Visualization of brain activity from in vitro to in vivo

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***Abstract*—In order to understand the function of neuronal circuits, the spatio-temporal activity of multiple neurons have to be measured. In this regards, imaging of neuronal activity using fluorescence dyes is one of the most promising techniques. We conducted imaging studies on nervous tissues of in vitro and in vivo preparations using several different fluorescence dyes.**

Ca^{2+} is an important messenger in signal transduction of neurons and the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is known to increase during the cell excitation. We made the Ca^{2+} imaging study in the brain slice preparations. $[Ca^{2+}]_i$ was measured using a high-speed cooled-CCD imaging system equipped with a excitation wavelength changer. In the basal ganglia striatal slices from mice, we observed the spontaneous $[Ca^{2+}]_i$ changes from individual neurons and glial cells. Long lasting spontaneous $[Ca^{2+}]_i$ changes, which lasted up to about 100 s, were found in both neurons and glial cells. In the visual cortical slice preparation, we measured the $[Ca^{2+}]_i$ changes of the neuronal population evoked by electrical stimulations. We could measure the signal propagation in the neuronal network of the visual cortex, and study the functional neuronal connections in the visual cortex.

The membrane potential changes of neuronal population can be imaged with a voltage sensitive dye. In vivo membrane potential imaging reveal how the visual signals are encoded in the visual cortex and how signals propagate in the intact visual cortex. A flash of light applied to contralateral eye induced focal activation in the primary visual cortex in accord with the retinotopic map.

In summary, imaging can visualize the population of the neuronal activity and have great potentials to reveal the spatio-temporal properties of the functional network from in vitro to in vivo.

I. Introduction

In the field of neuroscience, the research goals are to understand how neurons communicate each other, and how signals are processed in the brain. Brain consists of tremendous number of neurons and a neuron is connected by synapses with other neurons (cf. [1]).

For measurement of cellular activity in the single or a few cells, intracellular recordings [2], [3], extracellular recordings (cf. [4]), or patch clamp recordings [5] have been performed with a microelectrode from the middle of the twentieth century. However, we could not understand the behavior of the neuronal circuit with the individual neuronal recording. To understand signal processing in neuronal networks, the activity of multiple neurons should be measured. As a direct approach to reveal the circuit dynamics, multielectrode recordings were conducted [6]–[8]. A multielectrode array is able to record the spikes or local field potentials from an ensemble of the neurons simultaneously, however, this approach has disadvantage of sampling a small population of the neurons, and also lack anatomical information about which cells were activated.

To overcome these problems, especially in a brain slice preparation, imaging study is feasible with fluorescent calcium (Ca^{2+}) indicators, because they produce the large signals (more than several percent changes in fluorescence), making it possible to detect the responses from single neurons or some cellular process (eg. the presynaptic terminal and the dendrite), and the signal from the entire population of cells in the specimen can be measured simultaneously, using the bulk loading method with acetoxymethyl (AM) esters of the indicators [9], [10]. In addition, this method enables to identify the cell types and cell morphologies in combination with the use of transgenic mice, which expressed fluorescence proteins in a specific type of cells and with post-hoc histological reconstruction of neurons. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) can be increased by subthreshold postsynaptic potentials in the dendritic spine, sodium or calcium action potentials in entire the cell, or Ca^{2+} release from the intracellular Ca^{2+} store, such as endoplasmic reticulum [9]–[11]. Therefore, using the Ca^{2+} imaging method, we can detect the many types of neuronal signals depending on the situation.

To reveal activity of the neuronal network in vivo, the optical recording also has a great advantages. Because of difficulties in bulk loading of AM-ester Ca^{2+} indicators (cf. [12]), two types of optical imaging are often conducted: monitoring of intrinsic signals and voltage-sensitive dyes [9], [13]. The intrinsic signal thought to be changed by consumption of oxygen in the blood, therefore, monitoring of intrinsic signal is not suited for recording with high spatial resolution and the signal changes slowly compared with neuronal dynamics. In contrast voltage-sensitive dyes directly translate the membrane potential into an optical signal. Therefore, this method enables to detect the site of changes in membrane potential [14].

In this paper, we introduce the some results obtained by imaging studies from in vitro to in vivo, and what can be observed by the optical imaging. The related results of this work have been reported [11], [15], [16].

