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Improved deep two-photon calcium imaging in vivo

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ABSTRACT

Two-photon laser scanning calcium imaging has emerged as a useful method for the exploration of neural function and structure at the cellular and subcellular level in vivo. The applications range from imaging of subcellular compartments such as dendrites, spines and axonal boutons up to the functional analysis of large neuronal or glial populations. However, the depth penetration is often limited to a few hundred micrometers, corresponding, for example, to the upper cortical layers of the mouse brain. Light scattering and aberrations originating from refractive index inhomogeneties of the tissue are the reasons for these limitations. The depth penetration of two-photon imaging can be enhanced through various approaches, such as the implementation of adaptive optics, the use of three-photon excitation and/or labeling cells with red-shifted genetically encoded fluorescent sensors. However, most of the approaches used so far require the implementation of new instrumentation and/or time consuming staining protocols. Here we present a simple approach that can be readily implemented in combination with standard two-photon microscopes. The method involves an optimized protocol for depth-restricted labeling with the red-shifted fluorescent calcium indicator Cal-590 and benefits from the use of ultra-short laser pulses. The approach allows in vivo functional imaging of neuronal populations with single cell resolution in all six layers of the mouse cortex. We demonstrate that stable recordings in deep cortical layers are not restricted to anesthetized animals but are well feasible in awake, behaving mice. We anticipate that the improved depth penetration will be beneficial for two-photon functional imaging in larger species, such as non-human primates.

Contents

Abbreviations: AM, acetoxymethyl; GCaMP, protein containing circularly permutated green fluorescent protein, calmodulin and calmodulin-binding domain from the myosin light chain kinase; GECI, genetically encoded calcium indicator; OGB-1, Oregon Green© 488 BAPTA-1, name of a fluorescent calcium dye.

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1. Introduction

Two-photon fluorescence laser-scanning microscopy is often the method of choice for in vivo brain imaging with single-cell and high temporal resolution in anesthetized or even awake behaving animals [1–3]. Specific populations of neurons and glial cells can be functionally assessed both in health (for review see [1]) and disease [4]. Light scattering and aberrations due to refractive index inhomogeneities in biological tissues limit the depth of two-photon imaging and cause a gradual decrease of two-photon excitation efficiency with increasing cortical depths [2,5–7]. Generally, two-photon imaging of cells, labeled individually for example with a calcium indicator, is feasible even in deep cortical layers of the mouse brain [8]. However, recording activity in dense neural populations consisting of multiple labeled cells is often restricted to the superficial cortical layers [3], because of the background fluorescence generated in labeled tissue above the focal plane [9]. Therefore, images recorded from deep cortical layers of the mouse brain often appear blurred and calcium signals nominally collected from individual cells are significantly contaminated by out-of-focus fluorescence fluctuations [6,7].

Several approaches can help increase the recording depth when using in vivo multi-photon brain imaging of activity in neuronal populations. For example, three-photon excitation of fluorescent indicators can be used to shift the excitation light to longer wavelengths and, thus, to reduce scattering effects [5]. However, the cross-section of three-photon absorption is quite low for commonly used fluorophores and high laser power values are required for the detection of biological signals in individual brain neurons [10]. The high laser power needed for three-photon microscopy can be achieved by using regenerative amplifiers, which can provide excitation laser light pulses with high peak intensities [11]. The direct use of a regenerative amplifier with standard two-photon microscopes is another approach that helps increase the imaging depth. With this method, GCaMP6s-based calcium imaging in layer 5 neurons of the mouse somatosensory cortex was shown to be feasible [12]. Another approach of calcium imaging in the deep cortical layers of the mouse brain, like layer 5 cells, involves the use of adaptive optics that reduce optical aberrations and, thereby, increase the efficiency of two-photon excitation in deep cortical layers [6]. Furthermore, the use of red-shifted variants of genetically encoded calcium indicators (GECIs), with longer excitation and emission wavelengths compared to their green-emitting counterparts, is another strategy for potentially increasing the depth of two-photon imaging. With such red-shifted GECIs, single cell activity was recorded successfully in cortical layer 6 of the mouse brain [13] and in the dentate gyrus of the hippocampus [14]. Finally, an invasive approach that finds increasingly more applications and that can, in principle, be used for recordings at any brain depth, is based on the use of gradient-refractive index (GRIN) lenses [15,16]. Generally, these methods require additional, often sophisticated instrumentation or, in the case of the red-shifted GECIs, advanced and time consuming methods of fluorescent sensor delivery. In the following we present a simple method of two-photon calcium imaging with improved depth penetration that can be readily implemented for the use with standard instrumentation in acute experiments.

