances. Amplitudes are relative to the maximum for each signal component.

vessels are not present (Temporal and Nasal), and (5) the entire optic disc (D). A diffuse flash of −0.7 log unit intensity was used for stimulation, and 17 consecutive trials with 3-minute intervals were averaged.

The light reflectance changes were especially large in the central region where the central retinal artery and vein pass through the optic nerve (three times larger than that in the whole region; Fig. 6B). The light reflectance changes over the superior branch of the central retinal artery and vein were 1.2 times larger than that of the whole region. Although the size of the intrinsic signals varied in different regions within the margins of the optic disc, the time course at each region seemed to be almost the same (Fig. 6C).

Focal Stimulation

The recording of the focal macular ERG is a technique used to measure the electrical responses in the macula by focally stimulating the macular region.[24,25] Focal flash stimuli can be given to the posterior retina with our recording system, however, our system is not setup to deliver a background illumination to suppress the rod responses and cannot measure the electrical activity in the stimulated region. We have stimulated focal regions of the posterior retina, and compared the time course of the intrinsic signals in both the stimulated and nonstimulated regions in dark-adapted conditions.

First, the macular area including the fovea was focally stimulated with an 8.8° circular stimulus (Figs. 7A, 7B). The light

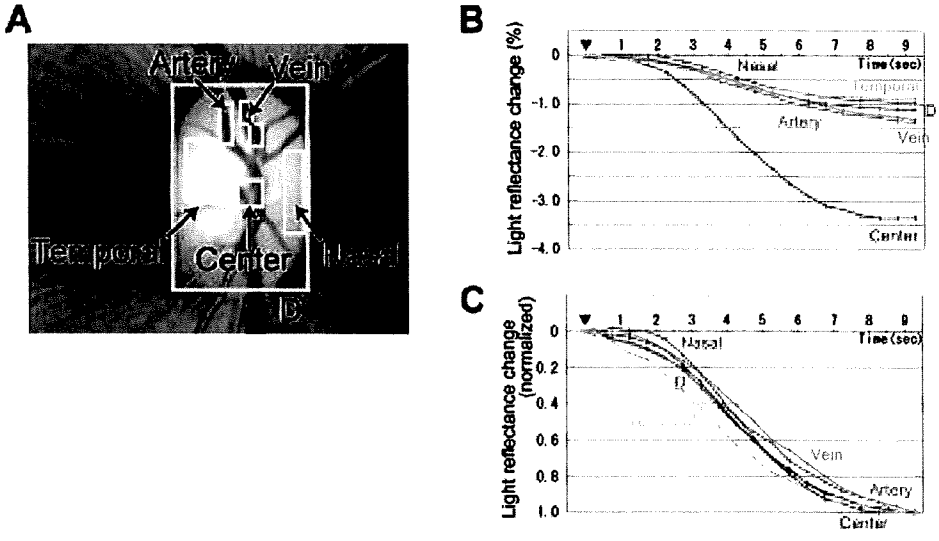

FIGURE 6. Time courses of intrinsic signals evoked by a diffuse flash from six regions of the optic disc. (A) Photograph of the optic disc showing the areas measured. D, entire optic disc; Center, central region where central retinal artery and vein run perpendicularly to the imaging plane; Artery, superior branch of central retinal artery; Vein, superior branch of central retinal vein; Temporal, temporal region, where large vessels are not present; and Nasal, nasal region, where large vessels are not present. (B) Plot of the time courses of the intrinsic signals measured at the six regions of the optic disc, presented as absolute values in light reflectance changes. (C) Plot of the time courses of the intrinsic signals, presented as relative to the maximum for each recording region.

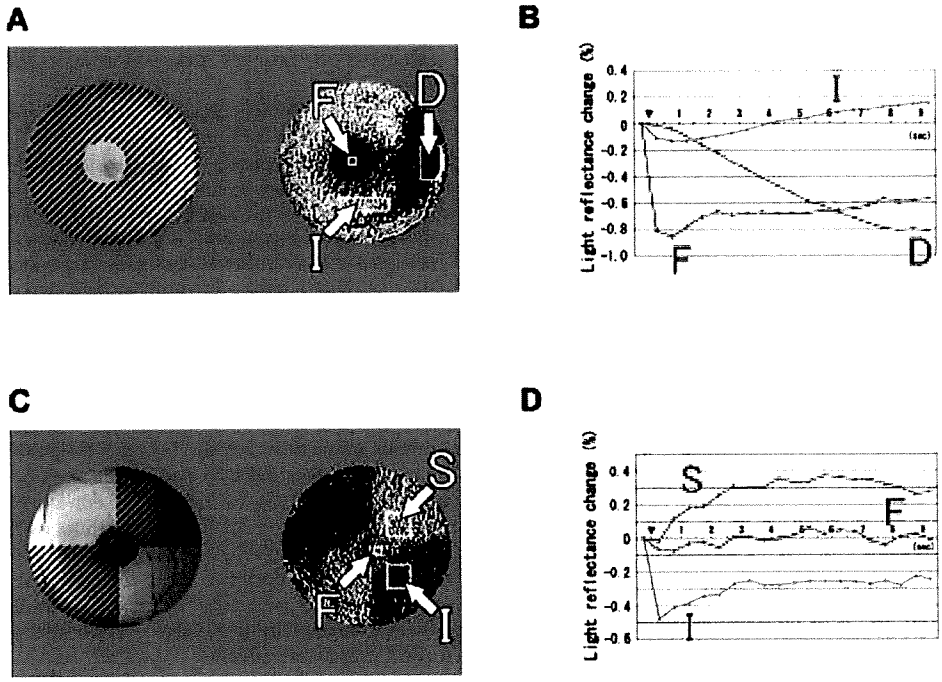

FIGURE 7. Effect of focal stimulus on the intrinsic signals. (A) *Left*: fundus image showing circular focal stimulus in the macula including the fovea (8.8° in diameter). The stimulus was blocked over the *hatched area*. Regions for time course analysis are indicated as F (fovea), D (optic disc), I (inferior retina within the vascular arcade, sparing thick vessels). *Right*: fundus image of intrinsic signal evoked by the focal stimulus, averaged from 6.5 to 9.5 seconds after stimulation. Data from four consecutive trials were averaged. (B) Plot of the time courses of light reflectance changes in a single trial after a focal flash at the three locations shown in (A). (C) *Left*: fundus image showing mosaic-like focal stimulus in the posterior pole, sparing central 8.0° including fovea. Regions for time course analysis are indicated as F (nonstimulated fovea), S (stimulated superior retina), and I (nonstimulated inferior retina). Right: two-dimensional image of intrinsic signal evoked by the focal stimulus, averaged from 6.5 to 9.5 seconds after stimulation (single trial). (D) Plot of the time courses of light reflectance changes in a single trial after a focal flash at the three locations shown in (C).

reflectance in the stimulated region decreased (Fig. 7B; F) and the region that darkened exactly matched the location of the stimulus (Fig. 7A). In contrast, the region without stimulation became brighter at the later phase of a recording trial (Fig. 7B; I). When two quadrants of the posterior retina were stimulated with the macula spared, the intrinsic signal showed exactly the same darkening pattern as the shape of the stimulus (Fig. 7C). The stimulated posterior retina (I) showed a negative **R1** and negative **R2** signal (i.e., a darkening; Fig. 7D). The nonstimulated area of the posterior pole (S) was brighter (positive **R2**), which is usually not observed under other recording conditions. The fovea (F), where the stimulus was masked, did not show any light reflectance changes after the flash.

DISCUSSION

The origin of the intrinsic signals in the cerebral cortices has been extensively investigated; however, most of the studies have dealt with the deoxygenation of hemoglobin.[8,9,11] The standard hypothesis is that the intrinsic signals in the cerebral cortex arise from light reflectance changes due to the many metabolic changes after neural activation. For example, the intrinsic signal measured at 570 nm is dominated by changes in the blood volume in the capillaries: That at 600 to 650 nm is dominated by the changes in the deoxygenation level of hemoglobin, and that in the infrared region is dominated by changes in tissue light scattering. Although different metabolic changes are highlighted at different wavelengths, the optical responses obtained at these wavelengths had nearly the same spatial pattern of activation as that of the activated neurons.[9,26] Whatever wavelength was chosen for the measurement of reflectance, the most critical premise for evaluating the intrinsic signal has been that it is the darkening (i.e., a decrease in light reflectance), that correlates with the local neural activity. This is more or less true of the intrinsic signal images in the retina[3]; however, the spatial distribution of the signals appeared to be more complicated when the retina is focally stimulated.

Our goal was to find out what each signal component represented by comparing the intrinsic signals with the ERGs recorded under the same conditions. Although the spatially localized responses of the intrinsic signals cannot be directly compared with the responses in full-field ERGs, this comparison may provide us with some keys to determine the possible mechanisms of the production of the intrinsic signals, because the neuronal mechanisms of the production of the ERGs have been well investigated.

