Functional imaging of neuronal activity of auditory cortex by using Cal-520 in anesthetized and awake mice

JINGCHENG LI,1,2 JIANXIONG ZHANG,1,2 MENG WANG,1 JUNXIA PAN,1 XIAOWEI CHEN,1,3 AND XIANG LIAO1,4

1Brain Research Center, Third Military Medical University, Chongqing 400038, China
2These authors contributed equally to this work
3xiaowei_chen@tmmu.edu.cn
4xiang.liao@aliyun.com

Abstract: The organization in the primary auditory cortex (Au1) is critical to the basic function of auditory information processing and integration. However, recent mapping experiments using in vivo two-photon imaging with different Ca2+ indicators have reached controversial conclusions on this topic, possibly because of the different sensitivities and properties of the indicators used. Therefore, it is essential to identify a reliable Ca2+ indicator for use in in vivo functional imaging of the Au1, to understand its functional organization. Here, we demonstrate that a previously reported indicator, Cal-520, performs well in both anesthetized and awake conditions. Cal-520 shows a sufficient sensitivity for the detection of single action potentials, and a high signal-to-noise ratio. Cal-520 reliably reported on both spontaneous and sound-evoked neuronal activity in anesthetized and awake mice. After testing with pure tones at a range of frequencies, we confirmed the local heterogeneity of the functional organization of the mouse Au1. Therefore, Cal-520 is a reliable and useful Ca2+ indicator for in vivo functional imaging of the Au1.

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1. Introduction

A sufficient sensitivity to detect single action potentials, a good signal-to-noise ratio (SNR), and no biased labelling for astrocytes are desired characteristics in general of Ca\(^{2+}\) indicators for studying neuronal physiology. A variety of synthetic and genetically encoded Ca\(^{2+}\) indicators (GECIs) have been developed and widely used for imaging neuronal activities [1–3]. The major advantages of the synthetic Ca\(^{2+}\) indicators over the GECIs is the high versatility for acute imaging experiments with rapid kinetics and a linear Ca\(^{2+}\) response [2, 4]. On the other hand, GECIs facilitate long-term repeated imaging of genetically specified neurons [2, 5]. Choosing a proper indicator for a particular study should be determined by the scientific goal and the neurons of interest [2].

So far, various Ca\(^{2+}\) indicators including rhod-2 [6], fura-2 [7, 8], OGB-1 [9–13], fluo-2 [14], fluo-4 [9, 15, 16], Alexa Fluor 594 together with fluo-5F [17, 18], and genetically encoded Ca\(^{2+}\) indicators (GCaMP3 [1] and GCaMP6s [3]) [14, 19, 20] have been widely used in studies of the Au1 with two-photon Ca\(^{2+}\) imaging in both in vitro and in vivo conditions. The in vivo experiments, particularly the functional mapping experiments, have provided important insights into the topographic organization of the Au1, but the degree of precision in tonotopic mapping has been controversial. The use of varying Ca\(^{2+}\) indicators (fluo-4, OGB-1 and GCaMP3) with different properties (e.g., detection sensitivity, signal-to-noise ratio, neuron-labeling quantity) may be a major cause of the discrepancies in these imaging results [5, 9, 15, 19, 21, 22]. Alternatively, different animal brain states (anesthesia versus wakefulness) may be another reason for the differing results [23–25].