2. In vivo two-photon imaging with the red-shifted calcium indicator Cal-590

The method of deep two-photon imaging with the red-shifted calcium indicator Cal-590 was recently introduced by Tischbirek et al. [17]. Here, we highlight the most important features of the method, include some extensions and demonstrate its feasibility for the use in awake, behaving animals. The original approach of bulk-loading was mostly used in combination with the fluorescent calcium indicator OGB-1 AM [3,18]. Subsequently, other blue or green fluorescent calcium sensors, such as for example Fura-2 AM, Fluo-4 AM, Indo-1 AM, were used (for review see [1]). It is important to note that the more recently introduced synthetic calcium dye Cal-520 AM reports calcium transients with a particularly good signal-to-noise ratio under many in vivo recording conditions [19]. However, a common limitation of the original method of bulk loading of calcium indicators was that two-photon imaging was largely restricted, for example in the mouse brain, to the analysis of upper cortical layers.

A significantly improved depth penetration can be achieved when using a red-shifted version of Cal-520, namely Cal-590, which has a peak emission wavelength of 590 nm. In our first description of the method [17], we had established that the optimal two-photon excitation wavelength of Cal-590 peaks around 1050 nm. It is noteworthy that our earlier attempts to use one of the 'classical' red-shifted calcium indicator dyes for in vivo two-photon imaging, including Calcium Orange AM, Fura Red AM and Calcium Crimson AM, were not successful because of multiple problems, including difficulties with dye loading and poor signal-to-noise ratios. These discouraging results were in line with observations by others [20]. In addition to unproblematic dye loading and a remarkably good signal-to-noise ratio, a convenient aspect of using Cal-590 for two-photon imaging is the availability of Ytterbium fiber lasers that emit light at 1050 nm. Compared to other pulsed lasers that are used for two-photon excitation, Ytterbium fiber lasers are (relatively) low-cost, robust and compact in size [21–23]. Besides using a laser with an appropriate excitation wavelength, only few changes are necessary to configure a two-photon microscope for deep-imaging experiments with Cal-590. It is useful to check that the optical components of the microscope, such as mirrors, lenses, the electro-optical modulator and the dichroic mirror are sufficiently suited for the excitation wavelength of 1050 nm (for details see [17]).

The basic approach of bulk loading with Cal-590 AM is in many ways similar to the original method of multi-cell bolus loading of fluorescent calcium dyes [3,24]. However, it involves some critical modifications when attempting deep two-photon imaging. For imaging cells in the upper cortical layers of the mouse brain, Cal-590 AM was delivered through a micropipette into the cortical tissue using a procedure that is identical to the one originally described for the use of OGB-1 AM [24]. Within 50–60 min following dye delivery, single cells of the neural population, after reaching stable levels of staining, become clearly discernible. Stained cells showed a characteristic, ring-shaped appearance because of the lower fluorescence levels in the nucleus compared to the cytoplasm. To relate calcium transients of single-cells recorded with Cal-590 AM to neuronal activity, two-photon imaging was combined with cell-attached patch-clamp recordings. Despite the relatively low Ca^{2+} affinity of Cal-590 [17], single action potential-associated somatic Ca^{2+} transients were readily detectable in most experiments and the fluorescence changes were linearly related to the number of action potentials [17]. Overall, Cal-590 works as least as good as OGB-1 when used for monitoring neuronal activity in upper cortical layers of the mouse brain.

For loading Cal-590 AM to deep layers of the mouse cortex, it is important to choose a cortical region free of large blood vessels, to avoid a too strong absorption of excitation and emission light. This is achieved by navigating the pipette through the brain tissue by continuously visualizing it using a procedure that is similar to that used for shadow patching [25]. For this purpose, we added Alexa 680 (10 μM; two-photon fluorescence excitation at 1050 nm) to the pipette solution. To avoid strong out-of-focus fluorescence, the dye creating the 'shadows' is solely applied to small volumes of tissue, such that the overlying tissue is only minimally contaminated.