II. METHODS

All experimental procedures were approved by the institutional animal care and use committee of Osaka University.

A. In vitro Ca^{2+} imaging in the striatal slice

The general procedures of the slice preparation and Ca^{2+} imaging were previously described [11], [15], except for the use of transgenic mice, which expressed green fluorescent protein (GFP) in astrocytes (GFAP-GFP mice [17]). Briefly, the postnatal day 10 (P10) to P21 GFAP-GFP mice were anesthetized with halothane and were decapitated. Then, the brain was rapidly isolated. Sagittal slices of striatum with cortex (corticostriatal slice), 300 μm thick, were prepared with a vibratome tissue slicer (Leica, VT-1000S) from a small block of brain containing the striatum and cortex in ice-cold artificial-cerebro spinal fluid (ACSF) bubbled with 95% O_2-5% CO_2. The composition of ACSF was (in mM): 137 NaCl, 2.5 KCl, 0.58 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 21 $NaHCO_3$ and 10 glucose.

The slices were incubated with 20 μM Fura-PE3-AM (Calbiochem) and 0.02% Chremophore EL (Sigma) in ACSF at 35 °C in the incubator that was humidified and continuously aerated with 95% O_2-5% CO_2. The slices were then washed in the incubator and transferred to a continuously superfused chamber on the stage of a epifluorescence upright microscope (BX51WI, Olympus).

The Ca^{2+}-indicator loaded slice was alternately excited at the wavelengths of 340 and 380 nm by the xenon light source through the excitation filter changer (DG-4, Sutter; exposure time was 100 or 200 ms on individual wavelengths) and fluorescence signals (F340 and F380) were captured every 2 s with a cooled-CCD through a 20x, NA 0.95 water-immersion objective and the barrier filter (center wavelength was 510 nm). The $[Ca^{2+}]_i$ was estimated by the fluorescence ratio (R = F340/F380) from each imaged cell. The experiments were performed at 29-31 °C (figure 1).

B. In vitro Ca^{2+} imaging in the visual cortical slice

The procedures were almost same with above description except for following points. Coronal visual cortical slices, 300 μm thick, obtained from P14 to P28 C57BL/6J mice were used. The slices were incubated with the loading solution containing 10 μM Oregon Green 488 BAPTA-1-AM. For fast imaging, the single wavelength excitation method was adopted to the visual cortical experiments. The center wavelength of the excitation was 475 nm and that of the emission wavelength was 525 nm. The sampling interval was 10 ms. For focal stimulation, biphasic current pulses (duration 0.2 ms) were applied with glass microelectrode (ϕ = 10-20 μm). The relative $[Ca^{2+}]_i$ changes were quantified as $\Delta F/F$, where F is the fluorescence intensity before stimulation and ΔF is the changes in the fluorescence intensity from F.

C. In vivo imaging with voltage-sensitive dye

Adult C57BL/6J mice (8-20 weeks) were anesthetized by intraperitoneal injection of urethane (1.25 g/kg). Atropine sulfate (0.01 mg, i.p.) was administrated, and then a tracheal cannulation was performed. A heating pad maintained the body temperature at 37 °C during the experiment. Right visual cortex was exposed by craniotomy in right parietal bone (1-4 mm from bregma and 1-4 mm from midline). The cortex was stained with voltage-sensitive dye, RH795 (0.7 mg/ml in saline, Invitrogen), by direct application on the cortical surface for 90 min, and then rinsed with saline. The recording was performed with CMOS imaging system (MiCAM ULTIMA, BrainVision). The excitation light (center wavelength was 530 nm) illuminated onto the cortex, and the emitted fluorescence through dichroic mirror and barrier filter ($>$ 590 nm longpass) was collected by CMOS based camera (100 $\times$ 100 pixels). The images were acquired at 1 kHz frame rate and collected and processed by PC. Visual stimulation to the left eye was

