Targeted bulk-loading of fluorescent indicators for two-photon brain imaging *in vivo*

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One of the challenges for modern neuroscience is to understand the rules of concerted neuronal function *in vivo*. This question can be addressed using noninvasive high-resolution imaging techniques like two-photon microscopy. This protocol describes a versatile approach for *in vivo* two-photon calcium imaging of neural networks, stained with membrane-permeant fluorescent-indicator dyes. It is based on a targeted pressure ejection of the dye into the tissue of interest and can be used for a large spectrum of indicator dyes, including Oregon Green 488 BAPTA-1 acetoxymethyl ester and Fura-2 acetoxymethyl ester. Through the use of dye mixtures and multicolor imaging, this technique allows the visualization of distinct neurons and glial cells up to 500 µm below the brain surface. It is suitable for staining the brain tissue of various different species (e.g., mouse, rat, cat and zebrafish) at all developmental stages. When combined with brain microendoscopy, it allows the monitoring of intracellular calcium signals in awake, behaving animals. The total time required to carry out the protocol, including dissection and cell staining, is ~2 h. Thereafter, imaging experiments might be performed for at least 6 h.

INTRODUCTION

This protocol describes an approach for *in vivo* two-photon Ca^{2+} imaging of large neuronal circuits at a single-cell level of resolution. Cells are stained by a brief 'bolus' injection of a membrane-permeant Ca^{2+}-indicator dye into the extracellular space (**Fig. 1**). The injected dye diffuses into the cells of interest, where it is hydrolyzed by intracellular esterases[1]. The activity-dependent Ca^{2+} transients in stained cells are then monitored using two-photon laser-scanning microscopy. This approach was initially developed for *in vivo* imaging of the mouse cortex, and is termed multicell bolus loading (MCBL)[2]. Over the past years, it has been successfully applied to the cerebral and cerebellar cortices of other species, such as rats and cats[3–6]. Interestingly, the same staining protocol is applicable to lower vertebrates and was used, for example, for *in vivo* imaging of the spinal cord[7], olfactory bulb[8] and tectal[9] neurons in zebrafish larvae.

MCBL differs from other staining methods utilizing membrane-permeant acetoxymethyl (AM) ester-based indicator dyes[10,11] in that the indicators are delivered for a short period directly to the target cells (see also the method of Regehr and Tank[12]). The targeted delivery minimizes the loss of the dye due to diffusion, nonspecific and/or glial uptake, and so on. MCBL can be applied at various developmental stages (**Fig. 2**). Remarkably, there is also efficient staining of neurons in the adult and aged brain. This is in contrast to the more conventional techniques of AM ester dye loading, which work well only in the immature tissue[10]. Additional advantages of MCBL include the need for only minor surgery, the possibility of re-staining neurons and, thus, the ability to conduct long-lasting, perhaps even chronic, recordings[13].

While MCBL allows many cell bodies to be imaged simultaneously, the method is not sensitive enough to be used for the analysis of subcellular structures. There are two obvious reasons for this reduced sensitivity. First, the image contrast is reduced due to the relatively high background staining of the surrounding neuropil. Second, the dye concentration in MCBL-loaded cells is low, on average 20 µM of the indicator dye[2]. These limitations restrict the use of MCBL to *in vivo* imaging of somatic Ca^{2+} transients. Note, however, that Ca^{2+}-indicator dyes also act as Ca^{2+} buffers, the presence of which within the cells perturbs the amplitude and kinetics of the Ca^{2+} signals under study[14]. From this point of view, low intracellular levels of indicator dyes (such as those achieved using MCBL) become an advantage.

To study neuronal Ca^{2+} dynamics in awake, behaving animals, the MCBL technique was recently combined with *in vivo* brain endoscopy[15]. A thin (diameter, 200 µm) optical fiber was implanted into the brain area with cells stained using MCBL. The optical fiber was used both for transmitting the excitation light and for collecting the emitted light. The authors detected fluorescence signals with a single photomultiplier and, thus, monitored the compound activity of a large neuronal population. Such an optical fiber can be implanted at any desired depth, allowing the detection of calcium responses in deep and/or hidden brain regions. This approach can be developed further by substituting a single optical fiber with fiber bundles and/or gradient refractive index (GRIN) fibers[16]. Such new approaches might soon allow the monitoring of behavior-related Ca^{2+} signals in individual neurons of any brain region.