It is important to understand that, in principle, the intrinsic signals are not necessarily produced by photoreceptors: There may be differences in the site of the photoreceptor and the site for producing the signals. The light reflectance changes reflect the summation of the stimulus-evoked metabolic changes happening in the 10 retinal layers, each of which may produce signals with different characteristics. The same difficulty arises when the origin of the different components of the ERGs is investigated. Thus, the type of activated photoreceptors and

the location where the signal is produced should be carefully separated.

D Signals

The threshold of the **D** signal was comparable to that of the ERG b-wave in the dark-adapted condition (Fig. 2).[19] The threshold was higher by 2.0 to 3.0 log units under light-adapted conditions (Fig. 3),[17] and the amplitude was greatly decreased in the presence of background illumination (Fig. 5).[22,23] These results indicate that the **D** signal evoked by a dim flash (weaker than −5.0 or −6.0 log units) under dark-adapted conditions reflects the activation of rod photoreceptors, and those evoked by stronger flash reflects the activation of both cone and rod photoreceptors. The **D** signal evoked under light-adapted conditions reflects mainly the activation of the cone photoreceptors.

The time course of the **D** signal is slow and is probably produced by a flash-induced blood volume or flow increase. The decrease in light reflectance is due to the increased light scattering of the red blood cells. Previous studies have shown that flashing lights can increase the blood flow at the optic disc of humans and cats.[27,28] An increase in blood volume is known to decrease the reflectivity of tissues due to the increased scattering of light by the red blood cells in the blood vessels.[11,29] Although a change in the blood volume or flow was not directly measured in this study, the results of measurements of the intrinsic signals on the optic disc support this (Fig 6). In the central region of the optic disc occupied by the central retinal artery and vein, the reflectance changes were three times greater than in other regions, although the time course was almost the same at any locations within the optic disc. The light-scattering changes induced by changes in the blood volume or flow may be most effectively observed when the vessels are perpendicular to the imaging plane. Even in the white regions (Fig. 6A, Temporal and Nasal), where large vessels are not present, the light reflectance changes showed a time course similar to that in the central region (Fig. 6C) indicating that this signal is derived from the blood volume or flow changes in the capillaries. As for the question of whether the blood volume or flow contributes more significantly to this signal, we do not have any evidence to conclude which has the greater role and recommend that the mechanism of blood-related light scattering changes be thoroughly investigated.

Neural activity in the optic nerve causes shrinkage of the extracellular space due to cellular swelling, and this was detected optically by intrinsic signal imaging in rats.[30,31] We believe that part of the scattering changes may be due to swelling of the axons of the ganglion cells or of the glial cells. Its contribution to the whole intrinsic signal, however, may be masked by the relatively large reflectance changes due to changes in blood volume or flow. A contribution by the changes in deoxygenated hemoglobin concentration in the capillaries to the intrinsic signal may also exist, although it is believed to be negligible compared with that of tissue light scattering under infrared light observation.[9,11]

R2 Signals

The properties of the **R2** signal were similar to those of the **D** signal, except that **R2** became very small and in some cases became positive under light-adapted conditions (Figs. 3B, 5B). The **R2** signal is probably a complex of different components and origins because the posterior retina is a complex layered structure, and its signal properties cannot be explained simply by the blood volume or flow changes in the capillaries.

We suggest that the inner retina may be the main contributor to the **D** and **R2** signals because this type of slow signal was not observed at the fovea, which lacks the inner retinal layers including the blood vessels.[18] Our data did not allow us to determine which type of cells contribute the most to the flash-evoked responses observed at the optic disc or the posterior retina.

We attempted to keep the systemic condition of the anesthetized monkeys as constant as possible during the data acquisition. In some trials, however, the heart rate became unstable and rapid changes occurred during consecutive recordings under the same stimulus conditions. For example, the heart rate increased from 120 per minute to 140 per minute during two consecutive trials in one monkey. Although such data obtained under unstable conditions were discarded, we did note that it was always the amplitudes of **D** and **R2** signals that were affected by the changes in the heart rate. In contrast, the amplitudes of **F** and **R1** signals were much less affected by changes in heart rate (data not shown). This observation suggests that the **D** and **R2** signals are related to blood-induced changes more than are the **F** and **R1** signals.

It was interesting that the amplitudes of **D** and **R2** signals were largest with 3- to 5-minute flash intervals (Fig. 4B). This finding is very different from the results of ERGs. It is possible that the mechanism by which neural activity is converted to the vascular response (i.e., neurovascular coupling)[32,33] is most effectively activated when the stimuli are given repeatedly at intermediate intervals. This possibility should be investigated more extensively.

F Signals

The **F** signal, which is the average of light reflectance changes within the central 300 μm in diameter was faster than the **D** and **R2** signals and reaches its peak within 100 to 200 ms (Fig. 1C). The threshold for the **F** signal was much higher than any other signals and was the same in both dark- and light-adapted conditions. The characteristic anatomic structure of the fovea (viz., the absence of rod photoreceptors, capillaries and other inner retinal layers), indicates that the **F** signal reflects the activation of cone photoreceptors under any recording conditions.

The light-scattering changes due to the microstructural changes after activation of the cone photoreceptors are probably the source of the **F** signals because the foveal avascular region is free of capillaries and not subject to the changes in hemoglobin concentration or blood volume after neural activation.[18] Recent functional OCT studies using blood-free slice preparations showed that the reflectance in the photoreceptor layer is strongly changed by neural activation followed by microscopic morphologic changes.[34,35]

R1 Signals

The amplitude of the **R1** signal increases with an increase in stimulus intensity under both dark- and light-adapted conditions as did the **F** signal. The threshold of **R1** lies between the threshold for the optic disc and fovea and was the same under both dark- and light-adapted conditions. This leads us to think that cone photoreceptors mainly contribute to the **R1** signal, because bleaching of rods in the bright condition did not change the **R1** threshold. It is difficult to assume, however, that rod and cone photoreceptors play different roles in light reflectance changes.

The property of the **R1** signal is complicated in another way. The abrupt darkening after a flash may well be explained by the photoreceptor responses like the **F** signal, but the results in Figure 4B strongly suggest that **R1** share the same signal origin with **D** and **R2** signals: The amplitude of **R1** signal did not increase with longer interstimulus intervals, but attained a maximum with 3- to 5-minute intervals as with the **D** and **R2** signals. We suggest that the **R1** signal is produced not

TABLE 1. Properties of Four Components in Retinal Intrinsic Signals

	Signal Components			
	D	**F**	**R1**	**R2**
Time to the peak in amplitude	Slow	Fast	Fast	Slow
Threshold in dark adaptation (log unit) (ERG a-wave, −6.7; b-wave, −7.8)	−7.8	−1.8	−4.8	−7.8
Threshold shift in light adaptation	++	−	±	++
Increase in amplitude with shorter flash intervals (3–5 min)	+	−	+	+
Decrease in amplitude by light adaptation	+	±	+	++
Possible sources				
Contribution of blood-related light reflectance changes	++	−	?	++
Contribution of inner or middle layer	++	−	+?	+
Contribution of outer layer	−	++	+?	+?

only by photoreceptors but also by other inner or middle layer structures, although our data do not provide any evidence for the exact origin.

A summary of the various properties in four signal components is shown in Table 1.

Focal Stimulation

Focal stimulation of the retina is one way to evaluate local neural activity in a dysfunctional retina and has been applied clinically with the focal macular ERG.[24,25] The intrinsic signals measured with focal stimuli showed that this technique can also be used to study local responses. The focally stimulated region showed a decrease in the light reflectance after the stimulus, and this darkened region exactly matched the location of the focal stimulus (Figs. 7A, 7C). It was striking that the nonstimulated posterior pole showed a slow light reflectance increase after a fast light reflectance decrease (Fig. 7B; I). In another case, the nonstimulated posterior pole showed only a light reflectance increase (Fig. 7D; S).

The brightening observed in the nonstimulated region in later phase (Fig. 7B, I; Fig. 7D; S) may be explained by (1) some type of horizontal interaction by, for example, horizontal cells, through which stimulated neurons could affect the reflectivity of the neurons outside the stimulated region, or (2) the spatial interaction in the intrinsic signals between the stimulated and nonstimulated regions via an inhomogeneous distribution of capillary blood flow.[8,9,11,36] These explanations, however, do not account for the strong and homogeneous brightening over the whole posterior region triggered by a small focal stimulus.[37] It is possible that the properties of the signals, such as polarity and threshold, are different in different retinal layers, and the difference in signal time course between stimulated and nonstimulated regions would reflect the difference of layers that mainly contribute to the light reflectance changes.

