Simultaneous recording of fluorescence and electrical signals by photometric patch electrode in deep brain regions in vivo

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Hirai Y, Nishino E, Ohmori H. Simultaneous recording of fluorescence and electrical signals by photometric patch electrode in deep brain regions in vivo. J Neurophysiol 113: 3930–3942, 2015. First published March 11, 2015; doi:10.1152/jn.00005.2015.—Despite its widespread use, high-resolution imaging with multiphoton microscopy to record neuronal signals in vivo is limited to the surface of brain tissue because of limited light penetration. Moreover, most imaging studies do not simultaneously record electrical neural activity, which is, however, crucial to understanding brain function. Accordingly, we developed a photometric patch electrode (PME) to overcome the depth limitation of optical measurements and also enable the simultaneous recording of neural electrical responses in deep brain regions. The PME recording system uses a patch electrode to excite a fluorescent dye and to measure the fluorescence signal as a light guide, to record electrical signal, and to apply chemicals to the recorded cells locally. The optical signal was analyzed by either a spectrometer of high light sensitivity or a photomultiplier tube depending on the kinetics of the responses. We used the PME in Oregon Green BAPTA-1 AM-loaded avian auditory nuclei in vivo to monitor calcium signals and electrical responses. We demonstrated distinct response patterns in three different nuclei of the ascending auditory pathway. On acoustic stimulation, a robust calcium fluorescence response occurred in auditory cortex (field L) neurons that outlasted the electrical response. In the auditory midbrain (inferior colliculus), both responses were transient. In the brain-stem cochlear nucleus magnocellularis, calcium response seemed to be effectively suppressed by the action of metabotropic glutamate receptors. In conclusion, the PME provides a powerful tool to study brain function in vivo at a tissue depth inaccessible to conventional imaging devices.

fluorescence calcium signal; local field electrical responses; acoustic local responses; brain activity in vivo; photometric patch electrode

HIGH-RESOLUTION MULTIPHOTON imaging has enhanced our understanding of signaling molecule operations within neural tissues in the brain in vivo and in regions as small as dendritic spines (Chen et al. 2011; Sabatini et al. 2002; Svoboda and Yasuda 2006; Yagishita et al. 2014). Spatiotemporal imaging of calcium ions is particularly important because of the versatility of calcium ions as intracellular signaling molecules (Berridge et al. 2000; Hagenston and Bading 2011). More specifically, the fluorescence signal of calcium ions has been interpreted to reflect the electrical activity of neurons in the brain (Chen et al. 2013; Grienberger et al. 2012; Ikegaya et al. 2004), and it has been successfully used to demonstrate the functional organization of the primary sensory cortex (Issa et al. 2014; Ohki et al. 2005). However, there are critical disadvantages to using multiphoton microscopy in vivo. First, because of the scattering of light in living tissues, high-resolution imaging is impossible in deep brain tissues (Helmchen and Denk 2005; Mittmann et al. 2011). Thus the depth is limited to a maximum 1 mm from the brain surface, and the majority of neural activity in vivo is beyond the range of microscope imaging. Second, simultaneous electrical recording and imaging in vivo is technically challenging (Kitamura et al. 2008; Margrie et al. 2003); however, electrical activity of neurons and neural circuits in the brain is a crucial step for elucidating brain function (Chadderton et al. 2014; Eccles 1964; Hubel and Wiesel 1959).

The depth limitation of imaging has been challenged by various approaches, including enhancing the penetration of light through brain tissue (Horton et al. 2013; Mittmann et al. 2011), using microendoscopy or fibered fluorescence microscopy (Flusberg et al. 2005; Jung et al. 2004; Vincent et al. 2006), and using a microprism to image multiple neurons across a large range of cortical layers (Andermann et al. 2013). Nevertheless, high-resolution imaging is extremely difficult at the deepest layers of the cortex, midbrain, or brain-stem nuclei in vivo. Microendoscopy that assembled multiple optic fibers and multiple metal electrodes was used to record electrical signals in vivo (Hayashi et al. 2012). However, the endoscope was not appropriately designed to track the precise time course for the electrical and fluorescence signals. Presently, the most sophisticated approach for the in vivo recording of neuronal electrical and fluorescence signals is a microprobe based on dual-core fiber optics, which uses an optical fiber core to excite and collect fluorescence and an electrolyte-filled hollow core to record electrical activity (Dufour et al. 2013; LeChasseur et al. 2011). However, because recording sites are separated by a distance of ~10 μm according to the parallel arrangement of two measuring cores, the device is not suitable for precise tracking and comparison of electrical and optical response of neurons.

In contrast to the aforementioned approaches, we used a patch electrode to measure both the electrical and the fluorescence signals; thus we have named the electrode a photometric patch electrode (PME). The PME enabled us not only to excite and collect fluorescence signals from dye-filled neurons, but also to apply chemicals locally in brain regions of varying depth. We measured fluorescent calcium signals and electrical signals in response to acoustic stimulation in several auditory nuclei across the auditory pathway from the cochlear nucleus magnocellularis (NM) in the brain stem to the auditory cortex (field L) of the chick in vivo. These nuclei are located at a depth of 3–7 mm from the brain surface. Moreover, we demonstrated how sound information is transformed into cal-
to measure fluorescence signals at faster speeds [1 ms or shorter sampling intervals; Fig. 1A(i)]. Here, signals were sampled by trigger and were ensemble-averaged optionally. The choice of detector depended on the type of experiment and the nature of the fluorescence signal, slow or fast kinetics. The PME is made of quartz capillary with a nickel (Ni)-coated exterior (Fig. 1B). We expected Ni coating to increase the reflection of light within electrode and to protect the electrode surface from stains. The proximal end of the PME was polished (Fig. 1C) to enhance transmission of light between the PME and the optic fiber bundle. Electrical output of the PME was made through a Teflon-coated silver wire and was connected directly to the headstage of a patch-clamp amplifier (Fig. 1B). The PME was filled with electrolyte based on artificial cerebrospinal fluid (ACSF; compositions are in a following section) with additional chemicals in some experiments to be ejected locally by pressure.

