A highly sensitive fluorescent indicator dye for calcium imaging of neural activity in vitro and in vivo

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Keywords: cerebellum, mouse, neocortex, population activity, two-photon imaging

Abstract

Calcium imaging of individual neurons is widely used for monitoring their activity in vitro and in vivo. Synthetic fluorescent calcium indicator dyes are commonly used, but the resulting calcium signals sometimes suffer from a low signal-to-noise ratio (SNR). Therefore, it is difficult to detect signals caused by single action potentials (APs) particularly from neurons in vivo. Here we showed that a recently developed calcium indicator dye, Cal-520, is sufficiently sensitive to reliably detect single APs both in vitro and in vivo. In neocortical neurons, calcium signals were linearly correlated with the number of APs, and the SNR was > 6 for in vitro slice preparations and > 1.6 for in vivo anesthetised mice. In cerebellar Purkinje cells, dendritic calcium transients evoked by climbing fiber inputs were clearly observed in anesthetised mice with a high SNR and fast decay time. These characteristics of Cal-520 are a great advantage over those of Oregon Green BAPTA-1, the most commonly used calcium indicator dye, for monitoring the activity of individual neurons both in vitro and in vivo.

Introduction

The activity of individual neurons induces calcium influx through voltage-dependent calcium channels, which can be monitored by using calcium indicator dyes. For the past few decades, various calcium indicators have been developed that have enabled the analysis of intracellular calcium dynamics (Grynkiewicz et al., 1985; Reggehr et al., 1989; Miyakawa et al., 1992; Eilers et al., 1995; Yuste & Denk, 1995; Svoboda et al., 1996; Finch & Augustine, 1998; Yasuda et al., 2004). Furthermore, these dyes can be used in combination with two-photon microscopy to monitor the activity of neuronal populations in vivo (Stosiek et al., 2003; Ohtani et al., 2005; Kerr et al., 2007; Sato et al., 2007; Sohya et al., 2007; Ozden et al., 2009; Schultz et al., 2009; Rochefort et al., 2011). Therefore, synthetic fluorescent calcium indicators have been a powerful tool to uncover the function of neural circuit dynamics and its underlying mechanisms in the intact brain from single synapse to single cell resolution (see, for review: Grewe & Helmchen, 2009; Grienberger & Konnerth, 2012).

Conventional synthetic calcium indicators can reliably resolve single action potentials (APs) and individual synaptic inputs in single neurons by perfusing the dye through an intracellular recording pipette in both brain slices and intact animals (Svoboda et al., 1997; Waters et al., 2003; Jia et al., 2010; Chen et al., 2011; Takahashi et al., 2012). However, bolus loading of membrane-permeable calcium indicators (acetoxymethyl ester derivatives of fluorescent indicators) (Tsien, 1981) has the great advantage that the activity of hundreds of neurons can be monitored simultaneously (Yuste & Katz, 1991; Stosiek et al., 2003). Although this method is sufficiently sensitive to detect single APs in both acute slices and organotypic slice cultures (Ikegaya et al., 2005; Yuste et al., 2011), the signal-to-noise ratio (SNR) is apparently lower in vivo such that single APs are often difficult to detect (Sato et al., 2007; Golshani et al., 2009) (but see Kerr et al., 2005; Grewe et al., 2010), presumably due to the scattering of fluorescence signals by thick brain tissues. This limitation is critical particularly for monitoring the activity of neurons with a low firing rate.

In this report, we demonstrate that a new BAPTA-based fluorescent calcium indicator, Cal-520, yields calcium transients with large amplitudes and high SNR in neocortical pyramidal cells and cerebellar Purkinje cells, which are sufficient to detect single APs reliably in the intact brain.

Materials and methods

The care of animals and experimental procedures were carried out in accordance with national and institutional guidelines, and all experimental protocols were approved by the Animal Experimental Committee of the University of Tokyo.

