Mapping Brain Circuit Function *In Vivo* Using Two-Photon Fluorescence Microscopy

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ABSTRACT Mapping the activity of neuronal circuits with high resolution in the intact brain is a fundamental step toward understanding brain function. In the last several years, nonlinear microscopy combined with fluorescent activity reporters has become a crucial tool for achieving this goal. In this review article, we will highlight the principles underlying nonlinear microscopy and discuss its application to neuroscience, focusing on recent functional studies in the rodent neocortex in combination with genetically encoded calcium indicators. *Microsc. Res. Tech. 00:000–000, 2014*.

INTRODUCTION

The mammalian brain is a network of more than 100 billion individual cells that can be divided into two main classes: neurons and glia. Each of these two categories includes multiple cell subtypes. For example, neurons can be classified based on their morphology, their electrical properties, the chemical transmitter that they release, the proteins that they express, or a combination thereof (Kandel et al., 2013). Similarly, glia are a heterogeneous population of cells that provides important feedback to neurons and includes many subclasses of functionally distinct cells (Kandel et al., 2013). Aside from the staggering number and diversity of cells, the complexity of brain behavior also depends on the precise connections that these cells form: a simple reflex response or a complex mental process is associated with the generation of elaborate spatial and temporal patterns of electrical activity in specialized brain areas. The generation of these patterns is strongly influenced by the functional connections between individual neurons, known as the functional connectome.

A first necessary step toward elucidating the basic principles underlying brain function is to precisely map the activity of its individual cellular elements in space and time. Given the importance of connectivity in shaping cellular responses, it is evident that this task should be addressed in vivo where connectivity is preserved and the activity of cells can be correlated with higher brain functions, such as perception, attention, motor coordination, and memory.

Electrophysiology has long been the preferred method for studying the central nervous system, because of its excellent temporal resolution and its ability to capture a wide range of neural phenomena, from the millisecond-precise spiking activity of individual neurons and small populations to the slower network oscillations (Buzsaki et al., 2012). Electrophysiological approaches in vivo, however, have limitations. Intracellular recordings can be made simultaneously in the form of either patch-clamp or sharp electrode recordings from a maximum of only a few individual cells (Chauvette et al., 2010; Margrie et al., 2002; Petersen et al., 2003). In contrast, extracellular recordings, which measure the activity of populations of cells, lack cellular specificity (Buzsaki et al., 2012). In an attempt to record from large networks of cells with cellular resolution, multielectrode arrays have been developed (Bareket-Keren and Hanein, 2012; Spira and Hai, 2013; Viventi and Blanco, 2012; Viventi et al., 2011). However, to measure the electrical signals from hundreds or thousands of cells in the intact brain, the recording devices used in this approach must be miniaturized to reduce tissue damage following probe insertion (Kim et al., 2013).

Whole-brain imaging techniques, such as fMRI, greatly contributed to our understanding of how neuronal circuits that are distributed over large brain areas respond to external stimuli, allowing researchers to directly and noninvasively visualize changes in the brain during normal and pathological conditions (Craddock et al., 2013; Ugurbil et al., 2013; Van Essen et al., 2013). However, these approaches have limited spatial and temporal resolution and do not allow the monitoring of brain dynamics at cellular and subcellular resolution.

In the last 20 years, the development of nonlinear microscopy in combination with fluorescent activity reporters has provided a valuable tool to overcome some of the limitations of the aforementioned approaches. First, fluorescence microscopy permits one to simultaneously visualize the structure and function of hundreds of cells with cellular and even subcellular resolution (Helmchen and Denk, 2005; Gobel et al., 2007; Svoboda and Yasuda, 2006; Stosiek et al., 2003). Second, because light goes through the tissue without causing mechanical disturbances, fluorescence microscopy allows the study of cells and their

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interactions with the external world with minimal invasiveness in the intact central nervous system (Helmchen and Denk, 2005; Svoboda and Yasuda, 2006).