MATERIALS

REAGENTS

- Experimental animals (see REAGENT SETUP)
- Anesthetic agent (e.g., isoflurane, ketamine/xylazine or urethane)
- Local anesthetic agent (e.g., lidocaine)
- Membrane-permeable calcium-indicator dye (e.g., Oregon Green 488 BAPTA-1 AM (OG-1 AM), Calcium Green-1 AM, Fura-2 AM, Fura-PE3 AM, Fluo-4 AM or Indo-1 AM from Molecular Probes or TefLabs)
- Sulforhodamine 101 (SR101; Sigma)
- 20% Pluronic F-127 in DMSO (e.g., 2 g Pluronic F-127 in 10 ml DMSO; Sigma) **! CAUTION** Strong detergent; skin, eye and respiratory system irritant. Wear suitable protective clothing
- Standard pipette solution: 150 mM NaCl, 2.5 mM KCl and 10 mM HEPES
- Standard external saline: 125 mM NaCl, 4.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM glucose, pH 7.4, when bubbled with 95% O_2 and 5% CO_2
- Low melting point agarose (Invitrogen GmbH)

EQUIPMENT

- Recording chamber with central access opening: custom-made from a standard tissue-culture dish (diameter, 35 mm^2)
- Glass capillaries (e.g., from Hilgenberg GmbH)
- Pipette puller (e.g., PP830 from Narishige)
- Brain atlas (e.g., from Academic Press)
- Surgical equipment, including a stereotaxic instrument, drill and warming blanket to ensure a constant body temperature; available from many providers (e.g., TSE-Systems)
- LN-Mini manipulator (Luigs & Neumann GmbH)
- Picospritzer II (General Valve) or pneumatic drug-ejection system (NPI)
- Patch-clamp amplifier (e.g., from HEKA)
- Two-photon laser-scanning microscope commercially available from several providers (e.g., Zeiss)
- Felt polisher (e.g., from Dr. Ihde Dental)
- Cyanoacryl glue (available from many providers; e.g., UHU GmbH)
- Millipore filter (Millipore)

REAGENT SETUP

Experimental animals So far, the protocol has been tested in mice, rats, cats and zebrafish. **! CAUTION** All experiments must be performed in accordance with the relevant authorities' guidelines and regulations.

EQUIPMENT SETUP

Anesthesia unit Including a chamber for pre-anesthetic medication, a flow meter and a vaporizer (the latter items are for volatile anesthetic agents only). Consult the literature (e.g., ref. 17) for the best choice of anesthesia for each species.

Anesthesia-monitoring system Used to measure the blood pressure, arterial oxygen concentration, body temperature, and respiratory and pulse rate of the animal. The monitoring equipment is available from many providers, but it is not always suitable for small laboratory animals (e.g., mice and neonatal rats). We use equipment from ADI Instruments to monitor the respiratory and pulse rate, body temperature and blood pressure in mice.

Manipulator and pressure-application device To inject the staining solution into the brain we use the LN-Mini manipulator and either Picospritzer II or the pneumatic drug-ejection system.

Patch-clamp amplifier Used to monitor the resistance of the pipette during dye injection. Monitoring of the pipette resistance is not mandatory, but is advisable because it allows pipette clogging to be recognized and the point when the pipette reaches the surface of the cortex to be estimated.

Two-photon laser-scanning microscope We use a custom-built microscope based on a mode-locked laser system operating at 710–920 nm wavelength (MaiTai, Spectra Physics) and a laser-scanning system (Fluoview, Olympus) coupled to an upright microscope (BX51WI, Olympus). Such a custom-built system can be assembled following the instructions in refs. 18,19.

Brain atlas Used to obtain stereotaxic coordinates of the respective brain region (for example, see refs. 20,21).

PROCEDURE

Preparation

1| Anesthetize the animal and place it onto a warming blanket (38 °C) in front of the oxygen supply[22]. Affix the sensors of the anesthesia-monitoring system. Ensure that the surgical level of anesthesia has been reached (e.g., by testing the pinch withdrawal and the eyelid reflex). We anesthetize mice by inhalation of isoflurane (1–1.5% (vol/vol) in pure O_2). Alternatively, adult mice can be anesthetized with either ketamine/xylazine or urethane (0.1/0.01 and 1.9 mg per g body weight, respectively, i.p.).