Recently, OCT imaging of neural activity has been demonstrated in the feline visual cortex,[38] isolated frog and rabbit retina,[34,35] and intact rat retina.[39] Functional OCT studies in slice preparations have revealed the complex nature of flash-evoked changes in the reflectances from various intraretinal layers: a decrease of near infrared scattering in the photoreceptor layer and an increase in the ganglion cell layer,[34] or a decrease in the photoreceptor inner segment and increase in the internal plexiform layer and photoreceptor outer segments.[35] Srinivasan et al.[39] first reported the results of functional OCT signals in the intact retina and demonstrated the flash-evoked reflectance increase in the photoreceptor outer segments. In these studies, the increase in light reflectance after a flash was mainly observed in the photoreceptor layer, whereas the decrease was mainly observed in monkey and human retinas.[3,6] This difference in signal polarity may be attributable to the difference in the methods used to measure the reflectances. In addition, other factors, such as the use of sectioned preparations that lack the RPE layer and blood supply, differences among species, and differences in the recording region in the retina, should also be considered.

The light-scattering changes after a flash observed in functional OCT is thought to be derived from the structural changes in the outer segment discs, membrane hyperpolarization, cell swelling, and changes in the composition of the interphotoreceptor matrix.[34,35] These sources can also explain the rapid light reflectance changes (**F** and **R1**) observed in our study. As suggested by our results and those of functional OCT studies of retinal sections, the characteristics of the light reflectance changes after a flash are different in different layers and different retinal locations and may be far more complex than the conventional idea of intrinsic signals mainly investigated in the cerebral cortex.[8,9,11] Interpretation of the retinal intrinsic signal is thus difficult, and maximum care should be taken in choosing the proper recording conditions and which signal is most closely correlated with the neural activities of the retina.

In conclusion, our results showed that the intrinsic signals in the retina are composed of several components of different origins, although the precise cellular mechanisms of signal production were not determined. The sensitivity of intrinsic signal images was high enough to detect weak neural activity in the retina (e.g., the slow signals in the posterior retina and the optic disc were as sensitive as the ERG b-wave in the dark-adapted condition). Moreover, the distribution of intrinsic signals reflects not only the cellular distribution in the retina but the current level of the activities. Although the source of the signal was much more complex than initially thought, by carefully selecting the proper recording condition, this imaging technique may have a potential to estimate the neural responses of different origins and obtain more useful information about various types of retinal disorders with different etiologies than the conventional electrophysiological examinations such as full-field ERGs, focal macular ERGs, and multifocal ERGs.

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今月の話題

新しい網膜機能のイメージング法 —網膜内因性信号計測法—

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光干渉断層計など眼科における画像診断技術は，近年めざましい進歩を遂げてきた。しかし，それらは解剖学的構造の把握を目的としており，視細胞をはじめとする網膜の神経活動を捉えることはできない。筆者らが開発した，新しい網膜機能のイメージング法である網膜内因性信号計測法について概説する。

はじめに

視機能の他覚的評価は，眼疾患の早期発見および治療効果の判定のために基本的かつ重要な課題である。眼科における画像診断技術（イメージング）は近年めざましい進歩を遂げてきた。例えば光干渉断層計(optical coherence tomograph：以下，OCT）は，検眼鏡によって捉えることのできない網膜微細構造の観察を可能にした。しかし，OCTや走査型レーザー検眼鏡(scanning laser ophthalmoscope：以下，SLO）などの画像診断法は，解剖学的構造の把握を目的としており，視細胞をはじめとする網膜の神経活動を捉えることはできない。そのため，網膜機能（神経活動）の他覚的評価のためには，電気生理学的検査である網膜電図(electroretinogram：以下，ERG)が今でも主要な役割を果たしている。

網膜，なかでもその中心に位置する黄斑部は，視力の維持のために重要な部位であり，同時に臨床的にさまざまな疾患が起こりやすい部位でもある。筆者らのグループでは，ERGとまったく異なる方法で神経活動を非侵襲的にイメージングする方法（網膜内因性信号計測法）を開発し，functional retinography（以下，FRG）と名付けた。そして，新たな眼科検査法としての実用化に向けた研究を行っている。

この技術により，従来は不可能であった錐体視細胞，桿体視細胞の機能的マッピングを高い空間解像度で行うことができるようになった。内因性信号計測法（intrinsic signal imaging）が眼底における機能計測に応用できれば，疾患の早期発見や早期治療につながることが期待される。

内因性信号計測法とは

神経活動に伴って神経組織の微細構造や光反射率が変化する現象は古くから知られている[1]。特に，神経活動に伴う代謝変化を光の反射率変化として捉える計測法は内因性信号計測法と呼ばれ，1990年以降この方法を用いて大脳皮質のさまざまな領域で機能的マッピングの研究が行われてきた[2~5]。

実際の計測法は，神経組織をcharge coupled de-

New technique of functional retinal imaging—Functional retinography

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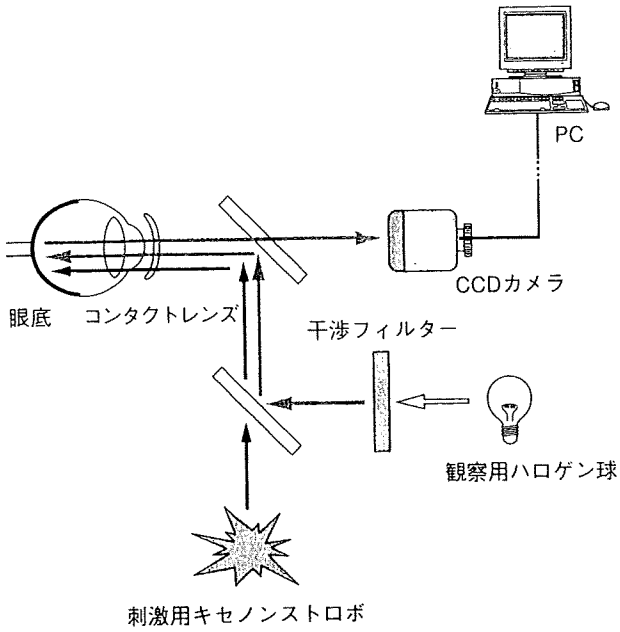

図 1　網膜内因性信号計測装置の概要

vice（以下，CCD）カメラで撮影し，刺激前と刺激後の画像を重ね合わせて比較するという非常に単純なものである。刺激後に画像の明るさが変化している部分が神経活動の起きた領域に相当し，通常は神経活動の高い領域が反射率の低下により暗く見える。信号の起源として主要なものには，神経活動に伴う組織の光散乱変化や血中ヘモグロビン飽和度の変化，毛細血管の血液量の変化がある。

ところで，大脳皮質において光学計測を行うためには脳表面を露出する必要があり，大きな侵襲を伴うものであるが，ヒトの網膜は内因性信号の計測に有利な特徴を備えている。まず，眼球の光学系（角膜，水晶体）を通して直接網膜を観察することができ，眼球全体が光学計測における理想的な観察用チェンバーとなるため手術的な侵襲がない。さらに，網膜外層では視細胞が密に一定方向に並んでいるため，光散乱変化による反射変化を捉えやすい。筆者らのグループではこの技術の眼科分野への応用に早くから着目し，網膜における実験を行ってきた[6~8]。

測定方法

FRG 開発のための動物実験では，ヒトの網膜とほぼ同じ解剖学的構造をもつニホンザル，アカゲザルを用いている。全身麻酔下において眼球運動を停止させ，眼底カメラを改良した観察系を用いて眼底を CCD カメラでモニターする（図 1）。

眼底観察用のハロゲン光は赤外線フィルター（840～900 nm）を透過して眼底後極部を照明する。眼底の観察に近赤外光を用いるのは，可視光で照明すると視細胞の色素褪色変化（bleaching）による眼底の光反射率の変化が起き，内因性信号と反対に明るくなる反応がみられるためである。解像度 640×480 pixels，毎秒 30 フレームの CCD カメラで眼底からの光反射率を持続的に記録する。測定開始から 0.5 秒後に眼底後極部全体を白色キセノンフラッシュ（1 ms）で刺激する。1 回の測定は通常 10 秒間行う。

刺激前 0.5 秒間の平均画像の反射率と，刺激後の画像における反射率との比をピクセル毎に計算し，その比を 256 階調にスケーリングし画像化する。

測定結果

1．明順応および暗順応下での網膜内因性信号

フラッシュによるびまん性刺激によって視細胞が活動すると，網膜全体の反射率が低下し画像では暗く描出される（図 2）。信号強度を疑似カラーで表示すると，明順応下では中心窩に内因性信号の急峻なピークを認め，周辺部に向かって減少するが（図 3a），暗順応下では中心窩に加えて周辺部にドーナツ状のピークを認める（図 3b）。内因