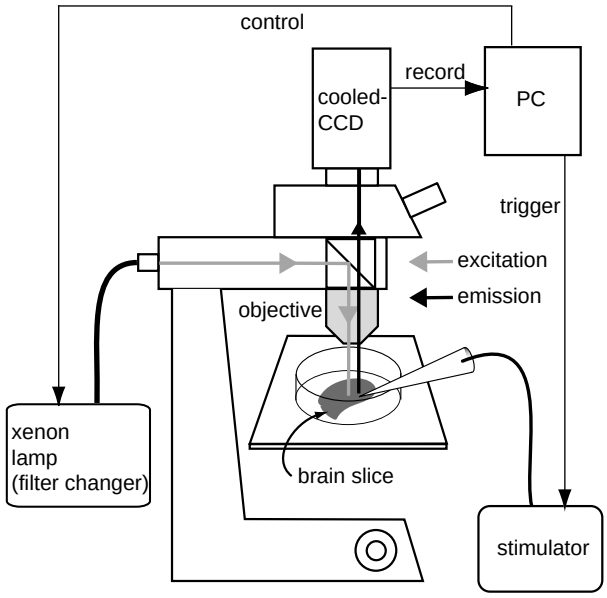

Fig. 1. Schematic diagram of Ca^{2+} imaging system for the slice preparation.

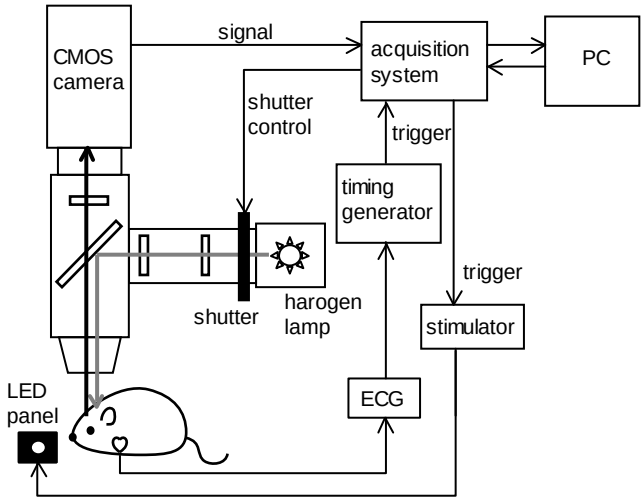

Fig. 2. Experimental setup for in vivo voltage-sensitive dye imaging.

performed with the spot of the white LED light (intensity: 365-560 nW/cm^2, duration: 20 ms) corresponding to ~7.5° field of view. To suppress the heartbeat interferes and some noise with optical recording in vivo, the onset of the data acquisition was synchronized with R-wave of the electrocardiogram (ECG), and the 16-32 consecutive acquisitions with or without the stimulation were performed. Then, the relative changes of fluorescence (ΔF/F) of the consecutive trials were averaged. The averaged ΔF/F with stimulation were subtracted by that of without stimulation (figure 2) [18].

III. Results

A. Spontaneous Ca^{2+} transients in striatal cells

At first, we introduce the results of in vitro imaging with single-cell spatial resolution. Figure 3(a) shows the pseudo color fluorescence image of the Fura-PE3 loaded striatal slice from GFAP-GFP mouse. GFP fluorescence is shown as green, and Fura-PE3 fluorescence excited by 380 nm excitation light is shown as red. A use of GFAP-GFP mice enables to discriminate between glial cells (astrocytes) and other cells (putative neurons) by monitoring of GFP fluorescence in a living tissue. In this slice, the long-lasting spontaneous $[Ca^{2+}]_i$ transients, up to about 100 s, were observed in both neurons and astrocytes. Figure 3(b) shows the typical spontaneous $[Ca^{2+}]_i$ transients in individual neurons and glial cells. Various patterns of the $[Ca^{2+}]_i$ transients were observed in both neurons and astrocytes. Some cells exhibited $[Ca^{2+}]_i$ transients with short (cell 2) or long durations (most of astrocytes in this figure), bursting activity (cell 1), and regular oscillatory activity (cell 3). During long-period recording, a mixed profile of these patterns was observed in many of cells. Administration of intracellular Ca^{2+} store depletor, thapsigargin, greatly reduced the $[Ca^{2+}]_i$ transient rate to 1.5 $\pm$ 1.0 % in neurons (n = 16 cells) and 0.0 % (n = 2 cells) in astrocytes (data not shown, cf. [19]). Therefore, these $[Ca^{2+}]_i$ transients mainly due to a Ca^{2+} release from the intracellular Ca^{2+} store.