**Bifurcated optic fiber bundle.** A bifurcated fused-end optical fiber bundle (3-mm core diameter, an assembly of 100 fine optical fibers of 0.22 numerical aperture; CeramOptec, East Longmeadow, MA) was used both to transmit laser light to dye-filled neurons and to detect fluorescence through the PME. A thinner branch (10% of fibers) delivered laser light to PME, and a thicker branch (90% of fibers) led fluorescence signal from the PME to the detectors (Fig. 1A).

**Laser.** A semiconductor 488-nm laser (50 mW; Excelsior 488; Spectra-Physics, Santa Clara, CA) was used to excite Oregon Green BAPTA-1 (OGB-1; Molecular Probes, Eugene, OR). The transmission of the laser light was shutter-controlled. The laser power measured at the distal common end of the bifurcated optical fiber bundle was 16 mW and was reduced to ~4 mW at the distal end of the PME holder. The laser power was reduced by inserting 25–50% neutral density (ND) filter and furthermore by a light-chopper (50% duty cycle) in photomultiplier tube measurements (Fig. 1A). In some experiments, a 532- or 445-nm laser was used to excite Alexa Fluor 555 dextran (Fig. 2B) or the enhanced cyan fluorescent protein (eCFP)-enhanced yellow fluorescent protein (eYFP) fluorescence resonance energy transfer (FRET) compound (Fig. 2D), respectively.

**PME.** PME was made from a quartz capillary (1.5-mm outer diameter, 1.0-mm inner diameter, 75-mm length; Medical Agent, Kyoto, Japan). The capillary exterior was Ni-coated using an autocatalytic chemical reaction (electroless Ni-plating method; Melplate MB; Meltech, Tokyo, Japan) except for the middle 10 mm because coating there was evaporated by heat during electrode pulling. Both ends of the quartz capillary were polished by an optical-fiber-polishing machine (Fig. 1C; SpecPro Polisher; Krell Technologies, Westford, MA). After washing and drying, the quartz capillary was pulled by a laser puller (P-2000/G; Sutter Instrument, Novato, CA) to make a patch electrode of approximately 2- to 3-μm tip diameter (approximately 1–3 MΩ when filled with ACSF; concentrations in mM: 125 NaCl, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, and 17 glucose, pH 7.4). Optical coupling was direct, without any lenses between the bifurcated optical fiber bundle and the PME (Fig. 1B).

The electrical signal from the PME is led by Ag wire (100-μm diameter) coated with Teflon (10-μm thickness; A-M Systems, Carlsborg, WA) to insulate the wire from the Ni-coated capillary surface (Fig. 1B). The electrode holder was designed to align the PME and optic fiber bundle (Fig. 1B). The holder assembly was made airtight to control the pressure within the PME using a gasket and a silicone tube that wrapped tightly around the electrode holder and the stainless steel (AP5), or 4-aminopyridine (4-AP) that were ejected from the tip locally by pressure (approximately 0.5–3 psi).

**Grating spectrometer.** The fluorescence spectrum was measured by a grating spectrometer (C5094), image intensifier (C7979-81), and CCD camera (ORCA-R2) in series [all from Hamamatsu Photonics, Hamamatsu, Japan; Fig. 1A(i)]. The signal-to-noise ratio of the spectrum was improved by parallel processing of multiple spectra and
Fig. 2. Recording of fluorescence signals in brain slices using a spectrometer. A: the PME assembly (right) and a laser-excited granule cell from the rat hippocampus (left). The inset shows the laser-illuminated neuron at a larger magnification with a calibration bar of 10 μm where the PME tip is indicated by dotted lines. B: 2 fluorescence spectra recorded from a dye-filled nucleus magnocellularis (NM) neuron of the chick by the on-cell and detached PME. Cells were labeled with Alexa Fluor 555 dextran (10-kDa mol mass) and were excited by a 532-nm laser. a.u., Arbitrary units. C: the percentage of the drop in fluorescence intensity after detachment of the PME was plotted for individual cells. After detachment of the PME, the fluorescence intensity dropped to approximately 42–88% (68 ± 9.5%, n = 115 cells). The difference signal was not affected by the type of fluorophore; thus data from various dyes were combined. D: a stacked display of time series spectra shows fluorescence resonance energy transfer (FRET) responses from a rat cortical pyramidal neuron after subtraction of the dark level (Dark in B). The spectrum was sampled at 20-s intervals, and every other data are plotted. Spectra before (dotted line) and after (thick black solid line) the bath application of a high-KCl medium (30 mM; arrow) are indicated in D and E. The background spectrum (BG; gray broken line) was overlaid on the 1st time series record. Arrows Y and C, peaks of enhanced yellow fluorescent protein (eYFP) and enhanced cyan fluorescent protein (eCFP), respectively. E: the BG was subtracted to show the spectrum change induced by KCl. Vertical gray bars indicate the wavelength ranges used to calculate eCFP and eYFP fluorescence intensity. In A and D, neurons were transfected by an eCFP-eYFP-based FRET compound and were excited by a 445-nm laser (Takatsuka et al. 2005).