Animal surgery for in vivo imaging

The C57BL6/J male mice (postnatal days 28–61) were anesthetised by intraperitoneal injection of 1.9 mg/g urethane (for neocortical...
imaging), or 100 mg/kg ketamine and 10 mg/kg xylazine (for cerebellar imaging). The depth of anesthesia was monitored regularly by observing whisker movements and the pinch withdrawal reflex of the hindlimb; additional doses of anesthetic were given as needed. Body temperature was kept at 37 °C with a heating pad (FHC, Bowdoin, ME, USA). The mouse was fixed on a custom stereotaxic apparatus under a surgical microscope (Leica). A skin incision was made along the midline and the skull was partly exposed. A stainless steel frame was glued to the skull with superglue and dental cement. A small craniotomy (~2 mm in diameter) was made to expose the left barrel cortex (3 mm lateral to the midline and 1.5 mm caudal to the bregma) or the left Crus IIa of the cerebellum (4 mm lateral and 2 mm posterior to the occipital bone line) using a high-speed drill (ULTIMATE500; NSK, Kanuma, Tochigi, Japan). The dura was carefully removed, and the craniotomy was filled with 1.5% agarose (type IIIa, Sigma-Aldrich) in a solution of the following composition (in mM): 150 NaCl, 2.5 KCl, 10 HEPES, 2 CaCl2, 1 MgCl2, pH 7.3. A coverslip was placed over the agarose to minimize brain movements. The mouse was then transferred to the animal stage under the two-photon microscope.

**Dye injection**

Multi-cell bolus loading of calcium indicator dye was performed as described previously (Stosiek et al., 2003; Sullivan et al., 2005; Mukamel et al., 2009; Ozden et al., 2009; Schultz et al., 2009; Hashizume et al., 2013). Cal-520 acetoxyethyl ester (Cal-520 AM) (AAT Bioquest, Sunnyvale, CA, USA; excitation/emission wavelength, 492/514 nm; spectra available on manufacturer’s website, www.aatbio.com; Kd, 320 nM; quantum yield, 0.75) or Oregon Green 488 BAPTA-1 acetoxyethyl ester (OGB-1 AM) (Invitrogen) was dissolved with 20% (for neocortical neurons) or 10% (for cerebellar Purkinje cells) w/v Pluronic F-127 (Invitrogen) in dimethyl sulfoxide. The dye aliquot was then diluted with the extracellular solution containing Alexa 594 fluorescent dye (20 μM; Invitrogen). The final concentration of the calcium indicator dye was 1 and 0.2 mM for neocortical and cerebellar neurons, respectively. The dye solution was sonicated for 1 min, filtered with a 0.2 μm syringe filter to remove dye aggregation, and then transferred to a glass pipette (2–7 MΩ).

Dye ejection was performed in layer 2/3 of the barrel cortex (200–300 μm from the surface) or in the cerebellar molecular layer (50–60 μm from the surface) at 5 psi for 3 min using Picospritzer (Parker Hannifin, Hollis, NH, USA). Successful dye ejection was monitored by two-photon imaging on the Alexa channel. When the pipette tip was clogged up during dye ejection, brief pulses of higher pressure (10–20 psi) were applied to recover the flow of dye from the tip of the pipette. Calcium imaging was performed more than 30 min after dye ejection.

**In vivo two-photon microscopy**

*In vivo* calcium imaging was performed by using a custom-built two-photon microscope (MOM, Sutter Instrument, Novato, CA, USA) controlled by ScanImage software (Pologruto et al., 2003). The brain was illuminated with a pulsed Ti:sapphire laser (MaiTai HP, 800–830 nm wavelength, 80 MHz repetition rate, 100 fs pulse width; NewPort Spectra-Physics, Santa Clara, CA, USA). The laser was focused through a 40 × water-immersion objective lens (LUMPLFLNIR40XW, Olympus) onto the tissue. The average laser power was adjusted to be < 15 mW at the specimen. Fluorescence signals of Cal-520 or Oregon Green 488 BAPTA-1 (OGB-1) and Alexa 594 were divided into green and red channels, respectively, by a dichroic mirror and emission filters (Chroma, Bellows Falls, VT, USA), and were detected by a pair of photomultiplier tubes (Hamamatsu, Shizuoka, Japan).

