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TECHNICAL NOTE

Improvement of the optical imaging technique for intact rat brain using a plano-concave lens

Minako Kawai · Noriyuki Hama · Shin-ichi Ito · Akihiko Hirota

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Abstract Use of a plano-concave lens improved the quality of optical signals from the rat cerebral cortex by improving the focus. When detecting neural activity from a curved surface of an in vivo brain by optical techniques, it is not possible to adjust the focus equally over the entire detecting area in the two-dimensional plane, since the active window of the optical detector is usually flat, while the intact brain surface is spherical. It has been known that the size of the optical signal is reduced as the distance of the real image to the active window of the detector increases; therefore, the level of the signal-to-noise ratio obtained from the unfocused area often becomes insufficient for quantitative physiological analyses. By placing a plano-concave lens on the cerebral cortex, we succeeded in obtaining a two-dimensional image that has no unfocused area over an entire image recorded by the detector.

Keywords Plano-concave lens · Optical recording · Noise reduction · Central nervous system

Introduction

Optical imaging of membrane potential using voltage-sensitive dyes has a great advantage in recording the electrical activity from many portions of the brain simultaneously at a high time resolution in the order of milliseconds [for reviews, see 1–5]. Since Orbach and Cohen [6] first demonstrated that optical signals could be obtained in vivo, the multiple-site optical detecting technique for studying neural networks has developed remarkably. However, the optical technique of membrane potential measurements has some difficulties and limitations in signal size. For example, the membrane potential-related signal is only of the order of 0.01–0.1 % change in background intensity for a 100-mV voltage change and there are many noise sources, especially during in vivo measurements. As a result, the signal-to-noise ratio (S/N) is very small and improvements in the S/N for quantitative analyses of physiological functions is required. We have previously developed original systems to record high-quality optical signals from the rat cerebral cortex [7–9], and reported several physiological studies of the neural activity in the rat somatosensory cortex [10–12].

When imaging the cerebral cortex in vivo, craniotomy is performed and the dura is exposed. In order to prevent the tissue from drying during recording, the cortical surface is conventionally covered with a liquid, such as artificial cerebrospinal fluid [13] or agarose [14], and a coverslip is placed on top. In the most commonly used optical system, the detector has a flat active window, while the intact brain surface is spherical. Therefore, it is not possible to adjust the focus equally for the entire brain surface in the two-dimensional plane of the active window. Salzberg et al. [15] and Cohen and Lesher [16] found that the size of the optical signal was reduced as the real image became unfocused, so that it is reasonable to consider that reducing the unfocused area would result in an increase in the number of pixel which has high S/N. Therefore, we hypothesized that placing a plano-concave lens, whose radius of curvature was the same as that of the intact

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M. Kawai · N. Hama · S. Ito · A. Hirota (✉)
Department of Neural and Muscular Physiology, Shimane University School of Medicine, 89-1 En-ya, Izumo, Shimane 693-8501, Japan
e-mail: hirophy2@med.shimane-u.ac.jp