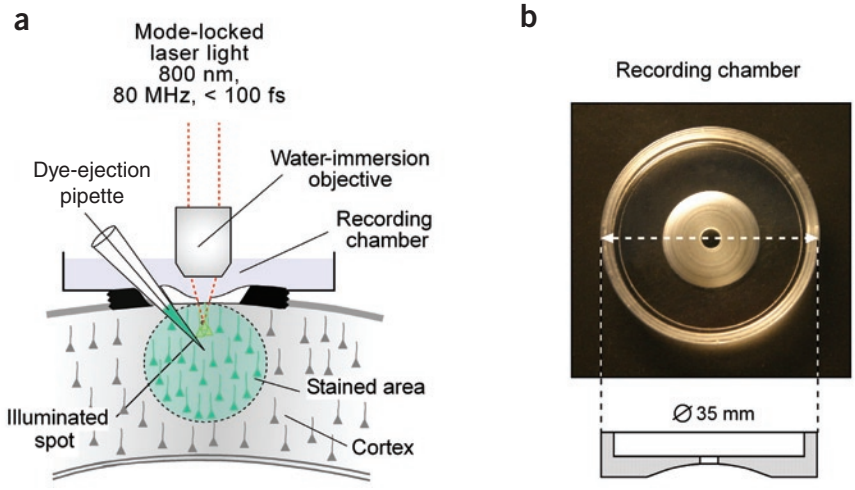

Figure 1 | Experimental arrangement for *in vivo* staining of neuronal populations with Ca^{2+}-indicator dyes. (**a**) A custom-made recording chamber is glued to the skull to allow head fixation and the use of a water-immersion objective. The chamber is perfused with warm (37 °C) standard external saline. Two small craniotomies are performed: one for the insertion of the staining-patch micropipette (obligatory) and one above the field of view for improvement of the imaging depth (optional). Cells, stained using MCBL, are shown in green. The stained area has a diameter of 200–400 µm. Modified from ref. 2. (**b**) Photograph of the recording chamber (upper panel) and schematic drawing of its cross-section (lower panel). Note that a circular region around the perforation is thinned to fit the curvature of the animal's skull.

2| Use a stereotaxic device to identify the location of the brain area of interest. Inject ~ 50 µl local anesthetic agent (e.g., 2% lidocaine; 20 mg lidocaine in 1 ml standard external saline) subcutaneously at the location where the skin is to be removed (optional).

3| Remove the skin above the desired brain area. Decide whether to image through the skull; the stability of the preparation is higher when imaging through the skull (lower impact of heart beat and breathing artifacts), but the efficiency of photon detection is higher when the skull is removed (images are crisper and imaging depth is greater).

4| Thin the skull and polish it with a felt polisher. Imaging through the thinned skull provides optimal results when the skull is thinned down to 10–20 μm^2. We suggest stopping thinning as soon as the bone bends when gently touched with the tweezers. If the skull is to be removed, less thinning is necessary.
▲ **CRITICAL STEP** The skull thinning must be performed as gently as possible; never press onto the skull. Inaccurate thinning often causes brain damage.

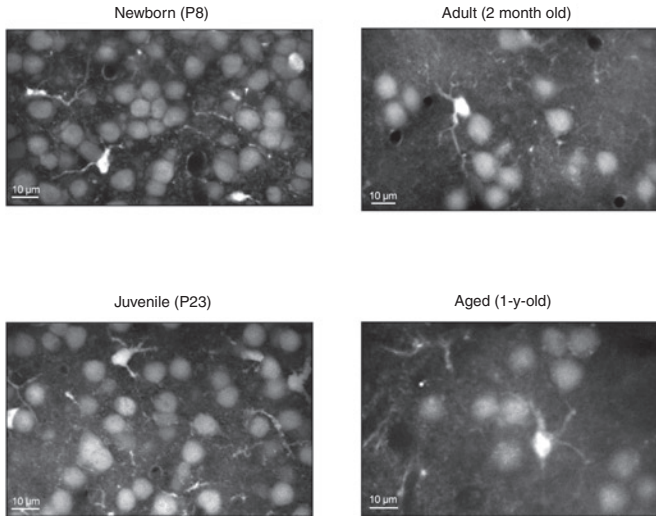

Figure 2 | MCBL allows staining of cortical tissue at various developmental stages. The microphotographs illustrate layer 2/3 cells in the visual cortex of mice at different ages. Cells were stained using OG-1 AM and images were taken using a 60× Nikon water-immersion objective (1.0 NA).

5| Use cyanoacryl glue to adhere the custom-made recording chamber to the skull.

6| Transfer the animal into the recording set-up (we use a set-up similar to that described in ref. 23, equipped with a two-photon laser-scanning microscope; see above) and place it onto a warming blanket (38 °C). Affix the sensors of the anesthesia-monitoring system. If necessary, adjust supply of anesthetic to keep physiological levels of monitored parameters.
▲ **CRITICAL STEP** The quality of recordings critically depends on the good condition of the experimental animal. Therefore, starting now, continuously monitor the following vital parameters: respiratory and pulse rate, body temperature, arterial oxygen concentration and blood pressure.