To obtain calcium transients from neuronal populations, images were acquired at a resolution of 128 × 128 pixels (sampling rate, ~8 Hz) for ~2 min. These image stacks were analysed offline. For high-speed imaging of calcium transients in bolus-loaded specimens, linescan (sampling rate, 500 Hz) was performed on somata of the cortical neurons or on dendrites of cerebellar Purkinje cells.

**Simultaneous calcium imaging and loose-seal cell-attached recordings in vivo**

A glass electrode (5–7 MΩ) filled with the extracellular solution containing Alexa 594 (50 μM) was inserted into the barrel cortex or cerebellar cortex and targeted to a soma that had been loaded with OGB-1 AM or Cal-520 AM. At about 10 min after the establishment of the cell-attached configuration, simultaneous loose-seal cell-attached recording and linescan calcium imaging (500 Hz) were performed on the soma of cortical neurons or on the dendrites of Purkinje cells at ~100 μm from the soma. The electrophysiological data were filtered at 10 kHz and digitised at 20 kHz by using Multiclamp 700B and Digidata 1322A (Molecular Devices, Sunnyvale, CA, USA), and acquired by AxoGraph X (AxoGraph Scientific, Sydney, Australia).

**Preparation of neocortical slices**

The 3–4-week-old C57BL6/J male mice were killed under CO2 anesthesia by cervical dislocation followed by decapitation. Whole brains were immediately removed in ice-cold oxygenated (95% O2 and 5% CO2) artificial cerebrospinal fluid containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 26 NaHCO3, 2 CaCl2, 1 MgCl2, and 25 glucose. Acute coronal brain slices containing the barrel cortex (250–300 μm thick) were prepared using a vibrating microtome (VT1200S; Leica). Slices were incubated for at least 30 min in oxygenated artificial cerebrospinal fluid at 30 °C and later stored at room temperature (23–25 °C) before transferring them to the recording chamber under the two-photon microscope.

**Simultaneous whole-cell recordings and calcium imaging in vitro**

Brain slices were mounted in the immersion-type recording chamber under the two-photon microscope and continuously perfused with oxygenated artificial cerebrospinal fluid at 30 °C during recordings. Individual layer 2/3 pyramidal neurons in the barrel cortex were visualised by detecting forward scattered infrared laser light with the transmitted light detector of the two-photon microscope (Wimmer et al., 2004). Patch pipettes were pulled from borosilicate glass capillaries on a vertical puller (PC-10; Narishige, Tokyo, Japan). The resistance of the patch pipette ranged from 4 to 8 MΩ when filled with the intracellular solution containing (in mM): 133 K-MeSO3, 7.4 KCl, 10 HEPES, 3 Na2ATP, 0.3 Na2GTP, 0.3 MgCl2, 0.05 Alexa 594, and either 0.1 Cal-520 potassium salt (AAT Bioquest) or 0.1 OGB-1 potassium salt (Invitrogen). Whole-cell current-clamp recordings were performed using an EPC10 amplifier (Heka Elektronik, Lambrecht, Rhineland-Palatinate, Germany), filtered at 10 kHz and digitised at 20 kHz. Calcium imaging was started at least 30 min after break-in to allow diffusion of the dye into the cell.

A laser scanning two-photon microscope (FV1000-MPE; Olympus) equipped with a pulsed laser (MaiTai HP DeepSee; NewPort Spectra-Physics) was used for two-photon calcium imaging with a
water-immersion objective lens (LUMPLFL/IR40XW, Olympus). The fluorescence signals of Cal-520 or OGB-1 and Alexa 594 were separated into green and red channels, respectively, and detected by a pair of photomultiplier tubes (Hamamatsu). Simultaneous whole-cell recording and linescan calcium imaging (714 Hz) were conducted on the soma of the pyramidal cells. APs were triggered by current injections (0.8 nA, 2 ms) through the patch pipette. Each trial was repeated at least 10 times and the mean value is presented.