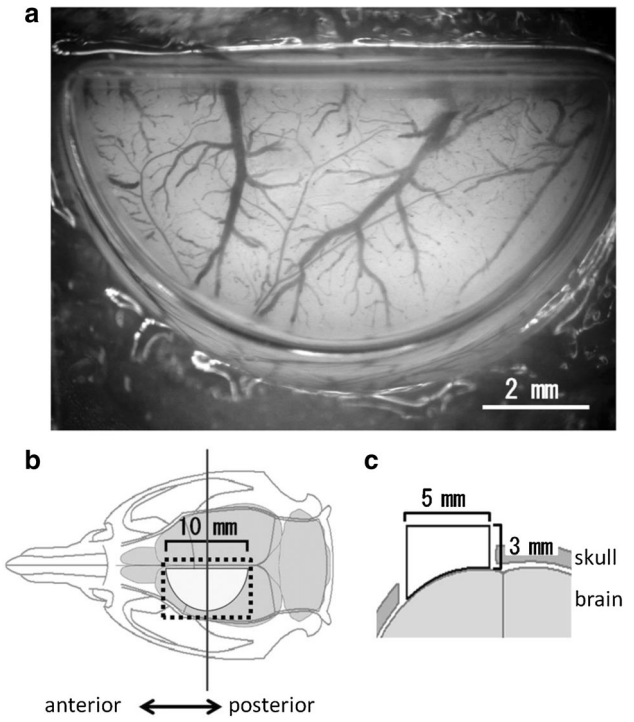

Fig. 1 **a** The dorsal surface of the rat cerebral cortex viewed through the plano-concave lens. After the left somatosensory cortex was exposed by craniotomy, the lens was placed on the cortex. To keep measurements of the area near the midline of the cerebral cortex as broad as possible, we used half of the lens. Despite the marginal zone of the cerebral cortex being curved, we succeeded in obtaining a two-dimensional image where focus was maintained over an entire image. **b** Relative location of the photographed area in the top view of the rat brain. The area surrounded by a *thick broken line* is the photographed area in (**a**), and the *semicircle* indicates the plano-concave lens. Dimensions of the plano-concave lens: 10 mm diameter; 3 mm center thickness; 20 mm radius of curvature of the curved surface. **c** A vertical cross-section of the brain with a plano-concave lens. The position of the section plane is the symmetry plane of the lens and indicated by *dotted line* in (**b**)

cortical surface, on the dura would allow imaging of the entire area with no unfocused part in the active window of the photodetector. In this article, we describe the effect of placement of a plano-concave lens for improving S/N of the optical signal at the marginal zone of the image and a quantitative analysis of this effect.

In all of the following, we assume that our optics is focused at the point where an optical axis and the object cross, and that the active window of the photodetector is placed on the image plane so as to fit the center of the window on the optical axis. Animal experiments were performed in compliance with the Guidelines for Animal Experimentation of the Center for Integrated Research in Science, Shimane University.

To begin with, we compared the S/N between two different conditions: using the plano-concave lens or using the agarose on the dura. Following our routine methods [7] and using adult female Sprague–Dawley rats ($n = 4$, weighing 220–230 g), the dura was exposed and the sensorimotor cortex was stained with a voltage-sensitive dye (RH414). A stable excitation light illuminated the stained cortex and fluorescence light was concentrated in the image plane using a tandem lens assembly. Light intensity was detected by a photodiode array. Optical measurements were carried out twice in the same individual at the determined point using the plano-concave lens (Fig. 1a) or using 4 % agarose (Agarose Low Melting Point; Sigma, St Louis, MI, USA) and a slide glass. Paired t test was used to assess significant differences in each element in the matrix array photodiode between the values recorded by the plano-concave lens and the agarose.

Table 1 gives the signal size, the peak-to-peak noise level, and background fluorescence intensity from 3 × 3 contiguous elements in the matrix array photodiode (Fig. 2) at the marginal zone of images obtained from the plano-concave lens or the agarose. The values in Table 1 are the averages and standard deviations over the four animals, each calculated from the average values of the eight trials in the individual animals.

In the optical recording of membrane potential, it is well known that the size of membrane potential change is proportional to the fractional change ($\Delta F/F$), i.e. change in fluorescent intensity (ΔF) divided by the background fluorescent intensity (F). Then, even if the background intensity changes, this ratio remains constant for the same membrane potential change. On the other hand, the dominant noise of our recording system is "shot noise", and the higher the background light intensity, the larger the noise [17]. In consideration that S/N is thus dependent on the background intensity value, the background intensity should be the same between the preparations to simply compare the S/N ratio of the optical signal between covering with the agarose and the plano-concave lens. It was practically impossible, however, to stain the cortex at the same strength even when we used the same concentration dye solution for the same staining time, and the background intensities differed among preparations. Therefore, we set the background intensity to have almost the same value at the center element (17–15; row–column number in Table 1) for all the recordings by regulating the excitation intensity.

Two typical optical traces are shown in Fig. 2. The signal size with the lens was larger than with the agarose significantly ($P < 0.001$), while there was no significant difference in the noise level (Table 1). It followed that using the lens increased S/N.