B. Recordings of the evoked signal propagation by Ca^{2+} imaging in visual cortical slices

Figure 4 shows the $[Ca^{2+}]_i$ transients evoked by 120 μA cortical layer 4 stimulation. The $[Ca^{2+}]_i$ transients were observed around the stimulus electrode immediately after the stimulation, and then the high $[Ca^{2+}]_i$ region spread vertically toward layer 2/3, but the horizontal spread was restricted. This $[Ca^{2+}]_i$ spread region was greatly reduced by blockade of ionotropic glutamate receptors with 10 μM CNQX [1] and 50 μM AP5 [2], and completely blocked by blockade of action potentials with 1 μM TTX [3] (data not shown). Therefore, evoked $[Ca^{2+}]_i$ transients were due to the signal propagation via excitatory synaptic connections in the visual cortical circuit. Time lapse images of $[Ca^{2+}]_i$ transients evoked by layer 1 stimulation are shown in figure 5. Unlike the case of layer

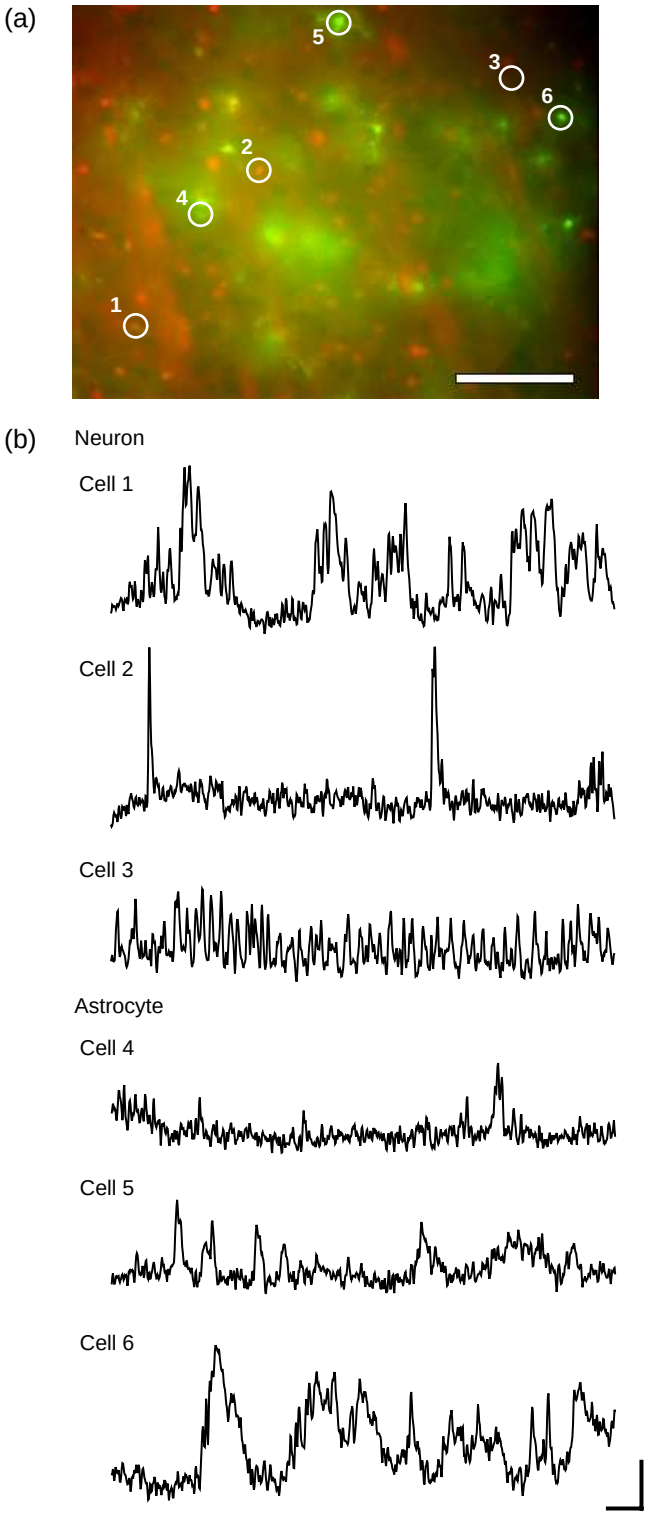

Fig. 3. Spontaneous $[Ca^{2+}]_i$ transients in the striatal cells. (a)Fluorescence image of the GFAP-GFP mouse striatal slice. This photomicrograph was obtained by overlaying the Fura-PE3 fluorescence image (red color) and the GFP fluorescence image (green color). Cells numbered 1-3 were putative neurons and 4-6 were astrocytes. Scale bar, 100 μm. (b) Time courses of the spontaneous $[Ca^{2+}]_i$ transients. Cell numbers correspond with the region numbers in (A). Scale bar, 100s, ΔR = 0.01.