**Data analysis**

All imaging and electrophysiological data were analysed using ImageJ (http://rsb.info.nih.gov/ij/), Igor Pro (WaveMetrics, Lake Oswego, OR, USA), MATLAB (MathWorks, Natick, MA, USA), and A xoGraph X. For population calcium imaging in the barrel cortex, regions of interest corresponding to individual neuronal somata were automatically defined by using the pixel correlation method (Smith & Häusser, 2010). The fluorescence signals of all of the pixels in each region of interest were averaged and calcium transients were detected by using a simple thresholding method.

For simultaneous calcium imaging and cell-attached recordings, detection of calcium transients was triggered by the onset of APs (for cortical neurons) or complex spikes (for cerebellar Purkinje cells) measured in the electrophysiological recording. Fluorescence signals in the linescan data were averaged over the entire width of the soma and dendrite. Calcium transients were calculated as $\Delta F/F = (F - F_0)/F_0$, where $F_0$ is baseline fluorescence and $F_b$ is background fluorescence for Cal-520 or OGB-1. The peak amplitude of calcium transients was defined as the difference between the baseline (average $\Delta F/F$ of 40 ms before the onset of APs or complex spikes) and the average of five data points around the peak. The SNR was calculated as the peak amplitude of calcium transients divided by the SD of the baseline fluctuation of the fluorescence trace. The calcium transient traces were smoothed with a sliding window of three to five points for presentation. The statistical significance of the data was tested by a two-sided Welch two-sample t-test unless otherwise noted, and all tests were performed using R (http://www.r-project.org/). All of the data are presented as mean ± SEM.

**Results**

**Imaging of calcium transients evoked by action potentials in neocortical pyramidal cells in vitro**

Calcium transients in response to APs were recorded in neocortical pyramidal cells in acute brain slices to quantify the signal amplitude and SNR. We first compared calcium signals detected by Cal-520 with those detected by OGB-1, a commonly used fluorescent indicator, which has a similar $K_d$ value (Cal-520, 320 nm; OGB-1, 170 nm). Pyramidal cells in the barrel cortex were whole-cell-patch-clamped and APs were evoked by injecting depolarising current pulses. Either Cal-520 or OGB-1 was loaded through a patch pipette and spike-induced calcium transients were assessed by high-speed linescan imaging (sampling rate, 714 Hz) (Fig. 1A). With the number of evoked APs used (one, two, four and eight APs at 20 Hz), the amplitude of calcium transients increased in a linear fashion in both Cal-520- and OGB-1-loaded pyramidal cells (Fig. 1B and C). Cal-520 showed a significantly higher amplitude and SNRs of calcium transients in all numbers of APs tested (Fig. 1C and D). It is of note that a single AP induced calcium transients with a larger amplitude (0.702 ± 0.140 $\Delta F/F$ for Cal-520 and 0.129 ± 0.030 $\Delta F/F$ for OGB-1; seven cells in five mice and seven cells in four mice, respectively, $P = 0.006$) and SNR (6.630 ± 0.668 for Cal-520 and 4.323 ± 0.522 for OGB-1, $P = 0.019$) in Cal-520-loaded cells, which enables the reliable detection of single APs. Although there was no statistical difference in the kinetic dynamics of both indicators (10–90 rise time: 0.0637 ± 0.0045 and 0.0854 ± 0.0314 s; seven cells in five mice and seven cells in four mice, respectively, $P = 0.518$; decay time constants: 0.691 ± 0.113 s for Cal-520 and 2.114 ± 0.955 s for OGB-1, $P = 0.188$) (Fig. 1E), these results show the superior sensitivity of Cal-520 over OGB-1 for the detection of calcium transients. The improved sensitivity of Cal-520 enabled the detection of discrete calcium transients induced by spike trains of higher frequencies up to 10 Hz (Fig. 1F).