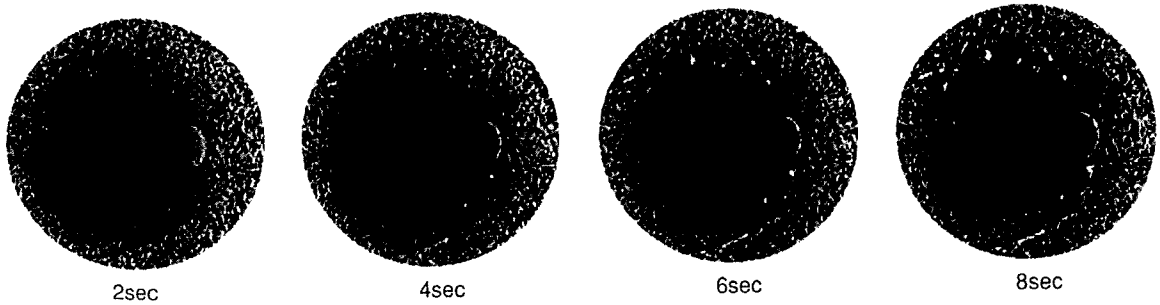

図 2　網膜内因性信号の時間経過

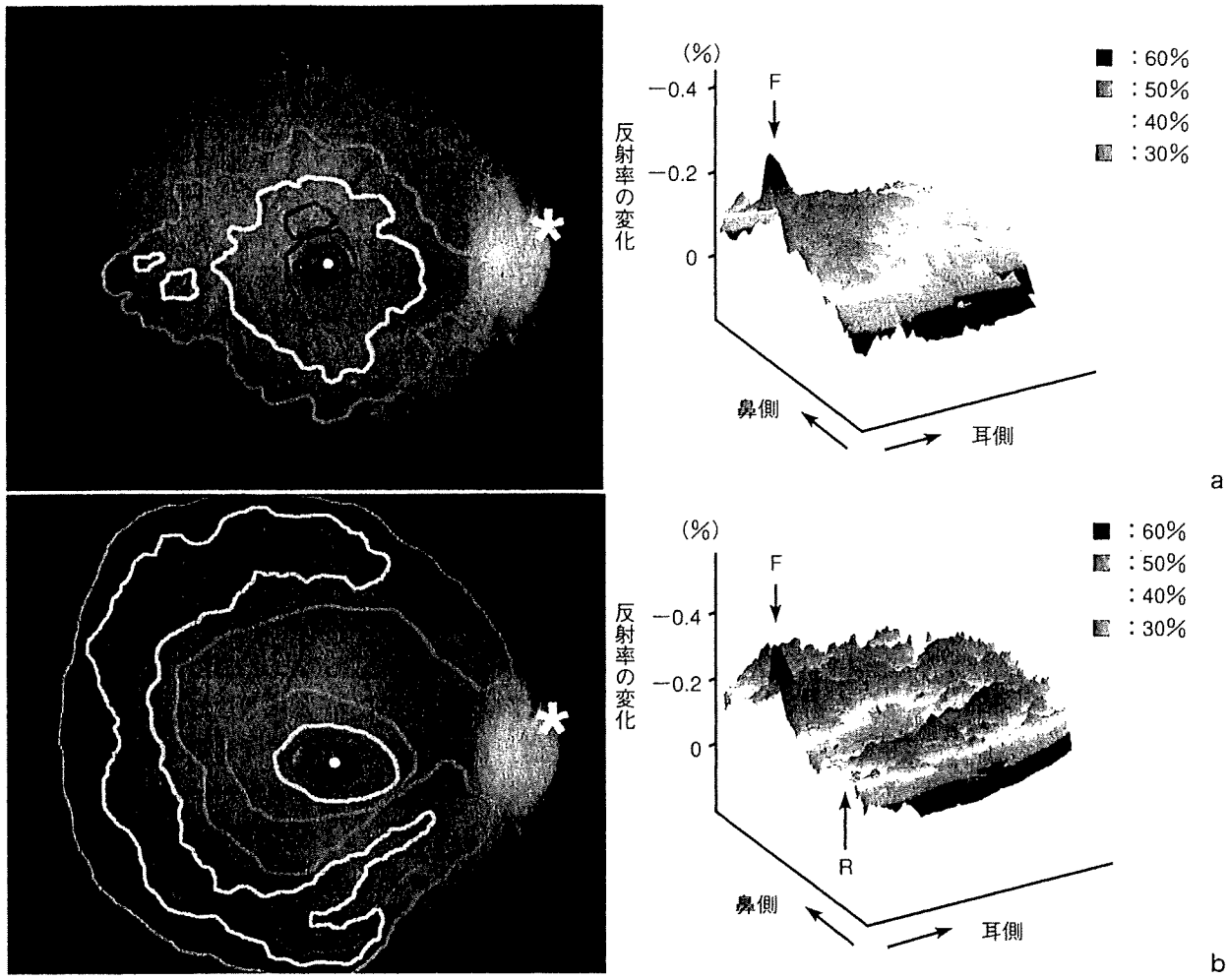

図 3　網膜内因性信号の 3 次元トポグラフィ
a：明順応下，b：暗順応下，＊：視神経乳頭部，F：中心窩，R：rod ring。

性信号のピークは中心窩では錐体視細胞に，周辺部では桿体視細胞の解剖学的な分布[9,10]（rod ring）によく一致しており，網膜内因性信号の発生には視細胞が大きく寄与していると思われる[6]。

2. 網膜部位と signal components

中心窩，後極部，視神経乳頭部の 3 か所で内因性信号を解析した（図 4）。図 5 にそれぞれの部位の反射率変化の時間経過を示す。

中心窩の反応（F signal，15×15 pixels）は網膜の中心，直径 300 μm の平均である。ここでは刺激後 100〜200 ms にピークをもつ早い反応がみられた。

次に視神経乳頭部に注目すると，ここでは網膜面とは異なりフラッシュ刺激後にゆっくりと信号が強くなり，5〜6 秒でピークに達する（D signal，70×50 pixels 以下）。これは刺激後の血流増加を反映した光散乱強度変化と考えられ[7,8,11]，中心動静

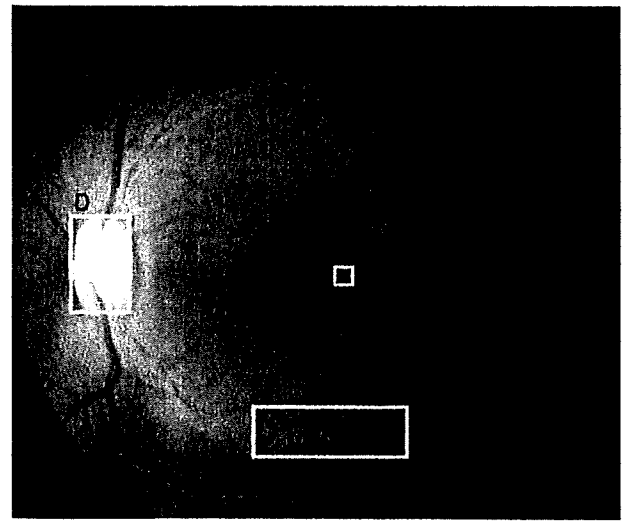

図 4　内因性信号解析部位
F：中心窩，R：後極部，D：視神経乳頭部。

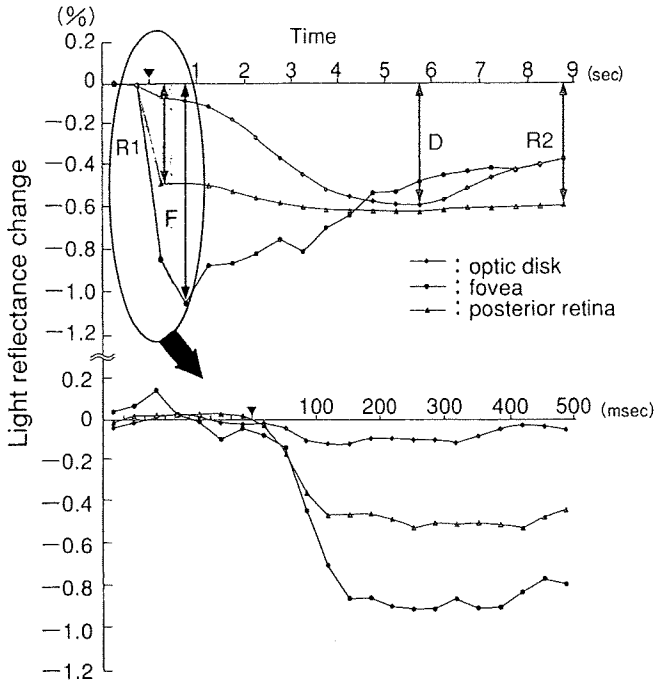

図 5　網膜内因性信号の時間経過
矢頭で示したフラッシュ刺激を 0 秒とした。下段にフラッシュ刺激から 500ms の時間経過を示す。F：中心窩の反応，D：視神経乳頭の反応，R1：後極部の早い反応，R2：後極部のゆっくりした反応を示す。