[1] 6-cyano-7-nitroquinoxaline-2,3-dione
[2] D,L-2-amino-5-phosphonovaleric acid
[3] tetrodotoxin

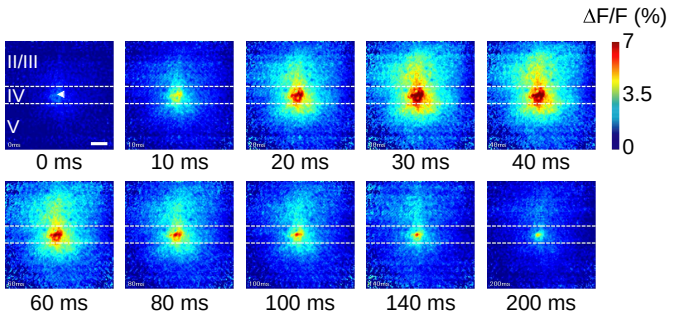

Fig. 4. Time lapse pseudo color images of ΔF/F evoked by layer 4 stimulation. At the bottom of each panel are the times in milliseconds after the 120 μA stimulation to layer 4. Stimulus position (white arrow head) and the number of cortical laminae are shown as roman numbers in the upper left panel (0 ms). Scale bar, 100 μm.

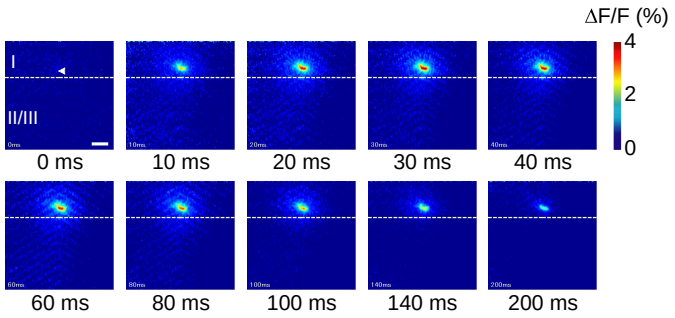

Fig. 5. Time lapse pseudo color images of ΔF/F evoked by 120 μA layer 1 stimulation. Figure is illustrated by same way as figure 4.

4 stimulation, the evoked $[Ca^{2+}]_i$ transients were observed only around the stimulus position and did not propagate. These results suggested that the functional synaptic connection manner was different between layer 4 and layer 1.

C. Recordings of propagation of neuronal activity in vivo visual cortex evoked by visual stimulation

The neuronal activity evoked by the visual stimulation was recorded by optical monitoring of the voltage-sensitive dye signals in the visual cortex (figure 6). The signals initially arose in the region indicated by black dot in upper left panel of figure 6(a), corresponding to the region within V1 [4]. And then the responsive region propagated to medial and lateral direction (figure 6(a)). The lateral responsive region might correspond to V2L [5]. Figure 6(b) shows time courses of ΔF/F obtained from three regions indicated by black, red (lateral region), and green dot (medial region) in upper left panel of figure 6(a). The visual evoked signals at the regions shown as black and red dot arose ~46 ms after the onset of visual stimulation and reached peak after ~140 ms, the signal at the medial region (green dot) arose after ~60 ms and reached peak at ~146 ms. All responses sustained during the recording period (512 ms). Averaged latency of