Bandyopadhyay et al. have used OGB-1 to map auditory stimulation-evoked activity in the Au1 in anesthetized mice [9]. However, this widely used synthetic indicator has insufficient sensitivity to detect Ca\(^{2+}\) transients evoked by single action potentials under in vivo conditions [26]. This sensitivity is extremely important in studies of the topographic organization of the Au1, because many cortical neurons respond to sound stimulation with only one or two spikes, particularly in layer 2/3 [27, 28]. In addition, Bandyopadhyay et al. and Rothschild et al. have imaged the mouse Au1 with fluo-4 [9, 15], a synthetic dye with a higher detection sensitivity and a better signal-to-noise ratio (SNR) than OGB-1 [9, 29]. However, fluo-4 has a bias toward staining glial cells, thus often inducing contamination to
the neuronal signal detection [30–34]. Moreover, fluo-4 labels much fewer neurons than OGB-1, thus leading to an incomplete activity map [35]. In addition, rapid advances in genetically encoded calcium indicators (GECIs) have provided promising tools to image Ca$^{2+}$ activity in living tissues and specific cell types in vivo. Issa et al. have previously used GCaMP3 in the Au1 to address the issue of topographic organization [19]. However, its sensitivity to single action potentials and the detection rate of active neurons are both inferior to those of the synthetic indicator OGB-1 [1, 3, 5, 36]. Thus, GCaMP3 does not appear to be a reliable indicator for effectively sampling active neurons in the Au1 and for detecting single spikes in cortical neurons during sound stimulation [27].

Therefore, an improved indicator with a sufficient sensitivity, a better SNR, and an unbiased capacity for labelling both neurons and astrocytes is needed to study the topographic organization of the Au1. Cal-520 is a recently reported synthetic Ca$^{2+}$ indicator that has such required properties [26]. It has been used in studies of the neocortex, such as barrel and auditory cortices in anesthetized animals [26, 37], subcortical regions such as the superior colliculus [38], and the cerebellum [26, 39]. In cultured human neuroblasts SH-SY5Y cells, Cal-520 has been demonstrated to be an optimal indicator for tracking localized subcellular changes, as compared with other synthetic Ca$^{2+}$ dyes including OGB-1 and fluo-8 and three GECIs (GCaMP6-slow, -medium and -fast variants) [40]. Thus, testing Cal-520 for the functional mapping of neuronal activity in the Au1 under both anesthetized and awake conditions is worth pursuing. Here, we performed in vivo two-photon Ca$^{2+}$ imaging using Cal-520 AM through bolus loading procedure [41]. The spontaneous and sound-induced responses of Au1 neurons were recorded in both anesthetized and awake mice. By testing with pure tones at different frequencies, we demonstrated the ability to use Cal-520 to investigate topographic organization at a fine scale under both anesthetized and awake conditions.

2. Materials and methods

2.1 Animals

C57/BL6J male mice (8 −12 weeks old; 22-26 g) were provided by the Laboratory Animal Center at the Third Military Medical University. All experimental procedures were performed in accordance with institutional animal welfare guidelines and were approved by the Third Military Medical University Animal Care and Use Committee. All mice were housed in a 12-hour light-dark cycle (lights on at 7 a.m.) with ad libitum access to food and drinking water.

2.2 Ca$^{2+}$ imaging in the Au1 in vivo

We exposed the right Au1 of each mouse for two-photon imaging under anesthesia, as described in previous studies [11, 15, 42, 43]. Briefly, each mouse was anesthetized with 1.5% isoflurane in pure oxygen and kept on a warm plate (37.5–38°C). After local lidocaine injection, the skin and muscles over the Au1 were removed. We then glued a custom-made plastic chamber to the skull with cyanoacrylic glue (UHU) and performed a small craniotomy (~2 × 2 mm) (the center point: Bregma −2.5 mm, 4.5 mm lateral to midline). Afterward, we transferred the mouse to the imaging setup under a lower level of anesthesia (0.4-0.8%) (Breathing rates 90-130 breaths per minute). The craniotomy was filled with 1.5% low-melting-point agarose. The recording chamber was perfused with normal artificial cerebral spinal fluid (ACSF) containing 125 mM NaCl, 4.5 mM KCl, 26 mM NaHCO$_3$, 1.25 mM NaH$_2$PO$_4$, 2 mM CaCl$_2$, 1 mM MgCl$_2$ and 20 mM glucose (pH 7.4 when bubbled with 95% oxygen and 5% CO2). We used the highly sensitive fluorescent Ca$^{2+}$ indicator Cal-520 AM [26] for multicell bolus loading [41] in the Au1. Cal-520 AM was dissolved in DMSO with 20% Pluronic F-127 to a final concentration of 567 μM with ACSF for bolus loading. The loading procedure was performed according to a previous study [41]. We performed the Ca$^{2+}$
imaging ~1 hour after dye injection, and the imaging lasted for up to 8 hours. The body temperature of the mouse was kept between 36.5 and 37.5 °C throughout the experiments.