7| Perfuse the recording chamber with a warm (37 °C) standard external saline.

8| Perform a craniotomy above an area devoid of large blood vessels. This should be small (≤0.5 mm) for imaging through the skull and larger (or two small craniotomies should be performed near each other; **Fig. 1**) for 'open-access' imaging.
▲ **CRITICAL STEP** Do not remove the dura mater; removal is not required for cell staining and strongly enhances movement artifacts. The stability of recordings also critically depends on the diameter of the craniotomy. Thus, openings larger than 1 mm in diameter are often accompanied by large movement artifacts occurring at the heartbeat frequency.

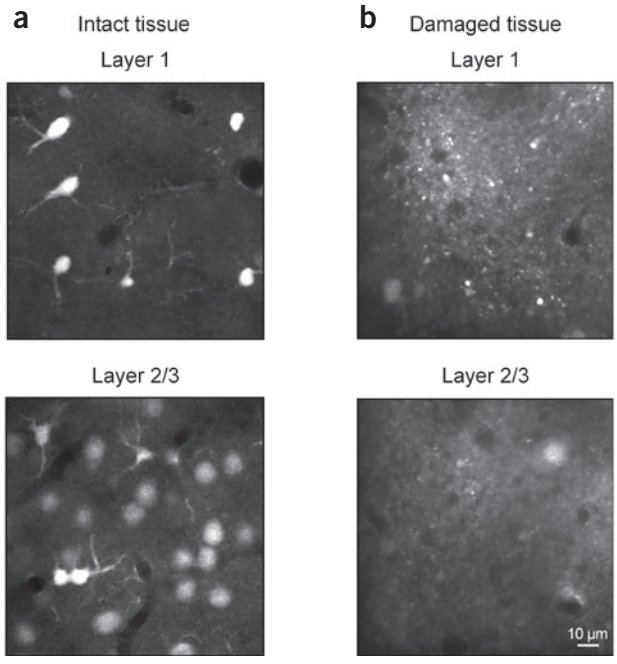

Figure 3 | Assessment of the staining quality. (**a**,**b**) High-magnification images of cells in layer 1 (upper panels) and in layer 2/3 (lower panels) taken at two different locations in the visual cortex of a 64-d-old mouse. Both areas were stained using the same protocol, but in (**b**) a large brain vessel was damaged prior to staining.

Staining neurons with a calcium-indicator dye

9| Dissolve AM ester of the preferred indicator dye (e.g., OG-1 AM) in the solution containing 20% Pluronic F-127 in DMSO (e.g., 2 g Pluronic F-127 in 10 ml DMSO) to yield a dye concentration of 10 mM. Dilute this solution 1/10 to 1/100 with the standard pipette solution to prepare the

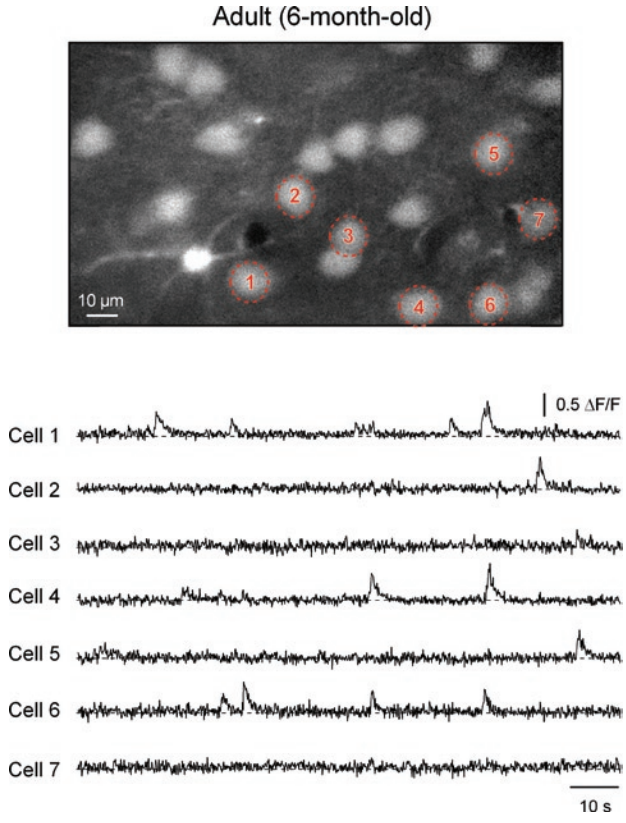

Figure 4 | Spontaneous Ca^{2+} transients in the visual cortex of an adult mouse. Spontaneously occurring Ca^{2+} transients (lower panel) in seven individual layer 2/3 neurons, marked with corresponding numbers in the upper panel. Data are derived from a series of time-lapse images recorded at 10 Hz.