[4]primary visual cortex
[5]secondary visual cortex, lateral area

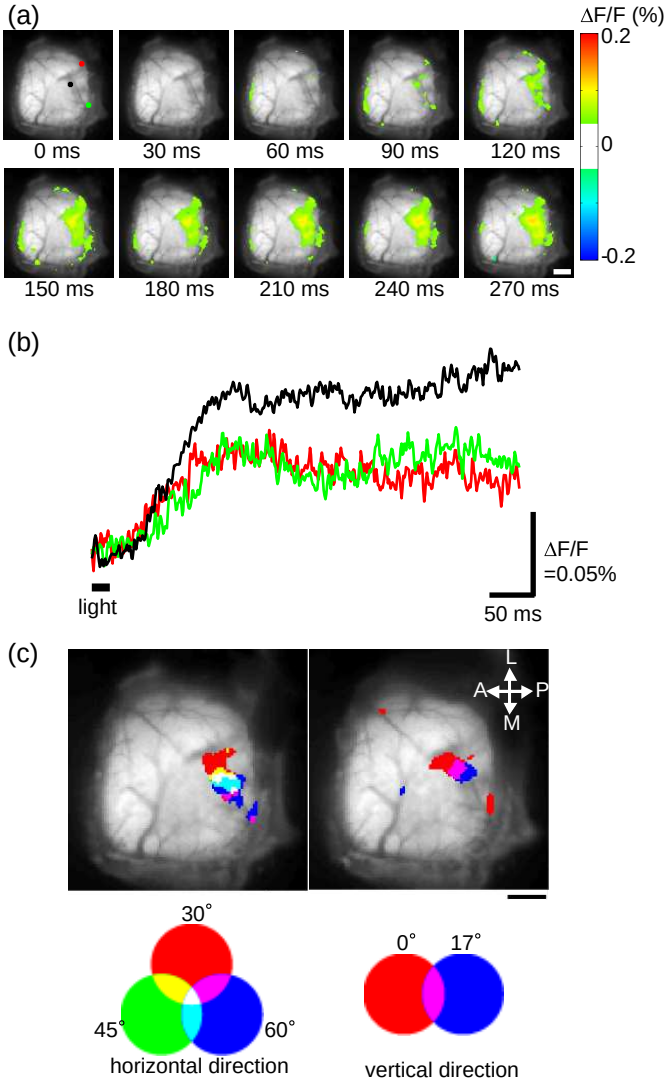

Fig. 6. Responses of visual cortex to visual stimulation. (a) Time-lapse images of voltage-sensitive dye signal changes (ΔF/F) in the right visual cortex. Less than 0.04% of fluorescent changes were cut off. (b) Time courses of the signal changes at the regions indicated in the upper left (0 ms) image in (a) (black, red, and green dot). (c) Extracted regions which responded different incident angle of the LED light. Colors corresponded with each color indicator below. A: anterior, P: posterior, L: lateral, M: medial. Scale bar, 1 mm.

the response at the initially responsive region in V1 was 39 ± 7 ms (n = 3). The region which potently responded to the stimulation was altered by displacement of the visual stimulus position (figure 6(c)). Horizontal displacement of the stimulus position (30, 45 and 60 degrees from midline) altered the responsive region from anterolateral to posteromedial in V1. Vertical displacement of the stimulus position (0 and 17 degrees from eye level) altered the responsive region from posterior to anterior. These displacement of the responsive regions corresponded with the retinotopic map of V1 shown by electrophysiological studies [20], [21] and intrinsic signal imaging study [22].

IV. Discussion

A. What can be revealed by optical recordings?

In this paper, we introduced some results of optical recordings: the spontaneous $[Ca^{2+}]_i$ transients in the striatal cells with single-cell spatial resolution (figure 3), evoked $[Ca^{2+}]_i$ transients in the visual cortical slice with high time resolution (figure 4, 5), and in vivo voltage imaging in the visual cortex with ultra-high time resolution (figure 6).

As shown in the striatal experiments, the Ca^{2+} imaging combined with a transgenic mouse, which expressed fluorescent proteins in a specific type of cells, enables monitoring the $[Ca^{2+}]_i$ transients in many of cells simultaneously under discriminating the cell types. The long-lasting spontaneous $[Ca^{2+}]_i$ transients in the striatum might be due to Ca^{2+}-release from the intracellular Ca^{2+} store (see Results A). This type of $[Ca^{2+}]_i$ transients concerned with signal transduction in the cell and related to modulation of the functions of proteins, such as enzymes and receptors, the gene expression, and morphological changes of cellular processes [23]. Hence, the spontaneous $[Ca^{2+}]_i$ transients thought to be concerned with the synaptic modulation and the neuron-glia communication [24], [25]. Therefore, study of the spontaneous $[Ca^{2+}]_i$ transients contributes to reveal the signal processing in the central nervous system.