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Evaluation of fluorescence signal. The fluorescence signal ($\Delta F$) was evaluated as a ratio relative to the base level at the start of each record (F). In spectrometer mode, a dark level was calculated at the wavelength range separated from the measured fluorescence and was subtracted from the denominator ($F - F_{dark}$). However, the dark level was not subtracted in photomultiplier tube mode because only the wavelength range of signal fluorescence was measured. $\Delta F$ was thus evaluated as $\Delta F/(F - F_{dark})$ for the spectrometer and $\Delta F/F$ for the photomultiplier tube.

Cross-correlogram to evaluate amplitude pairs of field current and fluorescence signals. Because fluorescence signals were delayed to current responses, corresponding peaks of fluorescence and current signals were identified by displacing the fluorescence signal by the amount of delay between the two signals. Delay was calculated from the correlogram by using the MATLAB function xcorr. Paired traces were overlaid, and the corresponding amplitude of the two peaks was evaluated as a ratio relative to the base level at the start of each record (F). In spectrometer mode, a dark level was calculated at the wavelength range separated from the measured fluorescence and was subtracted from the denominator (F – Fdark). However, the dark level was not subtracted in photomultiplier tube mode because only the wavelength range of signal fluorescence was measured. $\Delta F$ was thus evaluated as $\Delta F/(F - F_{dark})$ for the spectrometer and $\Delta F/F$ for the photomultiplier tube.

Background fluorescence light. The level of background fluorescence light was high with the PME recording system and on average occupied 68% (approximately 42–88%) of the total fluorescent light detected from the dye-filled single neurons (Fig. 2C). The background fluorescence was likely attributable to the PME assembly including electrode holder, PME, and optic fiber bundle itself as illuminated by strong laser light (Fig. 1B). However, the background level was stable, provided it was illuminated by a constant laser power. A brighter light emission from the neuron increases the fluorescence light level captured by the PME. Consistently, the resolution of signal fluorescence was improved when the fluorescence light level was increased severalfold of that on the brain surface in the recording from the dye injection site in vivo (Fig. 3, B and C). The fluorescence light captured by the PME in these in vivo experiments may reflect the number of neurons in ensemble-synchronized activity or the amount of the dye that filled local neurons.

Animals. Chicks approximately 2–6 days after hatching (approximately P2-6) were used for in vivo experiments and chicks (approximately P2-3) and rat pups (P9) were used for slice experiments as detailed in the following sections. All experimental procedures were reviewed and approved by the Animal Experimentation Committee of the Kyoto University.

Recording from neurons in vivo and acoustic stimulation. Chicks were anesthetized with an intraperitoneal injection of urethane (1 mg/g body wt). Additional injection of urethane or chloral hydrate was made to maintain the level of anesthesia. Surgical procedures and devices used for acoustic stimulation were previously described (Fukui et al. 2006; Nishino et al. 2008).

Bolus injection of OGB-1 AM (1 mM in ACSF; Murayama et al. 2007; Stosiek et al. 2003) was made by using a glass electrode with a tip diameter of approximately 20–30 µm. A tungsten wire (15 µm, Teflon-coated) was inserted into the electrode to detect the field current responses, corresponding peaks of fluorescence and current were overlaid, and the corresponding amplitude of the two peaks was evaluated as a ratio relative to the base level at the start of each record (F). In spectrometer mode, a dark level was calculated at the wavelength range separated from the measured fluorescence and was subtracted from the denominator (F). In spectrometer mode, a dark level was calculated at the wavelength range separated from the measured fluorescence and was subtracted from the denominator (F).
vertically to the field L at a depth of ~3 mm from the cerebral surface, 1.5 mm lateral, and approximately 1.2–1.5 mm rostral of the lambdoid suture; the inferior colliculus (IC) at a depth of ~7 mm from the cerebral surface, approximately 3.7–4.0 mm lateral, and approximately 1.7–2.0 mm rostral of the lambdoid suture; or the NM at a depth of ~7 mm, approximately 1.3–1.4 mm lateral at the rostrocaudal level of the inion through the cerebellum. The NM was approached by tilting the head with the beak downward by 30°. After 1 h of OGB-1 AM injection, the PME was inserted vertically (Fig. 3A).

The electrode was advanced by a motor-driven micromanipulator (DMA-1050; Narishige, Tokyo, Japan). While the PME was approaching, acoustic search stimulus of white noise burst (80-ms duration with 5-ms rise and fall envelope) was applied every 5 s, and fluorescence signal was measured (Fig. 3B and C). The OGB-1 fluorescence response to acoustic stimulus was tested when the fluorescence level became stable. Binaural acoustic stimulation of 80-dB white noise was generally applied unless otherwise specified. Sound stimulation and data acquisition were controlled by customized software written in MATLAB. All experiments were performed in an electrically shielded soundproof chamber placed within a soundproof booth. Local field currents were low-pass filtered at 15 kHz (2-pole) and were recorded using a patch-clamp amplifier (MultiClamp 700B; Axon Instruments, Union City, CA).

**Brain slices.** Chicks were injected with OGB-1 AM as described above and decapitated after 1 h in deep anesthesia, and brain-stem slices at the level of the NM (thickness 300 μm) were made as previously described (Kuba et al. 2003). After the incubation of slices at 35°C for approximately 30–45 min in high-glucose medium (75 NaCl, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, and 100 glucose, pH 7.4), electrical and fluorescence recordings were made at room temperature (approximately 25–27°C) in ACSF by using an upright microscope (BX51WI; Olympus, Tokyo, Japan). In some brain slice experiments, NM neurons were loaded with Alexa Fluor 555 dextran (10-kDa mol mass) retrogradely by dye injection to the contralateral side a day before the experiment (Fig. 2B). Slices of rat cerebral cortex or hippocampus were similarly made for experiments in Fig. 2, A, C, and D. These neurons were labeled with the eCFP-eYFP compound (Takatsuka et al. 2005).