In two-photon imaging experiments for awake mice with head fixation, we used a custom-made plastic chamber designed for experiments on head-fixed mice. After surgery as described above for gluing the chamber to the skull of the mouse, the mouse was allowed to recover for 5 days. During this period, the mouse underwent head-fixation training for 3-5 days (from 1 to 4 hours per day). The head and the body of the mouse were rotated approximately 70° on the basis of the craniocaudal axis to keep the surface of the Au1 perpendicular to the microscope objective. Mice gradually adapted to this posture and were able to sit comfortably for 4 hours after these head-fixation training sessions. A small craniotomy (~2 × 2 mm) was performed on the recording day under local anesthesia, and this was followed by bolus loading of Cal-520 AM. A camera (frame rate 30 Hz) under infrared illumination was used to monitor the status of the mice.

A custom-built two-photon microscope system based on a 12-kHz resonant scanner (model “LotosScan 1.0”, Suzhou Institute of Biomedical Engineering and Technology) was used in our two-photon imaging, similarly to a setup described previously [44, 45]. The excitation light (wavelength 920 nm) was delivered by a mode-locked Ti:Sa laser (model “Mai-Tai DeepSee”, Spectra Physics) through a 40 × /0.8 NA water-immersion objective (Nikon). Depending on the depth of imaging, the two-photon laser power delivered to the brain was controlled in the range of 30 to 160 mW. Images of 600 x 600 pixels were acquired at a 40-Hz frame rate. The typical size of the field-of-view was ~200 µm x 200 µm. Three planes of imaging on average were acquired for each mouse.

2.3 Auditory stimulation

As described previously [42], an ED1 electrostatic speaker driver and a free-field ES1 speaker (both from Tucker Davis Technologies) were used to present sounds. The speaker was placed ~6 cm away from the contralateral ear of the mouse. Background noise (~60 dB SPL) was dominated by low-frequency components, comparable to the setup used previously [11, 42, 43]. For more details of the background noise, in the low frequency range (0 to 10 kHz), the main peak of power is below 1 kHz with spectral density of 33 dB/sqrt(Hz). It drops to below −3 dB/sqrt(Hz) at about 5 kHz and reaches floor level. There are harmonic peaks at scattered frequencies (3.9 kHz, 7.8kHz) below 5 dB/sqrt(Hz), corresponding to an energy of ~25 dB SPL. In the middle frequency range (10-40 kHz), the baseline noise remains about −5 dB/sqrt(Hz). The spectrum is full of harmonic peaks and narrow bands of higher level. The most important one is around 19 kHz. Its peak spectral density is 23 dB/sqrt(Hz), and its total energy is about 46 dB SPL. In the high frequency range (40-80 kHz), there are also narrow harmonic peaks whose level remains below 15 dB/sqrt(Hz). The notable peaks that are substantially higher are at 48 kHz and 54 kHz, with a spectrum level of ~10 dB/sqrt(Hz) (about 33 dB SPL total energy). This is below the typical sound levels used in the experiments. Therefore, the low-frequency peak was of no concern, because it was below the hearing range of the mice. We used custom-written software based on LabVIEW 2012 (National Instruments) to generate the broadband noise, which was transduced to analogue voltage via a PCI 6731 card (National Instruments). The duration of each sound stimulus throughout this experiment was 100 ms.

The sound levels were measured by placing the microphone ~6 cm away from the loudspeaker. We calibrated all sound levels with a ¼-inch pressure prepolarized condenser microphone system (377A01 microphone, 426B03 preamplifier, 480E09 signal conditioner, PCB Piezotronics Inc, USA), as previously described [46]. We sampled the data at 1 MHz via a high-speed data acquisition board USB-6361 from National Instruments and analyzed them by using a customized LabVIEW program. We applied broadband noise (bandwidth 0-50 kHz) at approximately 76 dB SPL, which was substantially louder than the background noise. For pure tone stimulation, the nominal 0 dB attenuation level corresponded to a sound level of
79 dB sound pressure level (SPL) for frequencies between 1 kHz and 10 kHz and to a sound level of approximately 81 dB SPL for frequencies up to 40 kHz.