**Multi-cell bolus loading of the acetoxymethyl ester derivative of the indicator dye was performed to monitor the population activity of neurons in the barrel cortex (Stosiek et al., 2003; Golshani et al., 2009). Cal-520 AM or OGB-1 AM was injected into layer 2/3 of the mouse barrel cortex (200–300 μm from the surface). At about 30 min after dye loading, Cal-520 or OGB-1 was penetrated into the neurons and glia. Spontaneous calcium transients in multiple neurons in the field of view were reliably detected using Cal-520 (Fig. 2A and B) as well as OGB-1 (Fig. 2C). The mean frequency of spontaneous calcium transients was indistinguishable between Cal-520 and OGB-1 (0.0458 ± 0.002 and 0.0410 ± 0.002 Hz; 171 cells in 12 mice and 12 cells in four mice, respectively, $P = 0.64$, Mann–Whitney U-test) (Fig. 2D). The average number of active neurons in a field of view of the Cal-520-filled cortex (147 × 147 μm), which showed at least one spontaneous AP in 2 min, was significantly larger than that of the OGB-1-filled cortex (7.77 ± 0.27 and 1.71 ± 0.14 cells/field of view, 12 and four mice, respectively, $P = 0.0002$, Mann–Whitney U-test) (Fig. 2E). This result reflects the fact that Cal-520-filled cells showed a larger amplitude of calcium transients than OGB-1-filled cells (0.318 ± 0.001 and 0.218 ± 0.008 $\Delta F/F$, 171 cells in 12 mice and 12 cells in four mice, respectively, $P = 0.03$, Mann–Whitney U-test) (Fig. 2F), and thus the mean SNR of individual calcium transients by Cal-520 was significantly higher than that by OGB-1 (14.62 ± 0.04 and 8.65 ± 0.28, $P = 0.0002$, Mann–Whitney U-test) (Fig. 2G). Calcium transients evoked by sensory stimulation to contralateral whiskers were also clearly observed in multiple neurons (Fig. 3).

To quantitatively compare the signal amplitude and SNR, we next performed high-speed linescan imaging (sampling rate, 500 Hz) (Fig. 4A and B). The mean amplitude of individual calcium transients using Cal-520 was significantly larger than that using OGB-1 (0.696 ± 0.010 and 0.434 ± 0.011 $\Delta F/F$, 49 cells in nine mice and 14 cells in four mice, respectively, $P = 0.02$, Mann–Whitney U-test) (Fig. 4C), which resulted in higher SNRs (5.390 ± 0.052 for Cal-520 and 3.465 ± 0.077 for OGB-1, $P = 0.0004$) (Fig. 4D). The kinetic properties of calcium transients were not different between Cal-520 and OGB-1 (10–90 rise time (Fig. 4E): 0.053 ± 0.001 and 0.099 ± 0.006 s, 49 cells in nine mice and 14 cells in four mice, respectively, $P = 0.09$; decay time constants by double-exponential fitting (Fig. 4F): 0.755 ± 0.009 and 1.055 ± 0.010 s for Cal-520 (46 cells in nine mice), 0.675 ± 0.037 and 1.196 ± 0.043 s for OGB-1 (12 cells in four mice), $P = 0.60$ and 0.28, respectively).

As both the mean amplitude and mean SNR for each calcium transient, which we quantified above (Figs 2F and G, and 4C and D), included different numbers of APs/transient, further quantification was required to compare the true performance of these indica-
Therefore, we performed simultaneous loose-seal cell-attached recordings and high-speed linescan imaging to clarify the relationship between the calcium transients and APs (Fig. 5A). The amplitude of the calcium transients linearly correlated with the number of APs (Fig. 5B), and the amplitude of calcium transients per spike, which was calculated as the peak amplitude divided by the number of APs, was significantly larger for Cal-520 than for OGB-1 (0.188 ± 0.008 and 0.052 ± 0.009 ΔF/F, nine and four cells, respectively, *P < 0.05, **P < 0.01). (E) Rise times and decay time constants of calcium transients induced by single APs. Error bars indicate SEM (n = 7 cells each). (F) Trial-averaged fluorescence transients of Cal-520 to five spike trains at different frequencies. Bottom: responses to 10 and 20 Hz spike trains are shown in an expanded time scale. Gray lines indicate individual sweeps (n = 10). Red thick lines indicate average traces. Stimuli are indicated by the black vertical lines.