**RESULTS**

**Validation of PME to measure fluorescence signals from dye-filled neurons in brain slices.** A beam of laser light from the tip of the PME excited the fluorescence of a neuron expressing a fluorescent protein (Fig. 2A, inset). The fluorescence was detected by a photomultiplier tube in gabazine (0.5 mM). A: local field currents (black) and fluorescence responses (gray) to acoustic stimuli (gray line at the bottom). Current and fluorescence responses were generally coupled. Uncoupled responses are indicated by ⊗ or • (see main text). B: the ensemble averages of fluorescence (top) and current (bottom) signals. Individual traces are overlaid. C: 2 examples of the cross-correlogram between current and fluorescence signals. From top to bottom, each subpanel illustrates the cross-correlogram, fluorescence, current traces, and acoustic stimulus. In the correlogram, t indicates the delay time, and x.c. represents the correlation coefficient between 2 traces. The short black line at the right end of the fluorescence signal indicates the time displaced (delay time) to align the current and fluorescence signals to measure the peaks of a pair (boxes). D: scatter plots of paired fluorescence (F) and current amplitudes. Both acoustic and spontaneous responses are included. Liner fit slope is 0.94%/nA (r = 0.58, lower group) and 0.60%/nA (r = 0.76, upper group).
long-pass filter and a side peak of the laser. By detachment and withdrawal of the PME out of the microscope view field, the fluorescence peak near 570 nm decreased to nearly 40% of the intensity detected by the cell-attached PME. Importantly, the profiles of the additional two peaks were not affected by touch or detachment of the PME (Fig. 2B). Similarly, the fluorescence intensity difference of eCFP, eYFP, and OGB-1 was measured between the cell-attached PME and after detachment of the PME, which was not different among dyes, and all data were plotted in Fig. 2C (eCFP, 70.0 ± 9.6%, n = 50 cells; eYFP, 66.5 ± 9.6%, n = 50 cells; OGB-1, 66.3 ± 7.9%, n = 15 cells; grand average is a decrease to 68 ± 9.5% of the cell-attached intensity, mean ± SD, range approximately 42–88%, n = 115 cells). Accordingly, an average of 32% of the fluorescence signal was captured from the dye-filled neuron, and the remaining background fluorescence was attributable to the PME assembly.

We further tested the spectrometer by measuring FRET responses because FRET-based indicators are useful and have diverse applications (Miyawaki 2003; Tantama et al. 2012). We expressed an eCFP-eYFP-based Ca2+ indicator protein in neurons of the rat cortex using the Sindbis virus expression system (Takatsuka et al. 2005). A time series of fluorescence spectra was measured in slices (300 μm) using a cell-attached PME filled with ACSF (Fig. 2D). The FRET response was induced by a high-KCl medium (30 mM). The fluorescence emission near 525 nm was reduced, and the intensity near 490 nm was increased, which corresponds with peaks of eYFP and eCFP, respectively. After subtracting the background spectrum that was measured after detachment of the PME, the eCFP signal increased (25%) and the eYFP signal decreased (27%; 35% change in FRET ratio; Fig. 2E).

Validation of calcium fluorescence measurements in auditory nuclei of the chick in vivo. The transfection efficiency of the FRET-based calcium indicator used in the rat cerebral cortex (Fig. 2D) was very low in the chick brain tissues. We therefore used a synthetic calcium indicator dye in the in vivo experiments conducted in avian auditory nuclei. Fluorescence calcium responses to acoustic stimulation were measured after a bolus injection of OGB-1 AM (Grienberger and Konnerth 2012; Murayama et al. 2007; Stosiek et al. 2003). The field L (auditory cortex; Biederman-Thorson 1970; Karten 1968), IC (midbrain auditory center through which all ascending and descending auditory pathways pass; Ehret and Romand 1997; Ono and Oliver 2014), and NM (brain-stem cochlear nucleus that receives time-coding inputs from auditory nerve fibers, ANFs; Carr and Code 2000) are major nuclei in the auditory pathway and located deep in the brain tissue (approximately 3–7 mm from the surface of the brain). We used these brain regions to test the PME because the neurons are stably activated by acoustic stimulation.

When the ACSF-filled PME vertically approached the OGB-1-labeled neurons in the field L (Fig. 3A), the fluorescence spectrum increased (Fig. 3B), and it reached a maximal signal ~3,000 μm from the brain surface (Fig. 3C). The acoustic local field response was monitored in parallel and was robust at this depth. The spectrum increased at wavelengths from 500 to 600 nm, but the laser excitation peak (488 nm; Fig. 3B) was not affected by the penetration. In the field L, spontaneous low-amplitude currents were observed, oscillating at ~1 Hz (Fig. 3, D and E). These currents are reminiscent of the spontaneous activity observed in the mammalian cortical network (Hromadka et al. 2013; Kraus et al. 2014; Steriade et al. 1993; Wang 2001).