To quantify the improvement produced by the addition of the plano-concave lens, we performed theoretical analyses (see Supplementary material). Figure S1 in the

Table 1 Parameters associated with optical recordings obtained from 9 elements at the marginal zone of the image with a plano-concave lens or 4 % agarose

Element no. (row–column)	Plano-concave lens			4 % agarose		
	Signal size (pA)	Noise level (pA)	Background intensity (nA)	Signal size (pA)	Noise level (pA)	Background intensity (nA)
16–14	71.2 ± 14.0	25.3 ± 3.5	44.9 ± 3.3	53.9 ± 9.8	25.2 ± 4.1	41.9 ± 4.8
16–15	68.3 ± 14.8	25.6 ± 4.4	44.7 ± 4.3	49.8 ± 7.7	22.4 ± 1.6	40.8 ± 4.4
16–16	60.3 ± 12.3	22.9 ± 4.4	40.9 ± 4.5	42.6 ± 6.0	18.3 ± 1.4	36.9 ± 3.4
17–14	55.8 ± 11.0	22.9 ± 3.5	36.3 ± 1.5	48.1 ± 9.4	24.6 ± 4.2	36.8 ± 1.8
17–15	51.9 ± 10.7	22.2 ± 3.9	35.0 ± 1.9	43.4 ± 8.0	21.6 ± 2.4	34.6 ± 1.5
17–16	48.4 ± 8.9	20.8 ± 3.6	33.7 ± 2.4	39.0 ± 7.0	18.6 ± 1.3	32.9 ± 1.2
18–14	45.2 ± 8.2	20.5 ± 3.4	30.3 ± 2.7	43.3 ± 10.1	23.5 ± 5.0	34.2 ± 3.0
18–15	42.1 ± 7.8	19.6 ± 3.2	29.2 ± 2.6	39.2 ± 9.2	21.2 ± 3.2	31.9 ± 2.6
18–16	40.6 ± 6.9	19.1 ± 2.5	29.3 ± 2.6	36.3 ± 8.8	18.5 ± 1.2	31.2 ± 2.2
Lens/agarose	1.22	1.03	1.01			

Numerals in columns 2–7 are the average and the standard deviation ($n = 4$) of photocurrent generated by fluorescent light with the plano-concave lens or 4 % agarose. Each parameter was measured trial-by-trial from a single sweep optical signal of neural activity induced by electrical stimulation to the forelimb, as shown in Fig. 2, and then averaged 8 trials in the same individual. Column 1 gives element position (row and column number) in the matrix array photodiode. As shown in Fig. 2, 3 × 3 contiguous elements, at the marginal zone of the image, were selected. Columns 2 and 5 give the change of fluorescence due to neural response activities in pA. Columns 3 and 6 give the peak-to-peak noise level in pA. Columns 4 and 7 give the background fluorescence intensity in nA. Lens/agarose represents the ratio of the data with lens to those with agarose, respectively. Paired *t* test for the values in each element of each animal (i.e. the comparison of 36 values from each 9 elements in respective 4 animals) showed that the signal size was significantly different between the plano-concave lens and agarose ($P < 0.001$) but the noise level was not ($P = 0.472$). The background light intensity was set to have almost the same value at the center element (17–15) for all the recordings by regulating the excitation intensity (see details in the text)