**Fig. 1.** Properties of Cal-520 examined in layer 2/3 pyramidal cells in the barrel cortex in acute brain slices. (A) Representative image of whole-cell patch-clamp recording from a pyramidal cell in the barrel cortex. Dashed lines indicate the patch pipette. A magnified image is shown in the right panel. The red line indicates the position of the linescan. (B) Representative traces of fluorescence response (ΔF/F) of Cal-520 and OGB-1 in response to one, two, four, and eight spike trains at 20 Hz. Mean amplitude (C) and SNR (D) of Cal-520 and OGB-1 in response to one to eight spike trains at 20 Hz. Error bars indicate SEM (n = 7 cells each, *P < 0.05, **P < 0.01). (F) Rise times and decay time constants of calcium transients induced by single APs. Error bars indicate SEM (n = 7 cells each). (F) Trial-averaged fluorescence transients of Cal-520 to five spike trains at different frequencies. Bottom: responses to 10 and 20 Hz spike trains are shown in an expanded time scale. Gray lines indicate individual sweeps (n = 10). Red thick lines indicate average traces. Stimuli are indicated by the black vertical lines.
of calcium transients (0.325 ± 0.003 and 0.291 ± 0.009 ΔF/F, P = 0.49, t-test) (Fig. 6C), despite an increase of baseline fluorescence intensity (52.8 ± 0.3 and 73.9 ± 0.8 F in 59 cells and 22 cells, respectively, P = 0.00002, t-test) (Fig. 6B). The frequency of spontaneous calcium transients was also unchanged (0.0457 ± 0.0006 and 0.0391 ± 0.011 Hz, P = 0.36, t-test) (Fig. 6D).

**Imaging of calcium transients evoked by climbing fiber input in cerebellar Purkinje cells in vivo**

We next compared calcium transients measured with Cal-520 with those measured with OGB-1 in cerebellar Purkinje cells in vivo. In the cerebellar cortex, spontaneous calcium transients in Purkinje cell dendrites have been shown to be triggered by complex spikes evoked by climbing fiber inputs (Ozden et al., 2009; Schultz et al., 2009; Kitamura & Häusser, 2011). Cal-520 AM or OGB-1 AM was bolus loaded into the cerebellar molecular layer of the cerebellar cortex (Mukamel et al., 2009; Ozden et al., 2009; Schultz et al., 2009; Hashizume et al., 2013). Cal-520 or OGB-1 was penetrated into various cell types, including Purkinje cells, molecular layer interneurons and Bergmann glia. The somata of Purkinje cells in the Purkinje cell layer (Fig. 7A, upper) and their dendrites extending along the rostro-caudal axis in the molecular layer (Fig. 7A, lower) were clearly labeled with both Cal-520 (Fig. 7A, left) and OGB-1 (Fig. 7A, right).

High-speed linescan imaging was performed on dendrites of the bolus-loaded Purkinje cells to analyse the properties of calcium transients (five cells in four mice for Cal-520 and six cells in two mice for OGB-1) (Fig. 7B and C). The mean amplitude of the calcium
Fig. 3. Sensory-evoked calcium transients in neocortical neurons labeled with Cal-520. (A) Configuration of the experiment. Brief airpuff stimuli (40 psi, 50 ms) were delivered to the contralateral whiskers while neuronal activities in the left barrel cortex were recorded. In frame scan imaging, stimuli (blue bars) were delivered at 5 s intervals during an imaging session of 125 s. In linescan imaging, stimulus was delivered every 8 s. (B) Sensory-evoked calcium transients in multiple neurons. Upper: the fluorescence changes in neurons were recorded while airpuff stimuli were delivered. Lower: representative traces of calcium transients from four regions of interest (#1–#4) shown in the upper left panel. Vertical broken lines indicate airpuff stimulation. Gray dots indicate calcium transients detected from the baseline noise. (C) Sensory-evoked calcium transients recorded with high-speed linescan imaging. Upper left: representative image showing the pyramidal cell labeled with Cal-520. Upper right: a scanned image (2 ms/line) recorded at the position indicated by the orange line in the upper left panel. Lower: calcium transients recorded in the cell shown above. Eight of 10 airpuff stimuli evoked calcium transients. Scale bars, 20 μm.