The fluorescence signal was processed from a time series of single-trial spectra averaged between 515 and 550 nm and overlaid with the electrical signal (Fig. 3F). Several peaks of local field current coincided with fluorescence signal peaks and acoustic stimulus. The current responded to the on-and-off timing of acoustic stimulus (Fig. 3, F and J), and the fluorescence signal changed approximately 1–2% from the base level [ΔF/(F−F dark)]. However, the coincidence of these signals was not obvious in the experiment conducted with the ACSF-filled PME because of noisy field current activity. The field currents became larger with a more regular timing when gabazine (5 mM in ACSF) was locally applied through the PME (Fig. 3G). Large field currents of >1 nA occurred at intervals of approximately 3–4 s, and large fluorescence signals (approximately 3–5%) were tightly coupled. Application of 6,7-dinitroquinoxaline-2,3-dione (DNQX) eliminated both the local current and the calcium signal (Fig. 3H). This result shows that the fluorescent calcium responses are of postsynaptic origin. Generally, the current waveform was not distinctly different between the spontaneous activity and the acoustic response (Fig. 3, J and K). Thus acoustic stimuli may have triggered the local activity that underlies the local responses. Local application of AP5 with gabazine did not affect the tight coupling of the field currents with the fluorescence signals; however, approximately 15–30 min after AP5 application, the falling phase of the current became sharp (Fig. 3K). Accordingly, N-methyl-D-aspartate (NMDA) receptor activation may not contribute either to the local current or the induction of calcium signals.

Validation of fast fluorescence sampling by a photomultiplier tube in vivo. Despite a high sensitivity to light, the spectrometer was too slow (0.3 s) to track the detailed time course of fluorescence signals in relation to electrical activity (Fig. 3, I–K, and Fig. 6E). Therefore, we recorded fluorescence signals using a photomultiplier tube with a higher time resolution [1 ms or less; Fig. 1A(ii)]. Fluorescence signals in the field L were enhanced by local application of low concentrations of gabazine (approximately 0.05–0.5 mM in ACSF). Higher concentrations of gabazine enhanced spontaneous activity so strongly that field currents induced by acoustic stimuli were masked (Fig. 3G). Without gabazine, the intense field activity caused a poor resolution of fluorescence signal (Fig. 3F).

With low concentrations of gabazine, both field currents and fluorescence responses generally occurred simultaneously on acoustic stimulation (Fig. 4A). When aligned to acoustic stimulation, field currents preceded fluorescence responses (23 ± 6.5 ms; Fig. 4B; average 54.2 ± 18.9 ms, 13 experiments). The current and fluorescence amplitudes appeared well-correlated. The degree of correlation was evaluated by using cross-correlation to estimate the delay in measuring corresponding amplitude peaks. The data were then used to create scatter plots. Figure 4C illustrates two examples of the cross-correlogram calculated from the pair of fluorescence signal and local current. A delay was taken from the peak shift as 80 and 70 ms from time 0, and the correlation coefficients were 0.84 and 0.82, respectively. Pairs of current and fluorescence signal amplitudes were measured from corresponding peaks (Fig. 4C).
after displacing the fluorescence trace leftward based on the delay time and were scatter plotted (Fig. 4D). The scatter plots showed that the data were distributed into two groups, and the size of the field current and the fluorescence response was positively correlated in each group. Field currents smaller than approximately 2–3 nA induced fluorescence responses <4% (ΔF/F, lower group). Field currents larger than ~2 nA generated large fluorescence responses (upper group). Here, the scatter plots had a shallower slope, and the size of the fluorescence response was nearly saturated at ~10%. Calcium fluorescence signals were saturated easily because of the nature of the indicator dye (K$_2$ 170 nM; Molecular Probes) or by some safety mechanisms intrinsic to neural activity (Berridge et al. 2000; Mattson et al. 2008). Field responses reflect synchronized activity of nearby neurons. We propose that the two response groups may arise from an ensemble of neurons at subthreshold or suprathreshold activity, respectively.

Uncoupled responses. Fluorescence and current responses occasionally occurred uncoupled. We observed that fluorescence responses failed to occur or their size diminished when the field current occurred in the falling phase of preceding fluorescence responses (▲, Fig. 4, A and C). This could be attributed to some refractoriness of fluorescence response. Uncoupling was also observed during fluorescence responses that occurred alone or with a very small electrical response. In the top trace of Fig. 4A, some medium-sized fluorescence responses occurred with only a small current response of the pair (○). The field current was notably small and was likely generated far from the tip of the recording PME. Accordingly, the fluorescence signal of the pair was also generated at a distance (see Discussion). This type of uncoupling was clustered primarily in the early phase of a recording session and was observed in 5 out of 13 experiments; however, the incidence was low (0.9 ± 1.4% pair of traces; 9 pairs out of 991 total pairs, across 13 experiments). Two experiments were excluded from the 13 experiments mentioned above. In 1 of these 2 experiments, the current and fluorescence signals were apparently uncoupled in 70 out of 334 consecutive records (21%). Half of the uncoupled traces (35 traces) occurred in the 1st 8 min of a 38-min recording time. In this experiment, medium-sized fluorescence signals occurred repeatedly with small or on-off-blip current responses. The electrode tip may have failed to hit the activity area within the field L. In the other excluded experiment, 125 acoustic response trials were recorded. Signals were well-coupled for the 1st 15 min (100 trials), and both local field currents (~1 nA) and fluorescence responses (approximately 3–4%) were large; however, local field currents suddenly diminished (approximately 0.1–0.3 nA) and eventually disappeared, whereas fluorescence responses markedly decreased but remained (approximately 1–2%). The PME was likely dislocated during the recording session and failed detecting signals from the activity center. These data with uncoupled responses were discarded.

Assay of acoustic responses in the ascending auditory pathway. We recorded and compared the calcium fluorescence response with acoustic stimulation in the NM, IC, and field L, which are part of the ascending auditory pathway to the cortex (field L). Local field current and fluorescence calcium responses were enhanced in most experiments by local application of 4-AP (approximately 5–20 mM in ACSF). In the field L, acoustic stimulation generated multiple peaks in field currents, and the number of current peaks increased with time after the application of 4-AP (Fig. 5A). The fluorescence response tightly followed the local field current response.
15 min, burst firing emerged. Occasionally, bursts continued for >70 s and occurred repeatedly at intervals of ~130 s (Fig. 5B). Acoustic responses were evoked by either binaural or contralateral sound stimuli (Fig. 5C). Whereas calcium responses were prolonged and robust in the field L, calcium responses in the IC were transient, and the calcium responses were minimal in the NM.