staining solution. The same recipe is used to dissolve other Ca^{2+}-indicator dyes (e.g., Fura-2 AM, Fluo-4 AM, Calcium Green-1 AM, Indo-1 AM, Fura-PE3 AM, Fura Red AM and Magnesium Green AM)[2,7].

▲ **CRITICAL STEP** Note that the standard pipette solution (a simplified Ca^{2+}-free Ringer's solution) is designed to minimize precipitation of the dye. Filter the staining solution if necessary (we use a Millipore filter with a pore diameter of 0.45 µm).

? TROUBLESHOOTING

10| Pull a staining micropipette from a glass capillary using a pipette puller (we use standard-patch pipettes with a resistance of 6–9 MΩ when filled with standard pipette solution). Fill with staining solution. Insert the pipette into the cortex (under an angle of, for example, 30°) while monitoring the pipette resistance with a patch-clamp amplifier. The pipette resistance can increase slightly (up to 15 MΩ) when penetrating the dura mater, but must decrease to the initial level either immediately thereafter or, at the latest, upon the application of ejection pressure (see below). Advance the pipette along its axis until it reaches the desired depth (**Fig. 1**). With the dye-application pipette located 150–200 µm below the cortical surface, all cortical cells between the surface and a 400-µm depth are stained[2]. For staining the cells in deeper cortical layers, the pipette is positioned 650–750 µm below the pia[3]. Apply a pressure pulse (1 min, 70 kPa) to eject ~400 femtoliters staining solution near the cells of interest (see ref. 3 for the slightly modified protocol for labeling deep cortical layers). Remove the pipette.

■ **PAUSE POINT** Wait ~1 h to obtain a stable maximal fluorescence level in stained cells[2]. This protocol yields a stained area with a diameter of 200–400 µm.

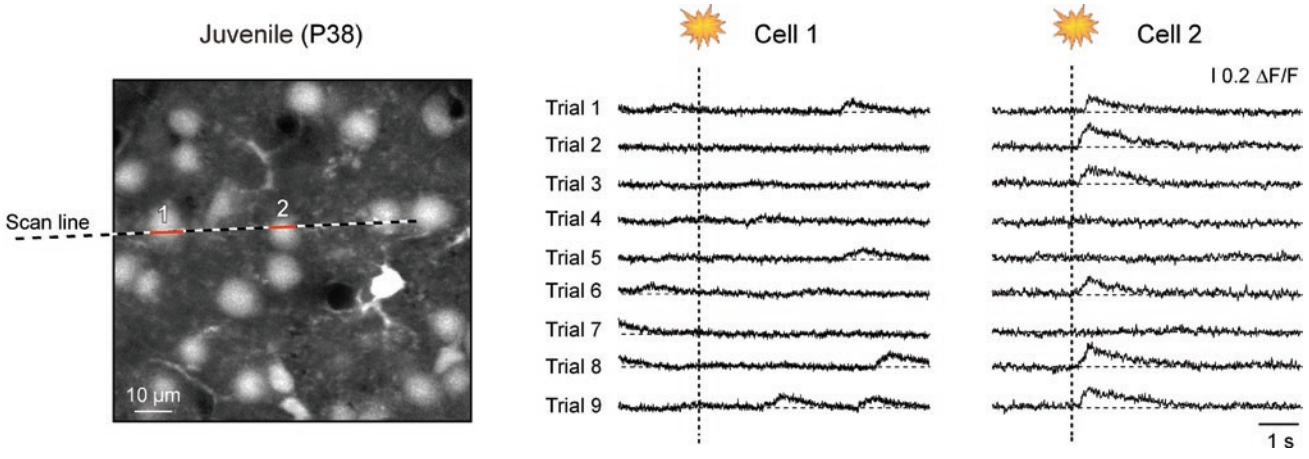

Figure 5 | Ca^{2+} transients in the individual cortical neurons evoked by sensory stimulation. Line-scan recordings of Ca^{2+} transients (sampled at 200 Hz; right panel) in two layer 2/3 neurons of the mouse visual cortex marked with the respective numbers in the left panel. The vertical broken line in the right panel marks the beginning of a brief (100 ms) light flash. Note that whereas cell 2 responded reliably to this stimulus, no responses were seen in the neighboring cell 1, which instead exhibited frequent spontaneous Ca^{2+} transients.