脈に相当する視神経乳頭中央部に急峻なピークがみられる（図 6）。

黄斑部を含まない後極部網膜の信号（60×40 pixels）は 100 ms 付近を屈曲点として，早い反応（R1 signal）とゆっくりした反応（R2 signal）に分けられた。これらの反応は中心窩を除く後極部全体で同じようにみられ，それぞれ主に網膜外層および内層の活動を反映していると考えられている[7]。

3. 網膜内因性信号と網膜電図の比較

角膜電極を用いて ERG を記録し，同一の刺激に対する FRG と比較した。

1）フラッシュ光強度と信号強度との関係（図 7）

暗順応および明順応の状態で，フラッシュ光強度を変化させていったときの網膜内因性信号，ERG の反応の大きさを比較した。

暗順応状態では，中心窩を除く網膜面の遅い反応と視神経乳頭部の反応閾値が最も低く，ERG の b 波の反応閾値とほぼ一致していた。それに対し，中心窩の反応域値は比較的高く，フラッシュ光強度の増加とともに反応も大きくなっている。臨床検査機器として確立している ERG と同程度に鋭敏であることがわかり，FRG のもつ可能性が改めて示された。

明順応状態では，網膜内因性信号，ERG とも暗順応状態と比べ，反応閾値が 2 log 程度高くなっている。しかし黄斑部の反応閾値のみ，暗順応状態と比較しても変化がない。これは，中心窩の内因性信号は網膜外層の錐体視細胞の神経活動を捉えているとする考えを支持している。

2）暗順応時間と信号強度との関係（図 8）

フラッシュ光刺激の間隔を変化させることで，暗順応時間と網膜内因性信号および ERG の反応の大きさを比較した。ERG では暗順応時間を長くすると a 波，b 波とも反応が大きくなり 30 分以降は一定となっている。網膜内因性信号では中心窩の反応は ERG と同じような時間経過をとるが，網膜面や視神経乳頭部の反応は 1〜3 分の暗順応時間で最も大きな反応を示す。その理由は明らかではないが，フラッシュ刺激により網膜の反応性の高くなる反応至適条件がある可能性がある。