The IC is located at a depth of ~7 mm from the cortical surface (Fig. 6A). Acoustic stimulation induced robust field responses (Fig. 6B), and sharp field currents occurred spontaneously when recorded with 4-AP. Fluorescence responses <1% were induced by acoustic stimulation and spontaneous activity. Ensemble-averaging demonstrated that acoustic responses were generated by onset type or primary-like neurons that responded to contralateral sound (Fig. 6C). Fluorescence calcium responses were fast rising and decayed without delay. The peak amplitude of current and fluorescence signals was not affected by the duration of acoustic stimulation (approximately 5–200 ms; Fig. 6D). When fluorescence signals were measured using a spectrometer recording system and were induced by a much longer acoustic stimulation of 4 s, signal size of peak fluorescence was not affected (Fig. 6E). We recorded single-unit responses to acoustic stimulation using the ACSF-filled PME. From these spectrometer recordings, it was also obvious that time resolution was low and failed to track fluorescence signals appropriately, particularly in the response to acoustic stimuli of short duration. Overall, fluorescence responses occurred quickly in the IC and disappeared shortly after the stimuli.

The NM is located equally deep in the brain stem (~7 mm from the brain surface), where ANFs make glutamatergic synapses (Fukui and Ohmori 2004; Trussell 1999). Despite enhancing the neuronal excitability by local application of 4-AP, it was difficult to detect fluorescence calcium responses to acoustic stimulation in the NM; however, field current responses were stably recorded (Fig. 7A). Small fluorescence calcium and field current responses to acoustic stimulation were observed after application of 4-AP, TEA, and gabazine (Fig. 7B). NM neurons were spontaneously active under these recording conditions, and individual local responses were accompanied with small fluorescent calcium signals (Fig. 7C). In brain-stem slice preparations including NM and nucleus laminaris (NL), small fluorescence responses were observed in the NM by applying antidromic electrical stimulation to the NM projection fiber to the NL (Fig. 7D) when slices were bathed in ACSF (0.14 ± 0.03%, n = 9 cells) or in 4-AP (approximately 0.25–0.5 mM in ACSF, 0.17 ± 0.11%, n = 11 cells). However, orthodromic stimulation of the ANFs failed to generate a fluorescence calcium response when slices were based in 4-AP (0.25 mM in ACSF; Fig. 7E). Small fluorescence calcium...
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Fig. 7. Nucleus magnocellularis (NM) responses to acoustic stimulation in vivo or to electrical stimulation in slices as recorded by a photomultiplier tube. A: ensemble-averaged acoustic responses of local field current (black) and OGB-1 AM fluorescence (gray) recorded in vivo with a 4-AP-filled PME (5 mM). B and C: acoustic responses recorded with 4-AP (10 mM), TEA (10 mM), and gabazine (0.5 mM) in B (ensemble-averaged) and C (single traces from the data set of B). D: the fluorescence signal induced by antidromic electrical stimulation of NM projection fibers to the nucleus laminaris was recorded in a brain-stem slice (300 μm) bathed either in ACSF (black) or in ACSF with 0.25 mM 4-AP (gray). E: the fluorescence signal was recorded in response to orthodromic electrical stimulation to auditory nerve fibers in a brain-stem slice bathed in ACSF with LY341495 (1 μM) and 4-AP (0.25 mM; dotted line, overlaid with individual traces in gray) or in 4-AP (0.25 mM in ACSF; solid line). Electrical stimulation (a train of 20 stimuli, 100 μs at 100 Hz) was applied in D and E, and signals were ensemble-averaged. OGB-1 AM was loaded by in vivo injection, and recordings were made using a cell-attached PME filled with ACSF in D and E.

responses (0.17 ± 0.06%, n = 8 cells) were observed in the presence of LY341495 (1 μM; antagonist for group II/III metabotropic glutamate receptor) and 4-AP in ACSF (Fig. 7E). These observations suggest that the calcium influx is inhibited during orthodromic synaptic transmission between ANFs and the NM.

DISCUSSION

We succeeded in using PME to record fluorescent calcium signals and electrical signals simultaneously in deep brain tissues in vivo. The fluorescence signal was sensed either by a spectrometer or a photomultiplier tube. The spectrometer may need a slightly faster sampling rate (presently 0.3 s), but it provided us a full-wavelength range spectrum and is suitable for the analyses of FRET or bioluminescence resonance energy transfer (BRET; Couturier and Deprez 2012) signals. By sensing signals with a photomultiplier tube in combination with a lock-in amplifier, we were able to trace the precise time course of calcium fluorescence with a time resolution of 1 ms or faster. Therefore, the PME is a useful recording system to monitor optical signal simultaneously with electrical signal in deep brain tissues.