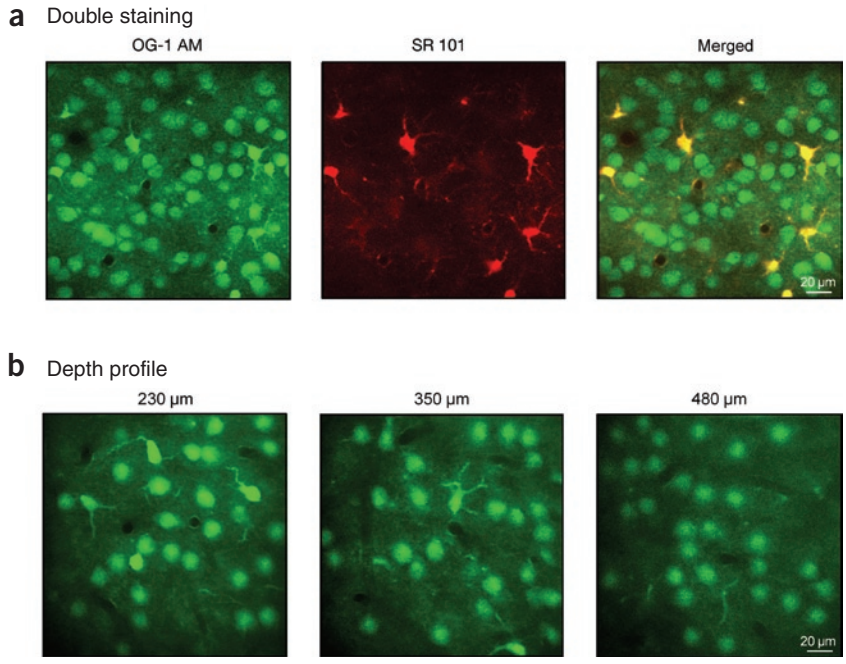

Figure 6 | Double staining and depth profile. (**a**) Microphotographs of layer 2/3 cells in the mouse visual cortex (30-d-old mouse) stained simultaneously using a dye mixture containing OG-1 AM and the glial marker SR101. The fluorescence of OG-1 AM was directed to the green channel (left) and the fluorescence of SR101 was directed to the red channel (middle). The merged image on the right shows neurons in green and glial cells in yellow. (**b**) Microphotographs of the visual cortex of a juvenile (32-d-old) mouse taken at different depths. The excitation light's pulse width under the objective was optimized by compensating group-velocity dispersion with a pair of prisms[28].

Two-photon imaging of stained cells

11| Choose the wavelength of the excitation light (depends on the indicator dye used). Consult ref. 24 for two-photon excitation spectra of common Ca^{2+}-sensitive dyes. Excitation light of 800 nm can be used for the initial visualization of neurons stained with the following indicator dyes: Fura-PE3 AM, Fura Red AM, Indo-1 AM, Calcium Green-1 AM, OG-1 AM, Fluo-4 AM and Magnesium Green AM.

12| Check the average power of the excitation light under the objective (must be at least 50–70 mW). Reduce the excitation power to the minimum and focus the objective (for example Nikon, 60×, numerical aperture (NA) 1.0, water immersion) of the two-photon microscope onto the brain surface. Raise the excitation power carefully (to avoid dye bleaching) and inspect the upper cortical layers using high-resolution settings (frame rate, 1 Hz). The following structures become clearly visible: large epithelial cells on the top of the cortex; layer 1 cells up to the depth of 100–200 µm, depending on the age of the animal (**Fig. 3a**, upper panel); and layer 2/3 cells (**Figs. 2–7**). When imaging through the