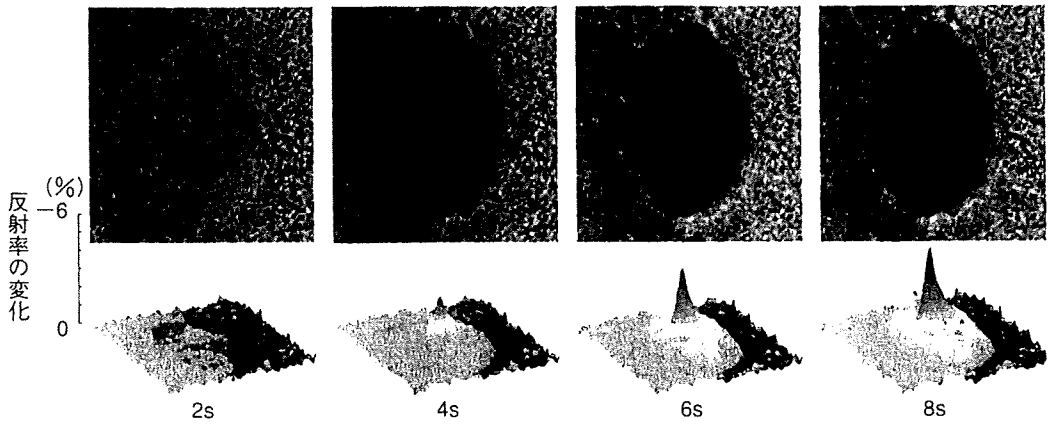

図 6　視神経乳頭部の内因性信号の 3 次元トポグラフィ

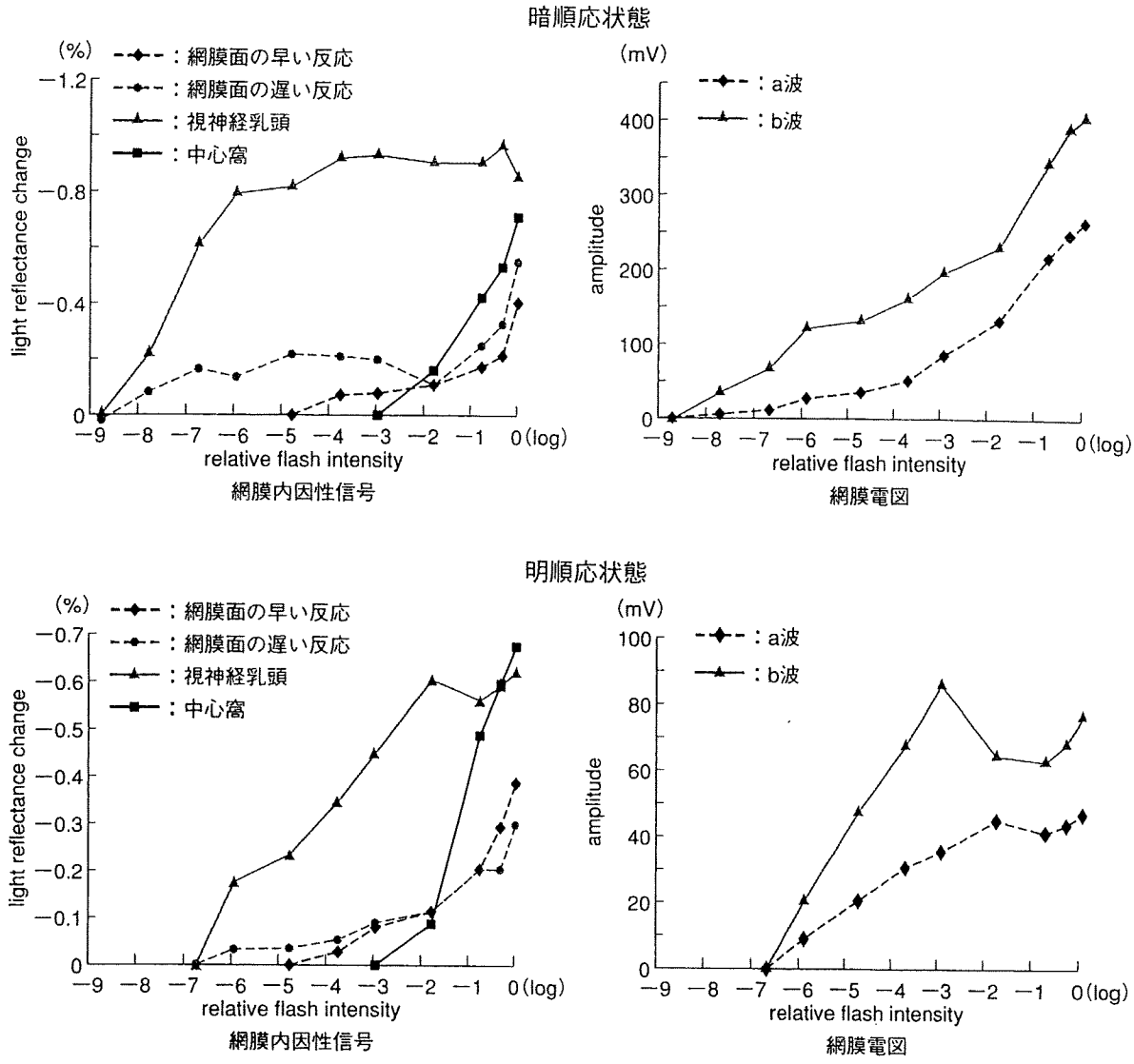

図 7　フラッシュ強度に対する網膜内因性信号と網膜電図の反応の大きさ

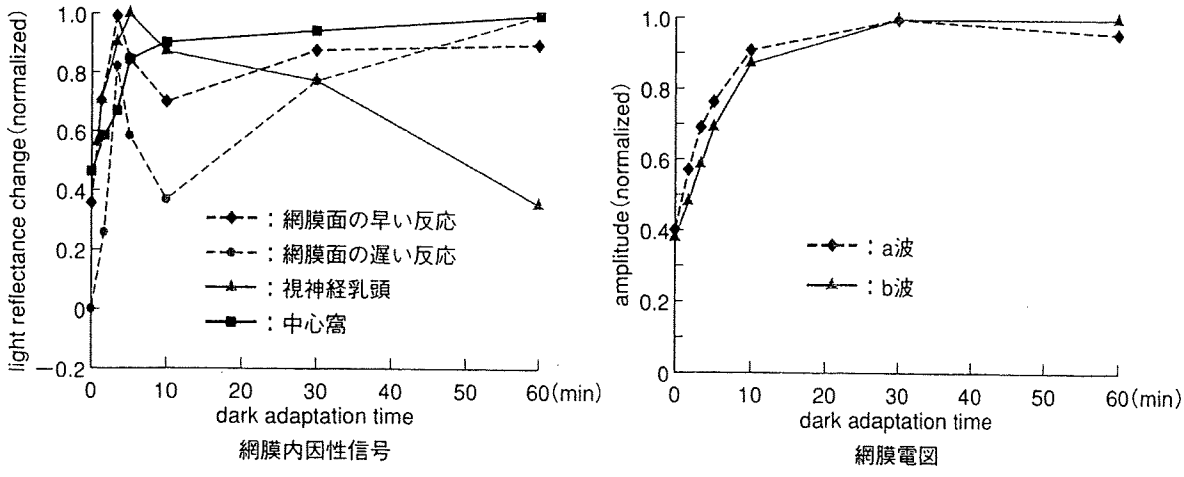

図 8　暗順応時間を変化させたときの網膜内因性信号と網膜電図の反応の大きさ

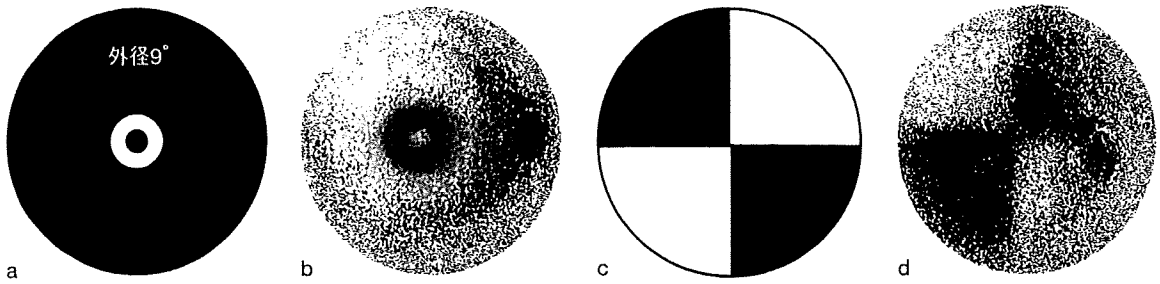

図 9　局所刺激による網膜内因性信号
a，c：フラッシュ刺激の形状，b，d：網膜内因性信号。

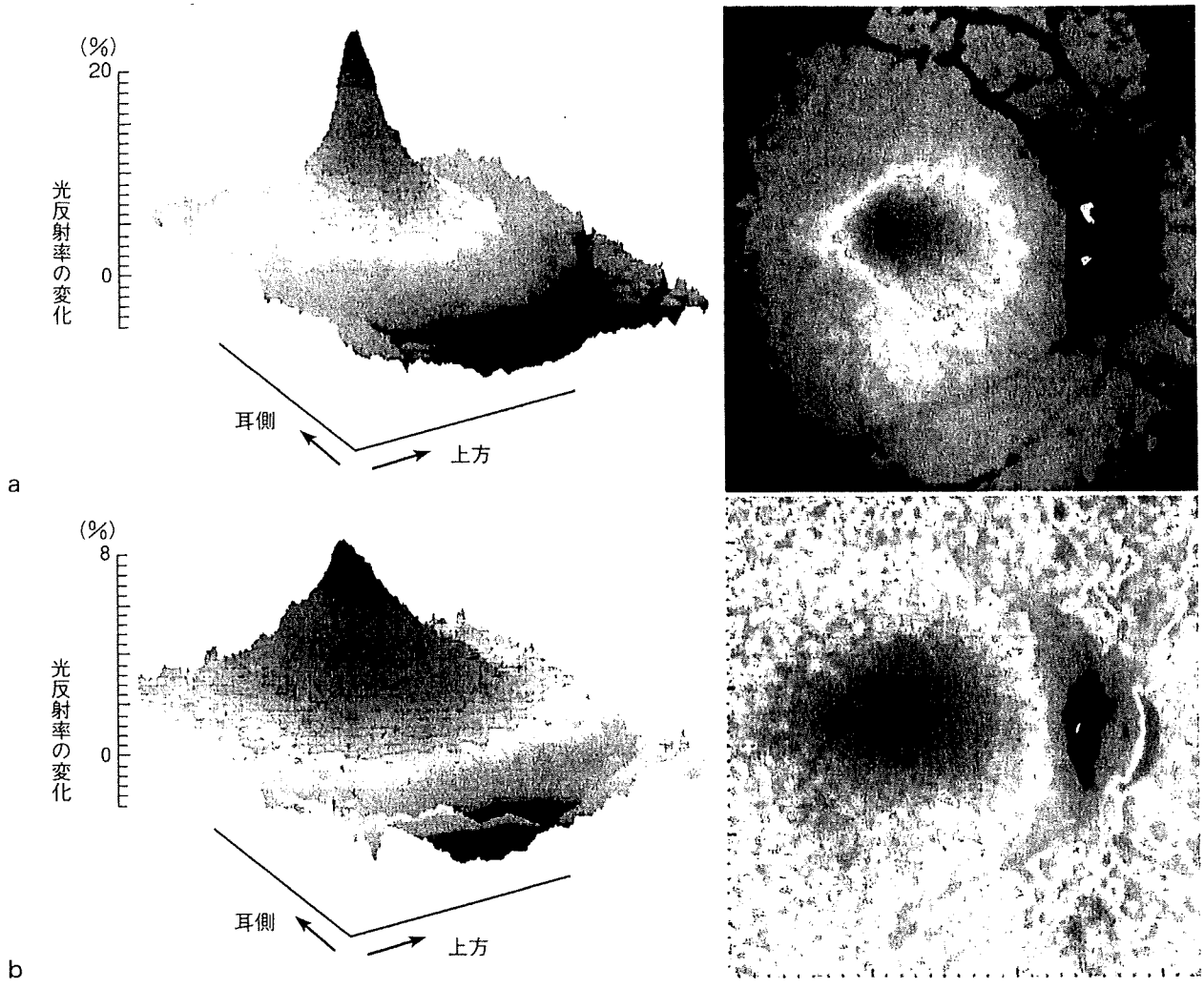

図 10　カラーフィルターを用いて測定した褪色変化のトポグラフィ
a：570 nm，b：630 nm。

さまざまな刺激方法による網膜内因性信号の測定

1．網膜局所刺激による網膜内因性信号（図 9）

網膜との共役面にフィルターを置いて局所フラッシュ刺激を行うと，刺激部位に相当する網膜から内因性信号を記録することができる。

2．可視光を用いた眼底撮影による網膜内因性信号

眼底観察光として可視光を用いることで視細胞外層の色素褪色変化（bleaching）を捉えることができる。これは，赤外光での内因性信号の測定とは異なり，褪色変化が起きた部分の網膜の反射率が上がる（明るくなる）反応であるが，570 nm の観察光では 20%（図 10a），630 nm の観察光では