2.4 Electrophysiological recordings in vivo

In cell-attached and whole-cell patch-clamp recording experiments in vivo, a shadow-patching procedure was used as previously described [11, 42, 43, 47, 48]. Recordings were carried out with an EPC10 amplifier (USB double, HEKA Elektronik). By using PatchMaster software (HEKA), the electrophysiological data were filtered at 10 kHz and sampled at 20 kHz. Glass electrodes containing normal ACSF and 50 μM Alexa-594 with a tip resistance of 5–8 MΩ were used for cell-attached recordings of Cal-520 AM-labeled neurons. For whole-cell recordings in awake mice, electrodes had a resistance of 5–8 MΩ with an internal solution containing 112 mM potassium gluconate, 8 mM KCl, 10 mM HEPES, 4 mM Mg-ATP, 0.375 mM Na2GTP, 10 mM sodium phosphocreatine and 50 μM Alexa-594. We monitored the series resistance of the electrode continuously, and the data were rejected for analysis if the resistance was higher than 30 MΩ.

2.5 Data analysis

All data analyses were performed using custom-written software in LabVIEW 2014 (National Instruments), Igor Pro 5.0 (Wavemetrics) and Matlab 2014a (MathWorks). From the observed Ca2+ imaging data, regions of interest (ROIs) were visually identified and drawn on the basis of the image intensity to extract the fluorescence time series. Then, the pixel values within each selected ROI were averaged to obtain the fluorescence changes (f). Relative fluorescence changes, Δf/f = (f-f0)/f0, of these ROIs were calculated as Ca2+ signals, and the baseline f0 was estimated as the 25th percentile of the fluorescence values within a sliding time window. In our analysis, glial cells were excluded on the basis of their morphology and the time course of the Ca2+ transients.

To automatically detect Ca2+ transients, we used a sliding time window (1 s) as the baseline and the following 500-ms detection window. First, the baseline of the Ca2+ signals was detrended using a linear fit, and then 3 times the standard deviation of the baseline activity was defined as the noise level. The difference between the averaged values (5 data points, 200 ms) around the peak in the detection window and the average of the baseline was considered as the amplitude of the Ca2+ transient candidate. Moreover, we calculated the first derivative of the Ca2+ signals in the detection window as the rate of the rise of the Ca2+ transients. Then, a signal was accepted as a transient only when both the amplitude and the maximum rate of the rise were greater than their corresponding noise level. After detection of the transient, the trace of the detected Ca2+ transient was extracted using exponential IIR filtering (200 ms window) and subtracted from the original signal. The residual activity was then used as the baseline for the next transient detection, as in the previously described peeling approaches [49]. The “success rate” was defined as the fraction of sound-evoked responses during 10 consecutive stimuli.

To analyze the frequency tuning property of neurons during pure tone stimulation (Fig. 5), the differences between the values of the detected Ca2+ transients (500 ms around the peak of the Ca2+ transient) and the average of the baseline (500 ms before the onset of the transients) were integrated to quantify the responses to each sound stimulus. The responses in the stimulation sequence were averaged for each frequency to construct the sound-evoked tuning curve. The average tuning curves across neurons were calculated first by normalizing each tuning curve with its highest amplitude, thus resulting in a range between 0 and 1. Then, the normalized tuning curves were aligned on the center frequency of their response bands and averaged. Tuning width was defined as the width of the tuning curve at half height, and the response band was defined as the corresponding range of frequencies [42]. For improving visibility, the average frequency tuning curves were fitted by a Gaussian function.
3. Results