Measuring optical signals in deep brain regions in vivo has been challenged using several optical devices, including multiphoton microscope (Andermann et al. 2013; Horton et al. 2013; Mittmann et al. 2011), microendoscope (Hayashi et al. 2012; Jung et al. 2004), or optical microprobe (DuFour et al. 2013; LeChasseur et al. 2011). However, multiphoton microscopy imaging has limited application in the deep brain region because of the scattering of light (Helmchen and Denk 2005). Microendoscopy using a GRIN lens coupled with multiphoton microscope imaging was successful in the deep brain regions (Jung et al. 2004). However, these imaging approaches do not allow the simultaneous recording of neuronal electrical activity. Optogenetic control and simultaneous recordings of neural electrical activity have been attempted using a microendoscope (Hayashi et al. 2012), patch electrode (Katz et al. 2013; Muñoz et al. 2014), or an optical microprobe (LeChasseur et al. 2011). Microendoscopic techniques were able to record light-induced neural activity from channelrhodopsin-2 (ChR2)-expressing neurons and light-induced single-whisker movement in a transgenic mouse (Hayashi et al. 2012). However, the microendoscope has a relatively large tip of several hundred micrometers diameter and can be invasive to brain tissues. A patch electrode holder was invented for the simultaneous recording of electrical signals and optical control of neurons by inserting an optical fiber into a glass electrode. This technique successfully recorded local field potentials and neural intracellular responses activated by photostimulation of ChR2 (Katz et al. 2013) or was used in vivo brain tissue to identify genetically defined neurons that expressed ChR2; in addition, these neurons were further labeled by injecting Neurobiotin through the pipette (Muñoz et al. 2014). The patch electrode is useful for light transmission and recording neuronal electrical activity. However, these experiments have not attempted to measure optical signals from the recorded neurons. Optical measurements were successfully conducted by using a dual-core microprobe that simultaneously measured the electrical activity of neurons with an electrolyte-filled hollow core and excited and collected the fluorescence signals with an optical core; moreover, neural activity was successfully controlled by optogenetics (LeChasseur et al. 2011). This electrode was recently improved to enable the recording of two types of electrical signals simultaneously; the local field potentials were recorded with an aluminum-coated microprobe tip, and single-unit activity was recorded using the electrolyte-filled hollow core (DuFour et al. 2013). This electrode detected fluorescence from distinct fluorescent proteins expressed in nearby cells, indicating the possibility of using a single probe to identify and record from multiple neurons in deep brain regions in vivo. However, the recording sites for optical signals and electrical signals are separated at the tip of the microprobe because of a parallel arrangement of the dual cores. Accordingly, detected optical and electrical signal peaks were separated by approximately 7–13 μm in the brain tissue (Figs. 3e and 4e of LeChasseur et
At the location of the maximum fluorescence signal, the spike amplitude was significantly reduced (Fig. 3e of LeChasseur et al. 2011). Therefore, a close correlation of the time course and size between the fluorescence signal and electrical signal of neurons was not evaluated.

In contrast to these previous approaches, the PME is a single patch electrode that can excite and collect the optical signal while simultaneously recording neuronal electrical signals. The electrical and the optical signals were generally tightly coupled (Fig. 4). However, we occasionally observed uncoupled responses of fluorescence and electrical signals (Fig. 4A). These uncoupled responses may be attributable to two cases of a distant signal source from the PME tip at least. First, the PME tip was longitudinally dislocated from the signal source. As expected, the electrical signal disappeared quickly by the detachment of PME from the cell surface, whereas the fluorescence signal partially remained when measured in slice preparations. When the PME was withdrawn by ∼50 μm, the fluorescence level was reduced to approximately half (46 ± 13%, n = 9 cells) of the fluorescence captured by the cell-attached PME. Second, some fluorescence responses that occurred along the PME may have been captured through the wall of the electrode. The electrical responses should be small as well.

Knowing the exact geometrical relationship between the PME tip and the source of the fluorescence signal in situ is important for the interpretation of the uncoupled and even the coupled fluorescence signals with local field electrical responses; however, we do not have the information, and we can only assume that the electrical recordings were done close to the source of the fluorescence signal. There are some reasons to believe that these signals were recorded in close proximity: 1) the electrical and fluorescence signals were highly correlated (Fig. 4); 2) there are several reports that a profile of emitted light near the tip of a patch electrode is sharply reduced with the distance in the brain tissue (Katz et al. 2013; Muñoz et al. 2014; Pisanello et al. 2014); and 3) we presume a similar situation of a limited excitation of fluorescence around the PME in the experiments in the brain tissue. We measured the fluorescence profile excited by the laser leak from the PME in a bathing solution containing Alexa Fluor 488 under several conditions of external coating of the electrode (Fig. 8). First, we found that the laser leak mainly occurred at the sharp tapered region of the Ni-coated PME (Fig. 8, A1 and A2). Despite a several millimeters separation of the PME tip from the sharp taper of the electrode, the background fluorescence was high around the tip in the bathing solution; accordingly, the tip image was hidden by the fluorescence (Fig. 8A4). The PME was Ni-coated, but the tapered region to the tip of the electrode was bare because the Ni coating was evaporated by the heat of electrode pulling (Fig. 1B; see MATERIALS AND METHODS). Second, we found that the site of the laser leak was displaced toward the tip by Sylgard coating of PME (Fig. 8B). Although Sylgard is a transparent resin, the laser leak was reduced, and the emission of laser light was visible as an intensified fluorescence spot at the tip (Fig. 8B2). Coating the PME with Sylgard mixed with soot greatly reduced the leak and the background fluorescence in the bathing solution. Here, the fluorescence spot was clearly visible at the tip of PME (Fig. 8C). Reduction of the laser leak by Sylgard coating may be attributed to the smaller refractive index of the silicone resin (approximately 1.40–1.43) that envelopes the quartz glass of higher refractive index (approximately 1.53–1.55). Indeed, this is the case for optical fiber glass. In the brain tissue, the fluorescence intensity around the PME should be reduced because of the scattering of light. Therefore, the PME likely measures fluorescence signals at the tip of the electrode where fluorescence signals and electrical signals are highly correlated.