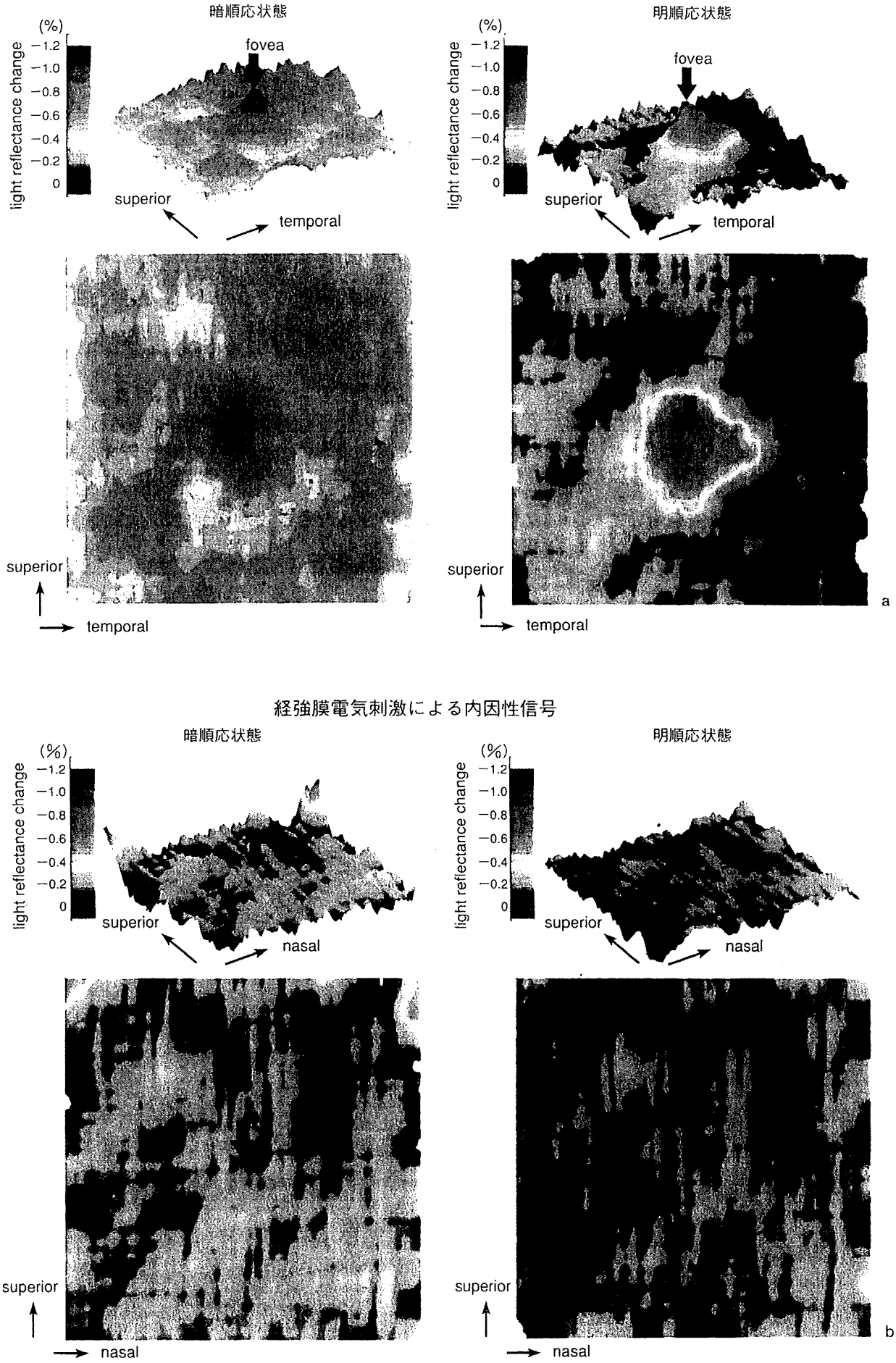

図 11　フラッシュ刺激と経強膜電気刺激による内因性信号のトポグラフィ

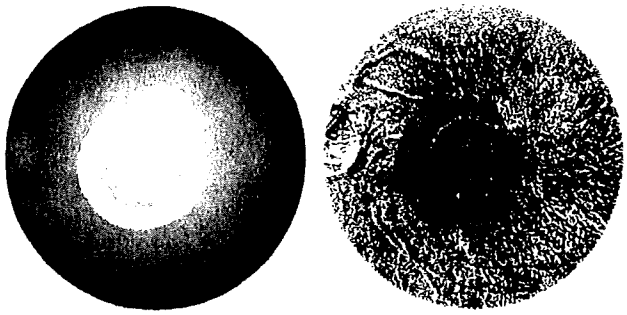

図 12　フラッシュ部分刺激に対する内因性信号
（ヒト網膜，覚醒下）

10%程度の大きな反射率変化が中心窩において認められる（図 10b）。

褪色変化自体は従来からよく知られているが，網膜内因性信号測定装置を用いることにより詳細なマッピングを得ることができる。この反応は信号の大きさが比較的大きく，信号起源が網膜視細胞であるとわかっているため，視細胞の機能評価として臨床応用に最も近いと考えられる。現在筆者らは，この信号の測定に特化した測定装置も作製しており，ヒトでの測定実験を行っている。

3. フラッシュ刺激と経強膜電気刺激による内因性信号（図 11）

では，フラッシュ光の代わりに電流によって網膜を刺激した場合はどのような反応が得られるのであろうか。経強膜的に網膜を電気刺激すると視神経および後頭葉視覚野の神経細胞が活動し，視野において白色の閃輝を自覚する。これは phosphene と呼ばれ，網膜外層の機能障害があっても内層の機能が保たれている場合には自覚することができる。このため phosphene の自覚（強さ，視野における範囲）を問診することによって網膜内層の障害をある程度推察することはできるが，実際に phosphene の程度を定量的に表現することは困難であり，生体網膜における刺激特性（反応閾値，刺激分布など）についての研究は少ない。

一方，経角膜あるいは経強膜的に網膜を電気刺激する治療法は，虚血性視神経症，網膜血管閉塞性疾患をはじめとする患者においてすでに国内でも試みられており，傷害された視野の改善をはじめとする治療効果が確認されている[12,13]。筆者ら[8]は経強膜電気刺激による網膜の反応分布を調べる目的で内因性信号を記録した。

二相性の経強膜電気刺激（current：500 μA, pulse frequency：20 Hz, stimulus duration：1.0 sec）により電気的に網膜を刺激すると，フラッシュ刺激でみられた中心窩の信号ピークがみられず（図 11a），網膜後極部全体でほぼ同程度の内因性信号がみられた（図 11b）。また，信号分布は明順応および暗順応の状態でも変わりはなく，信号発生の起源として網膜内層の寄与が大きいと考えられた[8,14]。

ヒト網膜における内因性信号測定

覚醒下のヒトでも，局所フラッシュ刺激により網膜内因性信号は測定可能であるが（図 12），現在のところ，詳細なマッピングを行うまでには至っていない。今後，測定技術および解析方法などのさらなる改良が必要である。

おわりに

FRG の利点は，赤外光の反射率変化を計測するために非侵襲的であること，空間分解能が高いこと，測定時間が短いことなどである。問題点は，ヒトの測定時に生じる固視微動などにより，画質が著しく低下することであり，現在それを克服すべく研究を行っている。将来，臨床応用が可能になれば，黄斑変性症や網膜色素変性症などさまざまな網膜疾患において精度の高い他覚的機能評価が可能になると期待されている。

さらに FRG 以外にも，近年は OCT を利用して神経機能評価を行おうという研究も行われている。これは 2002 年に理化学研究所の Maheswari ら[15,16]によって初めて提唱された Functional OCT という概念であり，網膜における応用に向けた研究が，Drexler，Fujimoto らなどすでに複数の研究者によって行われている[17,18]。網膜の神経機能をイメージングするという研究は新しい診断法として高く注目されており，将来は新たな網膜機能評価法として確立される日がくることが期待される。

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Distribution of Retinal Responses Evoked by Transscleral Electrical Stimulation Detected by Intrinsic Signal Imaging in Macaque Monkeys

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Purpose. The distribution of the electrical current over the retina when electrical pulses are delivered transsclerally has not been clearly determined objectively and quantitatively in humans. The purpose of this study was to determine the pattern of electrically evoked neural activity in the monkey retina by using intrinsic signal imaging.

Methods. The intrinsic signals of monkey retinas were recorded as changes in the reflectance of infrared light from the retina after transscleral electrical stimulation by DTL electrodes. The effects of changing the stimulus parameters (e.g., intensity, duration, and frequency) of the electrical current, were investigated.

Results. Electrical stimulation evoked a uniform change in the reflectivity across the posterior pole of the retina; that is, the intrinsic signals changed uniformly. A peak of the intrinsic signal was not observed at the fovea. The threshold of the intrinsic signal was not significantly different for the macula, perimacula, and optic disc, and the threshold did not differ under dark- and light-adapted conditions. The strength of the signals increased with longer stimulus durations, and the maximum signals were obtained when the stimulus frequency was between 15 and 20 Hz.

Conclusions. Intrinsic signals of the monkey retina evoked by transscleral electrical stimulation are elicited uniformly across the posterior pole of the fundus and most likely arise from activation of the inner or middle layers of the retina. These functional measurements could serve as a diagnostic tool for mapping the inner retinal activity, by which the site of a lesion can be noninvasively imaged. (*Invest Ophthalmol Vis Sci.* 2008;49:2193–2200) DOI:10.1167/iovs.07-0727

Electrical stimulation of the retina excites the neural pathway and evokes light sensations, called electrical phosphenes, and electrically evoked responses (EERs) from the occipital lobe.[1–4] Miyake et al.[5–9] have applied this technique for clinical diagnosis and have explored ways to evaluate the function of the inner retina of patients with total retinal detachment, night blindness, central artery occlusion, and optic nerve diseases objectively. With the increasing interest of retinal prosthesis,[10–12] the effects of electrical stimulation of the retina have drawn increasing attention because understanding the neuronal properties of the retina after electrical stimulation is essential for the development of retinal prosthesis[13,14] and determining residual retinal function in patients with inherited and acquired retinal degeneration should be known before a retinal prosthesis is implanted.[15,16] More recently, based on the results of several investigations on the neuroprotective effect of electrical stimulation,[12,17,18] electrical stimulation of the retina has been used to treat optic nerve diseases, such as nonarteritic ischemic optic neuropathy, traumatic optic neuropathy,[19] and longstanding retinal artery occlusion.[20]

Distribution of the electrical current across the retina, however, has not been definitively determined. Although electrical phosphenes are perceived homogeneously and continuously over the visual field when the retina is stimulated with a DTL electrode,[16] there is no way to evaluate the current distribution over the retina objectively, quantitatively, and noninvasively in humans. Electroretinographic techniques cannot be used to evaluate the retinal activity evoked by electrical currents because of the large electrical artifact from the stimulus.