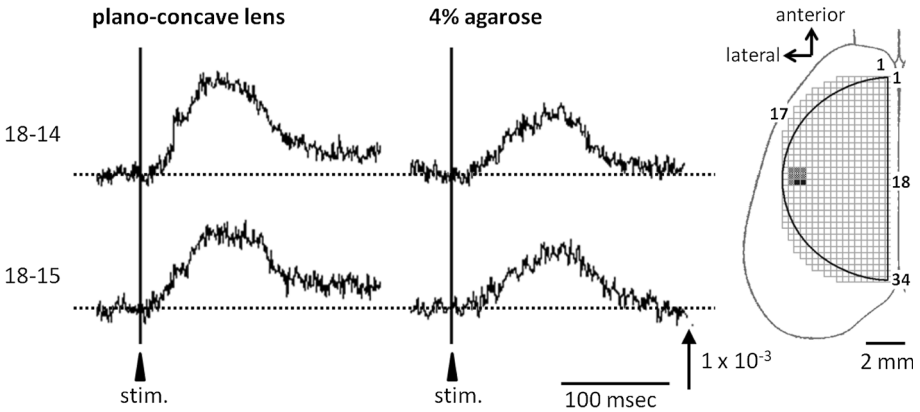

Fig. 2 Two examples of a single sweep optical signal from the rat cerebral cortex obtained with the plano-concave lens (*left*) and with 4 % agarose (*right*). Somatic evoked cortical response was induced by electrical stimulation (0.5 ms, 1 mA) of the contralateral forelimb. The *vertical line* indicates the timing of the electrical stimulation. The *direction of the arrow* in the lower-right corner indicates a decrease in fluorescence (corresponding to depolarization) and the *length of the arrow* represents the stated value of the fractional change (change in fluorescence intensity divided by the resting fluorescence intensity). The *right drawing* indicates relative position of the matrix array photodiode and that of the plano-concave lens to the image of the left cerebral cortex. Each square corresponds to each element in the photodiode array used in our laboratory. The 3 × 3 *gray squares* indicate the position of the nine contiguous photodiode elements at the marginal zone of the image used for analyzing in Table 1. Two *black squares* among the *gray* ones indicate the positions in the matrix where the optical signals shown in the *left* were recorded. The notation 18–14 or 18–15 indicates the element position in row 18 and column 14 or 15 of the matrix array, respectively

Supplementary material is a schematic drawing of our optics for detecting small intensity changes in the rat cerebral cortex stained with a voltage sensitive dye (RH414) using the photodiode array described above.

The theoretical analyses in the Supplementary material showed that the distance between the image plane and the real image at the most unfocused site on the object (ℓ_1' in Supplementary material) is 15.46 mm, without the plano-

concave lens or the agarose, i.e., there is only air on the object. When the plano-concave lens is placed on the cerebral cortex, this distance is 10.53 mm (ℓ_2' in Supplementary material). Therefore, the focus at the most unfocused site on the object is improved by approximately 32 % due to placement of the plano-concave lens. Since changing the focus by 300 μm led to a 50 % reduction of signal size [15], it is evident that the increase in the signal size that is yielded by improving the focus contributes significantly to the increase in S/N.

In the conventional treatments used in other laboratories, the space between the cortex and the coverslip is filled with liquid [13, 14]. Since the shape of this liquid-filled space closely resembles a plano-concave lens, this liquid may also have the same effect. However, from our quantitative analysis, it is evident that the higher the refractive index (RI), the more effective the liquid is at improving the focus. By substituting 1.3 (the approximate RI value for the agarose) for n in Eq. (26) in the Supplementary material, ℓ_2' becomes about 12.06 mm and the effect of agarose is evaluated to be an approximately 22 % improvement compared with air. Therefore, we estimated that exchanging the agarose for the plano-concave lens would further increase the signal size by about 10 %. There was a small tendency that, at the marginal zone, the background light intensity with the agarose was somewhat larger than that with the plano-concave lens; in contrast, the former was somewhat smaller than the latter at the central zone. Since the agarose was slightly cloudy and the excitation light intensity on the stained cortex was smaller at the marginal than at the central zone in our equipment, we assumed that stray light due to the light scattering from the central direction affected background intensity at the marginal zone. When the background light intensity was set to the same value at the marginal zone, the stray light also contributed to the value, in the case with the agarose. Since the signal size is proportional to the intensity of the background light excluding stray light, the presence of the stray light may decrease the signal size. This results in a further increment of the signal size with the plano-concave lens at the same background light intensity. Actually, the signal size was 22 % larger with the plano-concave lens than with the agarose (Table 1), exceeding the theoretical value.

In addition, there are some other advantages in using the plano-concave lens. The conventional treatment is used not only for dryness prevention but also for reducing the vibration noise derived from respiration and pulsation and the noise derived from free-surface interface between water and air. The plano-concave lens is also valid for reducing these sources of noise. Moreover, by gluing the lens to a slide glass attached to a manipulator, we were able to quickly release the lens from the cortical surface. Therefore, it is possible to remove and recover the lens to provide an access to the cortical surface during recording, for administering drugs directly in order to examine pharmacological effects and/or handling unexpected contingencies such as sudden bleeding from the dural surface of the brain.

Since the tandem lens system can be replaced by any ideal convex lens in the quantitative analyses described above, it is of utmost importance that, if any optics are employed, using the plano-concave lens improves the quality of optical signal by improving focus. This is especially true for the marginal zone of the active window of the photodetector. Therefore, this lens is a useful tool not only for the rat cerebral cortex but also for any other imaging technique that detects optical signals from spherical objects. We believe that this improvement in optics will contribute to the investigation of neural network function by optical techniques.

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Conflict of interest The authors declare that they have no conflict of interest.

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