3.1 $\text{Ca}^{2+}$ imaging of Au1 neurons in vivo using Cal-520 AM

Figure 1(A) illustrates the schematic of staining Au1 neurons in vivo with Cal-520 AM. We labeled the Au1 neurons and glial cells by using a bolus loading procedure with Cal-520 AM [26, 41] in isoflurane-anesthetized mice (Fig. 1(A)). Glial cells were distinguished from neurons on the basis of their morphology and $\text{Ca}^{2+}$ signal dynamics, as further confirmed by using SR 101 [50] (Fig. 1(B)). We restricted our analyses to neurons throughout this work. To examine the fidelity of the fluorescence changes reflected by Cal-520 relative to the number of action potentials, simultaneous recording of somatic action potentials and $\text{Ca}^{2+}$ transients in Cal-520-labeled neurons was performed in vivo (Fig. 1(C)). We found that the Cal-520 signals reliably reported single action potentials at a rate of nearly 100% (95.9% ± 2.3%; Fig. 1(D) and 1(E)).

![Fig. 1. Two-photon $\text{Ca}^{2+}$ imaging of the Au1 in vivo by using Cal-520 AM. (A) Schematic demonstrating the protocol for loading Cal-520 AM into the mouse Au1. (B) A merged image showing Au1 L2/3 neurons stained with Cal-520 AM in green and glial cells stained with both Cal-520 AM and sulforhodamine 101 (SR101, red) in yellow. (C) Simultaneous cell-attached recording and two-photon $\text{Ca}^{2+}$ imaging. The glass electrode is indicated by yellow dashed lines. (D) $\text{Ca}^{2+}$ transients (lower) and their corresponding action potentials (upper) from the neuron are indicated in panel C. The numbers of action potentials are indicated above the electrical trace. (E) Detection rate of $\text{Ca}^{2+}$ transients for different numbers of action potentials (n = 6 neurons). The time window of detection was 200 ms. Error bars represent SEM.](image)

3.2 Spontaneous and sound-evoked responses of Au1 neurons in anesthetized mice

One common issue in population $\text{Ca}^{2+}$ imaging using bolus loading is that neuronal signals can often be contaminated by adjacent neuropil [51]. To ensure that Cal-520 loading was not prone to such contamination, we randomly sampled several neurons (Fig. 2(A)) and their adjacent neuropils’ signals (Fig. 2(B), upper traces). The signals detected in neurons were not observed in their nearby neuropils. In addition, we also selected small regions of neuropils and compared their signals with those of the adjacent neurons (Fig. 2(B), lower traces). Similarly, we found no contamination of neuronal signals by nearby neuropils.
As shown in Fig. 2(C), we observed both spontaneous and sound-evoked activities in Au1 L2/3 neurons in anesthetized mice. Consistently with previous studies in neocortical L2/3 neurons (see review e.g., [28]), in Cal-520-based Ca$^{2+}$ imaging, the frequency of the spontaneous activity of Au1 L2/3 neurons was rather low. The distribution of spontaneous Ca$^{2+}$ transients was far from a normal Gaussian distribution, with most clustered at the left side with very low rates less than 0.1 Hz (Fig. 2(C) and 2(D); left). In addition, the response of Au1 L2/3 neurons to broadband noise stimulation had success rates varying from 0 to 100% (mean 43%; 1263 neurons from 12 mice; Fig. 2(C) and 2(D); right): 45% of the recorded neurons showed responses with success rates higher than 50%, 43% had success rates lower than 50%, and 12% were silent.

3.3 Spontaneous and broadband noise-induced responses of Au1 neurons in awake mice

General anesthesia is widely accepted to affect many aspects of cortical dynamics under conditions with and without sensory stimulation [23–25]. Therefore, we next tested whether Cal-520 has good imaging performance in awake animals. To keep the brain surface of the Au1 perpendicular to the microscope objective, we developed a plastic chamber and a matched fixing apparatus (See Materials and methods). While animals were awake, eye opening, whisking, and grooming were clearly observed under the camera recording. Whole-
cell current-clamp recordings of L2/3 neurons revealed that small, high-frequency membrane potential fluctuations with a unimodal distribution prevailed in the awake state (n = 4 cells) (Fig. 3(A) and 3(B)). These patterns differed from the large, slow fluctuations during slow oscillations under anesthesia [11, 42], thus further confirming the awake state of the mice.