Intrinsic signal imaging is a well-established imaging technique recently applied to translate neural activities elicited by photic or electrical pulses into the minimal visible changes of the appearance of the retina.[21–24] This has been used to assess the cone- and rod-induced responses in the retinas of macaque monkeys[25] and humans.[26,27] Recently, we reported that retinal intrinsic signals are composed of several components with different properties, and some of the components are strongly correlated with the neural activity of the inner retina.[28]

The purpose of this study was to determine the distribution of the electrical currents across the posterior retina after an electrical pulse. To accomplish this, we measured the changes in the retinal intrinsic signals evoked by transscleral electrical stimulation. A DTL electrode was used to deliver the electrical pulses, and different recording conditions, such as light- and dark-adapted states, along with different electrical current parameters, such as intensity, duration, and frequency, were studied. Results indicated that the electrical stimuli elicited responses uniformly across the posterior pole of the ocular fundus and activated mainly the inner or middle layers of the retina.

Methods

The principles of measuring intrinsic signals have been described in detail.[25,28] The experiments were performed on two rhesus monkeys (*Macaca mulatta*) under general anesthesia. After intramuscular injection of a mixture of atropine sulfate (0.08 mg/kg), droperidol (0.25 mg/kg), and ketamine (5 mg/kg), the monkeys were paralyzed with

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vecuronium bromide (0.15 mg/kg per hour) and were artificially ventilated with a mixture of 70% N_2O, 30% O_2, and up to 1.5% isoflurane. Electrocardiograms, expired CO_2, and rectal temperatures were monitored throughout the experiments. Before the recordings, one pupil was fully dilated with topical tropicamide (0.5%) and phenylephrine hydrochloride (0.5%). A hard contact lens was placed on the eye to prevent the cornea from drying.

The experimental protocol was approved by the Experimental Animal Committee of the Riken Institute, and all experimental procedures conformed to the guidelines of the Riken Institute and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Intrinsic Signal Imaging

A digital fundus camera system (NM-1000; Nidek, Aichi, Japan) was modified to record the light reflectance changes of the macaque retina over 45° of the posterior pole. The fundus reflectance was continuously monitored with light from a halogen lamp bulb filtered through an infrared interference filter (840–900 nm). The camera was focused on the macular vessels, and the images were recorded with a charge-coupled device (CCD) camera (PX-30BC; Primetech Engineering, Tokyo, Japan). The images were digitized with an IBM-compatible computer equipped with a video frame-grabber board (Corona I; Matrox, Quebec, Canada; gray level resolution, 10 bits; spatial resolution, 640 × 480; temporal resolution, 1/30 second; Fig. 1A). The respirator was stopped during the recordings to reduce the respiration-induced motion artifacts.

For recordings in the light-adapted condition, half an 80-mm diameter white polyethylene ball was placed between the fundus camera and the eye. The ball was illuminated by two halogen lamps through fiber optics so that the luminance in the center was 30 cd/m^2. The ball was removed a few seconds before data acquisition.

Electrical Stimulation of Retina

DTL electrodes were used to deliver the electrical pulses. The DTL electrode was selected for the measurements of intrinsic signals because the noise of the intrinsic signal is much lower with a DTL electrode than with a contact lens electrode such as the Burian-Allen electrode and because electrical phosphenes were perceived to be more homogeneous and continuous with a DTL electrode than with a contact lens electrode.[16]

The fibers of the DTL electrode were placed on the lower bulbar conjunctiva 5 mm from the corneal limbus. The conjunctiva was covered with 3% hyaluronic acid and 4% chondroitin sulfate (Viscoat; Alcon Japan, Tokyo, Japan), and the reference electrode was placed on the ipsilateral wrist (Fig. 1A).

Biphasic electrical pulses were used for all experiments (Fig. 1A, inset). The stimulus consisted of a positive current for x msec followed by a negative current for x msec and then a rest period for $3x$ msec. In most of the experiments with a pulse frequency of 20 Hz, x was set to 10, but in the experiment in which the pulse frequency was changed, x varied from 40 (5 Hz) to 2 (100 Hz) to keep the total current constant.

Stimuli were delivered 0.5 second after the initiation of data acquisition for 1 second, except in the experiment in which stimulus duration was changed from 0.5 second to 7 seconds. Pulse duration and frequency were controlled by a function generator (Multifunction Synthesizer WF 1944B; NF Corporation, Yokohama, Japan). Timing of the data acquisition and stimulus delivery were under computer control.

To compare the responses evoked by light flashes, white light stimuli were obtained from a xenon strobe (duration, 1 msec) embedded in the fundus camera. The light stimulus was given to the entire posterior pole of the ocular fundus, 0.5 second after the initiation of data acquisition. Flashes were delivered either as flickering flashes (20 Hz, 1 second, Fig. 2A) or as a single flash (Fig. 3). Flash intensity measured at the cornea was 6.07 cd · s/m^2 for the flickering flashes, and 140 cd · s/m^2 for a single flash (measured at 50.2 mm from the object lens by a photoradiometer; IL-1700, International Light Technologies Inc., Peabody, MA).

Data Analysis

After electrical or light stimulus, the light reflectance from the ocular fundus decreased and the fundus image became darker. The intrinsic

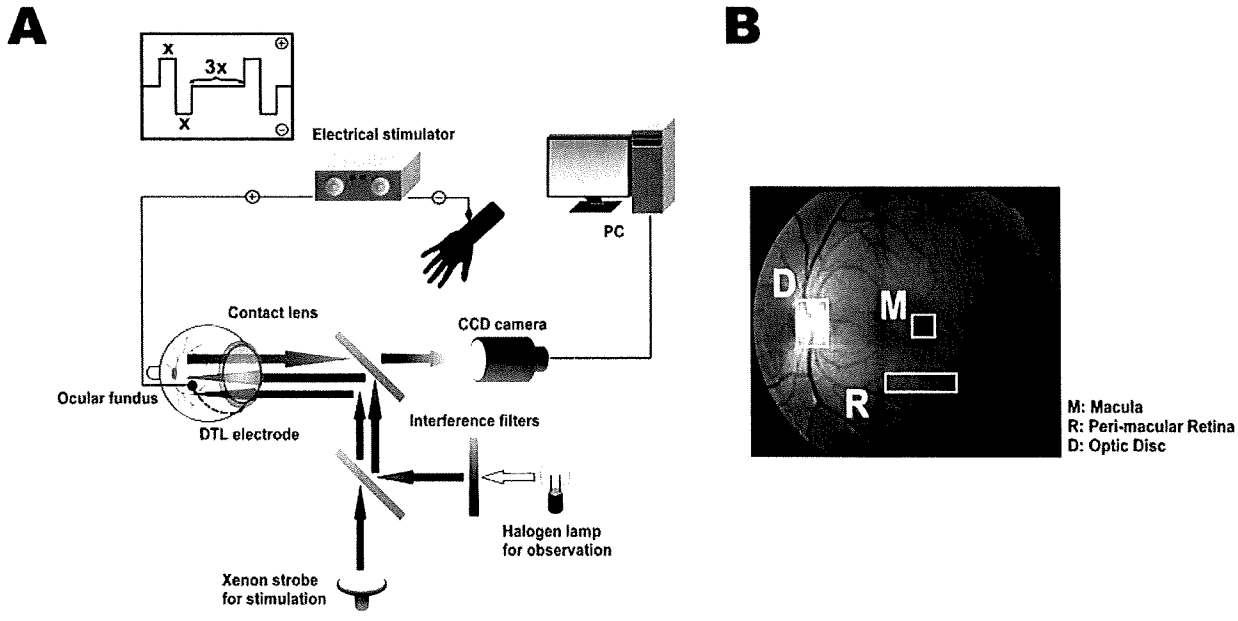

FIGURE 1. Schematic drawing of the experimental setup, pattern of electrical pulses, and fundus photograph. (A) Ocular fundus was illuminated by the light from a halogen lamp filtered through an infrared interference filter and monitored by a CCD camera during the recording trial. Stimulus was given by a xenon flash through the objective lens of a fundus camera or by electrical current through a DTL electrode placed on the conjunctiva. *Inset*: biphasic pulse current with a duration of x is followed by a resting period as long as $3x$. As the frequency is changed from 5 to 100 Hz, x is changed from 40 to 2 msec, respectively. (B) Fundus photograph of normal retina showing the locations to be analyzed.