As shown in the visual cortical slice experiments, Ca^{2+} imaging combined with electrical stimulation revealed the signal propagation in the neuronal circuit. The evoked $[Ca^{2+}]_i$ transients in cell somata thought to be caused by the action potential generation [9], [10]. Unlike the electrophysiological studies, Ca^{2+} imaging can observe the activities of whole population of neurons in the observation area. Therefore, optical monitoring of the $[Ca^{2+}]_i$ transients enables recordings of the activity of the huge neuronal population, simultaneously, and analyzing the functional network in the neuronal circuit.

We showed that the optical recording with voltage-sensitive dye could follow the membrane potential changes in the visual cortex caused by sensory stimulation in vivo (figure 6). For in vivo experiment, extracellular recordings were often conducted to record field potentials. However, field potentials are affected by currents in the extracellular space. Thus, field potentials are not restricted to the site of changes in membrane potential, whereas optical signals are restricted to the site of membrane potential change. Therefore, high speed optical imaging with a voltage-sensitive dye provides a much better spatial resolution than electrical recording of field potentials [14].

B. Limitation of the optical recordings

As described in the introduction, Ca^{2+} imaging can monitor various types of neuronal activity. It is very useful for understanding the cellular physiology, but there are somewhat difficulties to determine the Ca^{2+} sources of the $[Ca^{2+}]_i$ transients. Especially in the recordings of the spontaneous $[Ca^{2+}]_i$ transients, we did not apply the stimulation, thus, we could not know which intrinsic stimulation arose the $[Ca^{2+}]_i$ transients. For resolving this issue, the pharmacological and/or electrophysiological experiments are needed. Therefore, combination of Ca^{2+} imaging and conventional physiological experiments should be needed for getting more credible insights.

Ca^{2+} imaging has high spatial resolution more than single-cell level. In spite of the nature, we could not distinct the cell location of which the evoked $[Ca^{2+}]_i$ transients were observed (figure 4, 5). The electrical stimulation to a massive tissue, like a slice preparation, causes the $[Ca^{2+}]_i$ transients in various depth of cells, thus the fluorescence of the Ca^{2+}-indicator comes from every depth in the tissue, and it prevents the detection of the location of the active cell somata. Therefore, if the single-cell spatial resolution is needed, we should use a laser scanning confocal microscope (cf. [26]), raising the spatial resolution, rather than a conventional epifluorescence microscope.

The voltage-sensitive dye imaging can record the membrane potential changes directly at just the site of the membrane potential changing. Unfortunately, voltage-sensitive dyes have small signals (usually less than 1%). Thus, we can get only low signal-to-noise ratio data. To raise the signal-to-noise ratio, we should average the many trials of acquisition. Although, in general, fluorescence changes of voltage-sensitive dyes may relate to the synaptic potential [14], [27], there are no guarantee that the optical signals measured are related to which type of membrane potential changes (eg. synaptic potential or action potential). Moreover, especially in slice or in vivo study, the voltage-sensitive dyes do not enable single-cell resolution recording, and as mentioned above, there are no information of the depth of the fluorescence signals. To confirm these issues, the electrophysiological measurement should be conducted.

C. Integration of the results from in vitro and in vivo experiments

In the visual cortex, we conducted the imaging studies in in vitro slices and in vivo preparations. The neuronal connections remain intact in in vivo preparation, therefore, the in vivo experiment has a great advantages, especially in the study concerned with the sensory system. Thus, we can know the neuronal coding manner or the neuronal representation to the sensory stimulation. However, it is hard to understand precisely the neuronal circuitry which is the basis of the observing signal. In the slice (in vitro) preparation, we cannot know the neuronal representation to the sensory input. But, we can observe the cellular activity with single-cell resolution and reveal the spatio-temporal properties of the signal propagation in the local neuronal circuit. Those observations lead to understand the detail mechanisms concerned with the signal propagation or the cellular interaction. Therefore, to reveal the neuronal information processing, we should consider the results obtained from both in vitro and in vivo. At present, we are accumulating the data from both in vitro and in vivo experiments. In the near future, we must analyze the in vivo data in combination with in vitro data.

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