Fig. 3. Whole-cell patch-clamp recordings in Au1 neurons in awake mice. (A) (A and B) Two example neurons from two different awake mice. Left, whole-cell recording. Right, distribution of the membrane potential (binned at 0.5 ms) from the corresponding neuron shown in the left panel.

Using Ca²⁺ imaging, we observed the spontaneous and broadband noise-evoked responses in Au1 L2/3 neurons (Fig. 4(A)). Similarly to the data under anesthetized conditions, the spontaneous activity of neurons in awake mice exhibited a low frequency in a range of 0 to 0.3 Hz (Fig. 4(B) and 4(C), left). During broadband noise stimulation, the average response rate of Au1 L2/3 neurons was 34%, with a range of 0 to 100% (Fig. 4(B) and 4(C), right). Here, 30% of the recorded neurons showed responses with success rates higher than 50%, 59% had success rates lower than 50%, and 11% were silent. Therefore, as compared to that under anesthesia (Fig. 2), in awake mice the total fraction of responsive neurons is similar (~88% versus ~89%). However, the fraction of cells with a success rate higher than 50% in awake animals (~30%) is lower than that in anesthetized animals (~45%). This could be in part due to the inhibition dominance in awake cortex during sensory responses as previously demonstrated in mouse visual cortex [25].

Fig. 4. Spontaneous and broadband noise-evoked responses of Au1 neurons in awake mice. (A) Two-photon image of Cal-520 AM-labeled neurons at a depth of 227 µm from the pial surface in an awake mouse. (B) Spontaneous activation (left) and sound-evoked responses (right) of five neurons as indicated in panel A. The vertical gray bars denote sound stimuli. (C) Distribution of spontaneous response frequency (left) and success rate for evoked responses to broadband noise (n = 855 neurons from 8 mice).

3.4 Pure tone-induced responses of Au1 neurons in anesthetized and awake mice

To test the ability of Ca-520 to be used in investigating the topographic organization of the Au1, we next recorded the neuronal responses to pure tone pips over a range from 2 to 40 kHz (each frequency was presented 20 times; 0 dB attenuation) in both anesthetized and awake mice. Figure 5(A) and 5(B) show two example neurons under anesthesia that responded to sound stimulation at multiple frequencies, with their largest responses at 8.9 kHz (Fig. 5(B) and 5(C)). Across populations in both anesthetized and awake mice, Au1
neurons showed strong responses to pure tones, with single-peaked tuning curves. However, the average curve had a large tuning width, because we used a relatively high sound level in this study (Fig. 5(D)).