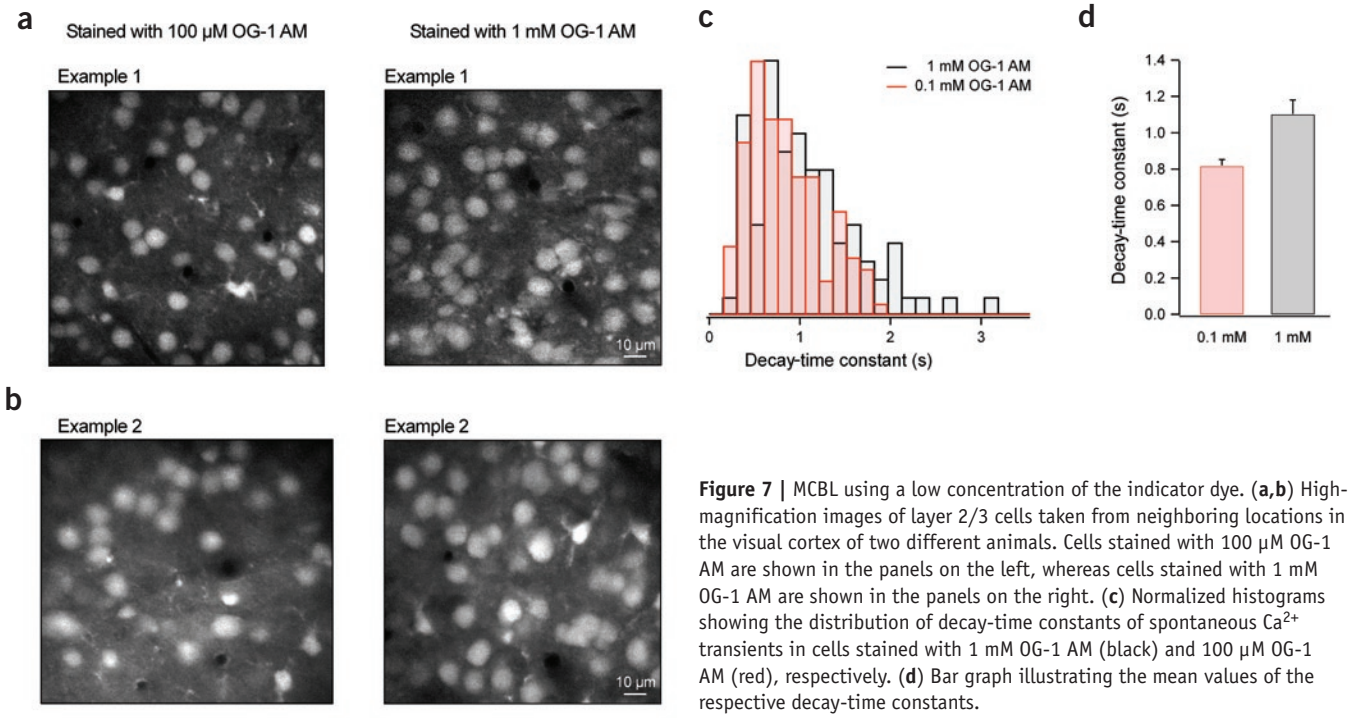

Figure 7 | MCBL using a low concentration of the indicator dye. (**a**,**b**) High-magnification images of layer 2/3 cells taken from neighboring locations in the visual cortex of two different animals. Cells stained with 100 µM OG-1 AM are shown in the panels on the left, whereas cells stained with 1 mM OG-1 AM are shown in the panels on the right. (**c**) Normalized histograms showing the distribution of decay-time constants of spontaneous Ca^{2+} transients in cells stained with 1 mM OG-1 AM (black) and 100 µM OG-1 AM (red), respectively. (**d**) Bar graph illustrating the mean values of the respective decay-time constants.

thinned skull, individual cells can be well resolved up to 250 µm below the cortical surface. Removing the skull above the imaging field further improves depth resolution. The maximal depth at which individual cells can be resolved depends on the shape and density of cells, the density of blood vessels in the imaged region and the age of the animal. In juvenile and young adult animals, the depth limit for single-cell imaging in the cortex is ~500 µm (**Fig. 6b**; consult ref. 25 for optimization of the imaging depth). In general, all cells in the preparation are stained; the only dark areas correspond to blood vessels, which are easily identified when focusing through the tissue. Furthermore, characteristic astrocytes often surround the blood vessels (see below).

? TROUBLESHOOTING

13| Monitor Ca^{2+} transients of interest, for example, with a frame rate of 10 Hz. Use online brightness-versus-time analyses to monitor photobleaching. Adjust excitation power to levels just below the bleaching 'threshold'. Use a commercially available software package for collection of time-lapse images and online data analyses.

? TROUBLESHOOTING

● TIMING

Dissection (Steps 1–8), 1h
Staining (Steps 9–10), 1h
Imaging (Steps 11–13), at least 6 h

? TROUBLESHOOTING

See **Table 1**.

TABLE 1 | Troubleshooting table.