Fig. 4. Properties of calcium transients in neocortical neurons in vivo measured with Cal-520 and OGB-1. Representative linescan imaging from somata of layer 2/3 neurons labeled with Cal-520 (A) or OGB-1 (B). Scale bars, 20 μm. Upper right: linescan images obtained at the position indicated by the orange lines in the upper left panels. Lower: spontaneous calcium transients recorded at the regions of interest shown as the rectangles in the upper right panels. Gray dots indicate calcium transients detected from the baseline noise. (C–F) Bar graphs showing the properties of calcium transients measured with Cal-520 (filled columns) or OGB-1 (open columns). Cal-520 showed higher mean amplitude of calcium transients than OGB-1 (C), resulting in higher SNR (D). Rise times (E) and decay time constants (F) of calcium transients were indistinguishable between Cal-520 and OGB-1. *P < 0.05, ***P < 0.001.
transients was significantly larger in cells labeled with Cal-520 than those labeled with OGB-1 (1.788 ± 0.500 and 0.275 ± 0.020 ΔF/F, respectively, \( P = 0.04 \)) (Fig. 7D), which resulted in much higher SNRs (87.834 ± 13.42 for Cal-520 and 18.001 ± 2.772 for OGB-1, respectively, \( P = 0.0004 \)) (Fig. 7E). The mean values of the 10–90 rise time were similar between the two indicators (0.013 ± 0.001 s for Cal-520 and 0.014 ± 0.001 s for OGB-1, \( P = 0.364 \)) (Fig. 7F), whereas the calcium transients measured with Cal-520 had shorter decay times than those measured with OGB-1 (0.011 ± 0.004 and 0.174 ± 0.083 s for Cal-520, 0.044 ± 0.007 and 0.918 ± 0.110 s for OGB-1, \( P = 0.0005 \) and \( P = 0.006 \), respectively) (Fig. 7G). These results indicate that Cal-520 could clearly detect calcium signals by single APs.

Discussion

In this article, we demonstrated that a new fluorescent calcium indicator dye, Cal-520, is highly sensitive and has an excellent SNR, which is sufficient to detect single APs both in vitro and in vivo. The amplitude of calcium transients is linearly correlated with the number of APs, and individual APs in a train up to 10 Hz can be resolved. These properties enable reliable high-resolution optical recordings of spatiotemporal activity patterns of neural circuits in the intact brain.

Calcium imaging of the spiking activity of single neurons in vitro and that of neuronal populations in vivo clearly showed that Cal-520
Cal-520 OGB-1 (C). Upper right: representative images obtained by high-speed linescan at the position indicated by the orange lines in the upper left panels. Scale bars, * times were shorter in dendrites labeled with Cal-520 than those with OGB-1 (G). Five cells in four mice for Cal-520 and six cells in two mice for OGB-1.

transients than OGB-1 (D), resulting in higher SNR (E). 10 ing the properties of calcium transients measured with Cal-520 (G). Recently, genetically-encoded calcium indicators have been exten-

d.s. the mechanism of this labeling specificity is unknown, this property might also contribute to better SNRs in population calcium imaging in vivo. In contrast, one disadvantage of Cal-520 was that the spread of dye solution was smaller than that of OGB-1 (data not shown) and thus multiple injections might be required for the imaging of large areas.

Acknowledgements
We thank Dr Taro Ishikawa for providing the analysis program (TaroTools for Igor Pro) and members of the laboratory of M.K. for discussion. This work was supported by Grants-in-aid for Scientific Research (23115504,
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