Fig. 5. Pure tone-evoked responses of Au1 neurons in both anesthetized and awake mice. (A) Two-photon image of Cal-520 AM-labeled L2/3 neurons in an anesthetized mouse. (B) Pure tone-evoked Ca$^{2+}$ transients (average of 20 trials) in two example neurons indicated in panel A. Eleven pure tones were presented. Each frequency was applied 20 times. (C) Frequency tuning curves of the two neurons in panel A and B. Data points are the mean integrals of the Ca$^{2+}$ responses (fitted with a Gaussian function). (D) The normalized and aligned frequency tuning curve of 361 neurons from anesthetized mice (left) and 194 neurons from awake mice (right). (E and G) Spatial distribution of responsive neurons to pure tones in two example imaged fields from two anesthetized (E) and two awake mice (G). The frequencies are shown on the right side of panel G. (F and H) Left: Distribution of the $\Delta$best frequency of imaged neurons as the function of their spatial distance in the anesthetized condition (F) or awake condition (H); Right: Distance distribution of the neuron pairs whose $\Delta$best frequency is less than one octave in anesthetized mice (F) or awake mice (H). (I and J) Left: Distribution of the $\Delta$best frequency of imaged neurons as the function of their spatial distance at sound intensity of $-20$ dB (I) or $-40$ dB (J); Right: Distance distribution of the neuron pairs whose $\Delta$best frequency is less than one octave at sound intensity of $-20$ dB (I) or $-40$ dB (J) ($n = 361$ neurons, 6 anesthetized mice).
The spatial distribution of the best frequency (BF) of the neurons was intermingled over a 200 µm × 200 µm scale: neighboring neurons were equally able to show the highest preference for any of the frequencies used here (see two example fields of imaging in anesthetized mice in Fig. 5(E) and in awake mice in Fig. 5(G)). We plotted the ΔBF (Δfrequency) as the function of the Δdistance between any two neurons in the same imaging plane. In both anesthetized and awake states, the Δdistance was distributed almost evenly at each data point of ΔBF. Notably, a shift in the BF of more than 4 octaves was observed among neurons whose Δdistance was less than 50 µm, thus indicating that there was no spatial clustering of neurons with a similar BF (Fig. 5(F) and 5(H), left). Moreover, the maximal distance of the neurons with ΔBF of 0 to 1 octave reached up to 200 µm in both anesthetized and awake mice (Fig. 5(F) and 5(H), right). This value is much larger than the largest distance of 100 µm in tonotopy previously reported in large-scale mapping experiments [52]. Furthermore, we performed the same experiments with two lower sound intensities (20 dB attenuation, −20 dB; 40 dB attenuation, −40 dB). As shown in Fig. 5(I) and 5(J), with the pure tones with lower intensities, the spatial distribution of the best frequency of the neurons was still intermingled at a fine scale. These results suggest that there is no smooth tonotopy at a 200 µm × 200 µm scale but instead suggest a heterogeneous organization of local circuits in the Au1 [9, 15].

4. Conclusion and discussion

In this report, we systematically validated the efficacy of a recently reported synthetic Ca²⁺ indicator Cal-520 AM for mapping experiments in the mouse Au1 [26, 41, 51]. We confirmed that Cal-520 AM in the Au1 performed well under both anesthetized and awake conditions. Cal-520 in the Au1 had a high SNR and a sufficient detection sensitivity for single action potentials. Furthermore, we re-investigated the topographic organization of the Au1 at a fine scale. We confirmed a heterogeneous organization of the Au1 in both anesthetized and awake mice as described previously using OGB-1 or fluo-4 [9, 15]. Therefore, our results with Cal-520 AM support the hypothesis of heterogeneity of the topographic organization in L2/3 local circuits in the Au1 [22], and provide a proof-of-principle for the use of Cal-520 for Au1 mapping experiments.

The inconsistent results in previous two-photon imaging-based mapping experiments in the Au1 can be explained by various reasons, including differences in recorded cortical areas, cortical layers, experimental methodologies, and species and brain states [22]. Among these reasons, the use of different indicators might be an important possibility, although this aspect seems to be often ignored in the community. The use of indicators with a low SNR and detection sensitivity, e.g., OGB-1 and GCaMP3 [14, 19, 20], may result in a low detection rate of responding neurons in the sensory cortex, thereby yielding an incomplete activity map. In addition, the use of fluo-4, a commonly used indicator for astrocytes [34], may cause a significant contamination of neuronal signals by astrocytes. Moreover, the use of indicators that have a non-linear Ca²⁺ response, e.g., mostly GECIs with four cooperative Ca²⁺-binding sites, may result in a significant change in the shape of tuning curves, thereby yielding an incorrect activity map. Our results provide evidence that Cal-520 AM is suitable for studying the functional organization of the Au1, because it has a good SNR, a high detection sensitivity and an unbiased labelling property for neurons. It should be noted that, due to the lack of direct comparison with any other indicators under the same experimental conditions, our results do not address the issue of inconsistent results in previous two-photon imaging-based mapping experiments in the Au1. However, by focusing on the technical side showing a series of proof-of-principle experiments, our results provide a potentially suitable choice of Ca²⁺ indicators for addressing this inconsistence issue. In the near future, it would be interesting to test the performance of the latest GECI GCaMP6s in the issue of topographic organization, because it has a much higher sensitivity than GCaMP3 to detect single action potentials as initially demonstrated in mouse visual cortex [3].
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