PROBLEM	SOLUTION
Step 9: the staining patch micropipette gets clogged	Clean glass capillaries used to manufacture the pipettes; dissolve Ca^{2+}-indicator dyes immediately before use; filter the staining solution before use (e.g., with a Millipore filter; pore diameter, 0.45 µm)
Step 12: cells are not, or are only poorly, stained	There are two likely scenarios, either (i) the tissue is intact but the dye delivery failed, or (ii) the tissue is damaged (**Fig. 3**); for (i) monitor the pipette resistance during dye injection (a value above 20 MΩ indicates that the pipette is clogged); for (ii) check the anesthesia monitoring parameters, try to thin the skull as gently as possible, avoiding damage to large blood vessels, and increase the speed of dissection and staining (the latter should be accomplished in less than 2 h)
Step 13: recording conditions are unstable; occurrence of high-frequency vibrations of cells and/or slow drift of the plane of focus	These problems are more profound when the skull is removed; cover the skull opening with 2% low melting point agarose (e.g., 20 mg in 1 ml standard external saline) and keep the temperature of the external saline stable (with a precision of 0.1 °C); the high-frequency vibrations are usually the result of heart-beat pulsation (compare with measurements of the anesthesia-monitoring system) and are more profound in regions with a high density of blood vessels

ANTICIPATED RESULTS

Figures 2–5 illustrate MCBL-stained layer 2/3 cells in the visual cortex of mice of different ages. At each age, stained cortical neurons show spontaneous (**Fig. 4**) as well as light-evoked (**Figure 5**) Ca^{2+} transients. Data shown in **Figure 4** are derived from a series of time-lapse images recorded at 10 Hz. Light-evoked responses in **Fig. 5** were recorded as a series of line scans at 200 Hz. Note that in each case, the signal-to-noise ratio is sufficient to allow individual non-averaged somatic Ca^{2+} transients to be distinguished clearly from the background. As was shown by Kerr *et al.*[3] in rat somatosensory cortex, spontaneous Ca^{2+} transients reflect action-potential firing. Also, in the mouse visual cortex, both spontaneous (**Fig. 4**) and light-evoked (**Fig. 5**) Ca^{2+} transients required firing of action potentials (O.G. and A.K., unpublished observations).

As mentioned above, the MCBL technique is not cell-type specific. It provides a relatively homogeneous staining of different cell types (although glial cells are generally brighter than neurons). An identification of the various cell types can be achieved using additional markers (e.g., cell-type specific expression of green fluorescent protein) and multicolor two-photon imaging. A versatile approach for distinguishing between neuronal cells and astrocytes was developed by Nimmerjahn *et al.*[5] and is based on the use of the astrocyte-specific marker SR101, which emits fluorescence light at

wavelengths (550–750 nm) that are significantly longer than those of common Ca^{2+} indicators. For targeted SR101 staining, we modify the protocol of Nimmerjahn *et al.*[5]. We dilute 1 mg SR101 in 4 ml standard pipette solution and use it to dilute the concentrated solution containing 10 mM Ca^{2+} indicator (Step 9). The combined staining cocktail, containing SR101 and the Ca^{2+}-sensitive dye, is then injected into the brain (Step 10). This results in a good loading of neurons and glia cells with the Ca^{2+} indicator, and specific staining of glia cells with SR101 in the same brain region (**Fig. 6a**).

The MCBL technique was originally designed to apply 400 femtoliters of the concentrated Ca^{2+}-indicator dye-containing solution (yielding a final pipette concentration of the dye of 1 mM). Because membrane-permeant Ca^{2+}-indicator dyes are dissolved in DMSO, the staining solution also contained 10% DMSO (vol/vol). Although similar and/or higher detergent concentrations are routinely used to stain intact tissues with indicator dyes[11,26,27], we recently found that such a high dye/detergent concentration is not needed. As shown in **Figure 7a,b**, the MCBL protocol can provide high-quality staining of the mouse cortex with 100 μM OG-1 AM and 1% DMSO (vol/vol)-containing pipette solution. However, the use of the lower dye concentration imposes higher demands on the quality of preparation. The time constants of spontaneous Ca^{2+} transients were, on average, 0.81 ± 0.04 s ($n = 123$) in cells stained with 100 μM OG-1 AM and 1.10 ± 0.08 s ($n = 88$) in cells stained with 1 mM OG-1 AM (**Fig. 7c,d**). This similarity suggests that the intracellular indicator levels obtained with 100 μM OG-1 AM-containing staining solutions are nearly as high as those reached with 1 mM OG-1 AM-containing solutions (in the range of 20 μM^2).

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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