The surface coating to the tip of PME makes the PME thicker (Fig. 8B); nevertheless, the coating may improve the sensitivity of optical monitoring, particularly in the recording from slice preparations, where the laser leak around the tip of PME would be expected to occur at the intensity observed in Fig. 8A. In slice experiments where labeled neurons were sparsely distributed, the standard Ni-coated PME likely collected optical signals from the tip of the electrode under the
cell-attached recording conditions; accordingly, the fluorescence signals were not seriously affected (Fig. 2).

In the present PME in vivo assay, we labeled auditory nuclei using a synthetic calcium indicator dye (OGB-1 AM) rather than one of the available genetic-encoded calcium indicator compounds (Akerboom et al. 2012). Advantages of using OGB-1 AM include reliable labeling by injection and a fast response time. Because stable labeling of targeted brain regions with a reporter compound was the most difficult step in the present experiments, finding appropriate labeling procedures and compounds was critical for the success of recording in the in vivo experiments.

We inserted the PME into the brain guided by the acoustic stimulation to detect field electrical responses and the stereotaxic axis. Nevertheless, our recording success rates varied daily. Highly correlated fluorescence and current signals were recorded from repeated insertions of the PME at nearby locations during a successful experimental session. We presume that the success rates depended strongly on how well the neurons were labeled. For localizing exact recording sites and tracking the activity of single neurons, it is desirable to use the genetic expression of bright indicator molecules to targeted neurons of a specific brain region in future projects (Akerboom et al. 2012; Matsui et al. 2012; Schecterson et al. 2012).

In most of the experiments in vivo, we have enhanced fluorescence calcium responses using pharmacological agents. We have tried to record fluorescence signals under normal physiological conditions using ACSF in the PME; however, the success of recording depended on the nucleus tested. The field L was too noisy to isolate single events under normal physiological recording conditions (Fig. 3, F and I), and pharmacological agents were effective in reducing the frequency of spiking activity and enhancing the response size. We occasionally achieved the recording of single-unit responses and correlated calcium fluorescence signals in the IC in vivo under normal physiological conditions (Fig. 6E). In slice preparations from the dentate gyrus of the mouse hippocampus labeled intensely by incubation with OGB-1 AM, large fluorescence calcium responses (~5% ΔF/F) were detected in the ACSF in response to a train of antidromic electrical stimulation (data not shown). Nevertheless, we need to improve the signal-to-noise ratio of the PME recording system and use brighter fluorescence reporter compounds that can be expressed reliably in the targeted neurons of the brain, which may improve the recording of fluorescence signals under normal physiological conditions in vivo.

We have studied the local responses to acoustic stimulation in several nuclei of the avian auditory pathways. We found different patterns of calcium responses in the NM, IC, and field L. The NM neurons hardly generated calcium signals in response to acoustic stimulation in vivo (Fig. 7, A and B) and in slice preparations (Fig. 7E). However, well-resolved transient calcium fluorescence responses to orthodromic electrical stimulation were reported in slice preparations where NM neurons were intensely labeled by incubation of the slice with fura-2 AM (Wang et al. 2012). Perhaps neuronal labeling with in vivo injection of OGB-1 AM was insufficient in our experiments because the NM is located in a region of the brain stem that faces the ventricular space; thus there is a chance the injected dye solution can be diluted by leakage into this space. For the NM neurons, we plan to apply targeted labeling with bright fluorescence compounds by a genetic manipulation in future experiments. Despite the difficulty of labeling neurons in the NM in vivo, we confirmed previous reports that a rise of intracellular Ca$^{2+}$ is likely inhibited by reducing Ca$^{2+}$ influx through voltage-dependent calcium channels due to the activation of metabotropic glutamate receptors or the high capability of Ca$^{2+}$ buffering (Lachica et al. 1995; Lu and Rubel 2005; Wang et al. 2012). NM neurons have a high rate of firing activity because of the spontaneous firing activity and drive of ANFs (Fukui et al. 2006; Kuba and Ohmori 2009; Sullivan and Konishi 1984) and are likely required to limit calcium influx to prevent apoptosis (Mattson et al. 2008). Calcium responses detected in the IC were transient and small (Fig. 6). The transient nature of the calcium response was consistent with recent calcium imaging experiments conducted in the rat IC dorsal cortex using Nipkow-disk confocal microscopy (Ito et al. 2014). These results may reflect a mechanism that limits the size and duration of calcium responses in the IC. The response pattern may further indicate that the IC sorts and extracts auditory features based on hard-wired circuits rather than operating as an auditory center with neural plasticity. However, the ascending auditory pathway is known to be calibrated and aligned with the visual space map during the critical period of the barn owl (Brainard and Knudsen 1993), and the IC is proposed as a target of descending cortical modulation (Bajo and King 2013). The response pattern of the field L is quite different from the other two nuclei we studied (Fig. 5). Although both electrical and calcium responses were enhanced using 4-AP, large and prolonged calcium signals followed the acoustic responses, and long-lasting burst activity repeatedly occurred (Fig. 5B). These observations are consistent with the idea that the field L is a site of plasticity (Berridge et al. 2000; Hagenston and Bading 2011; Hell 2014). Thus the pattern of calcium responses to acoustic stimulation might indicate distinct roles for each nucleus in the processing and transformation of neural information in the ascending auditory pathway.

In conclusion, the simultaneous measurement of optical and electrical signals is important to understand the role of signaling molecules in neural activity. We propose that the PME recording system is a useful and powerful tool to address the relationship between electrical activity and optical signals from a variety of reporter compounds in brain regions in vivo. The PME can also be used in combination with molecular biology or genetics that use light to control and analyze neuronal activity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).