Two-Photon Fluorescence Imaging in the Intact Brain

Visualizing how neuronal networks respond, process and integrate incoming stimuli in the intact brain is a challenging task. First, neuronal networks comprise thousands of different neurons located in a three-dimensional volume, whose connections can extend over hundreds of microns up to several millimeters and whose cellular processes can have submicron dimensions. Thus, there is a need for imaging both with submicrometer resolution and over fields of view of several hundred microns in three dimensions. Second, neurons generate very fast (~1 ms) electrical signals (called action potentials) which propagate along cellular processes to target other neuronal cells. Ideally, a detection system with high temporal resolution is desired. Third, the brain is a spatially nonhomogeneous medium that contains structures with different refractive indexes and highly scatters and absorbs light. Thus, imaging is increasingly difficult as the depth within the sample increases.

Two-photon microscopy combined with fluorescent activity reporters has proven to be a valuable approach to investigate the intact central nervous system at cellular resolution (Grienberger and Konnerth, 2012; Grewe and Helmchen, 2009). In general, fluorescent microscopy is based on the transition of an electron to the excited state through the absorption of a photon of energy equal to the energy gap between the excited and ground states. Nonlinear, and in particular two-photon, fluorescence microscopy is based on the observation that the transition of an electron to the excited state can be obtained by the near-simultaneous absorption of two photons of energy, each equal to half the energy gap between the excited and ground states (Denk et al., 1990; Diaspro, 1999; Diaspro and Sheppard, 2002). Given that the energy of each photon is smaller, the light wavelength is longer. The success of two-photon microscopy for neuroscience applications is due to the fact that the longer wavelengths (700–1000 nm) that are commonly used for excitation are less sensitive to scattering than visible light, allowing fluorescence imaging in deeper brain regions. Moreover, because the absorption of two photons is required in two-photon imaging, the probability of absorption is a quadratic function of the light intensity (Helmchen and Denk, 2005). This implies that the probability of efficiently exciting the fluorophore in the two-photon regime is constrained in the z dimension, as compared to single-photon excitation. This intrinsic property of two-photon excitation drastically reduces out-of-focus fluorescence excitation, thus preventing probe photobleaching and tissue photodamage in out-of-focus regions (Denk and Svoboda, 1997; Zipfel et al., 2003). Furthermore, because two-photon excitation is spatially confined, all emitted fluorescence photons that are collected by the microscope objective can be directly deflected to the photo-detector with a dichroic mirror. This configuration, referred to as nondescanned, increases the signal-to-noise ratio of the emitted signal (Helmchen and Denk, 2005). However, to efficiently excite molecules using a two-photon process, a high temporal density of photons at the focal plane is needed. For most neuroscience applications, mode-locked laser sources that emit ultrashort (~140 fs duration) pulses with a large peak power and a fast (~80 MHz) repetition rate are commonly used. Ti:sapphire lasers, which can be tuned over a relatively large spectral range (680–1080 nm) are widely used for this type of application (Helmchen and Denk, 2005).

The typical optical set up of a two-photon microscope is shown in Figure 1. The intensity of the laser beam is first modulated (intensity modulation unit, Fig. 1A,B) and then directed on to a fast deflection system based on the scanning mirrors (galvanometric or resonant, Fig. 1A) (Denk et al., 1990) or acousto-optic devices (AODs, Fig. 1B,C) (Cotton et al., 2013; Duemani et al., 2008; Grewe et al., 2010; Katona et al., 2012). The scan and tube lenses form a telescope, which matches the dimensions of the laser beam with those of the back-aperture of the objective lens. When acousto-optic devices are used, a dispersion compensation unit is also inserted in the beam path (Fig. 1B). Emitted fluorescence signals are usually detected in the episcopic configuration. As explained above, to maximize the signal-to-noise ratio, the nondescanned configuration is most often utilized in combination with low-magnification, high numerical aperture objectives.

Imaging in three dimensions (3D) is fundamental to fully reconstruct the anatomy and function of cellular networks within the brain. Different experimental strategies have been developed to achieve the movement of the focus position along the axial direction, including: the mechanical motion of the objective by means of piezoelectric translators (Callamaras and Parker, 1999), variable focus lenses (Oku et al., 2004), deformable mirror devices (Qi et al., 2004), acousto-optic deflectors (Reddy and Saggau, 2005), small light-weight mirrors (Botcherby et al., 2008) and spatial light modulators (SLMs) (Daria et al., 2009; Dal Maschio et al., 2011). However, a few of these approaches have, so far, been applied for 3D fluorescence imaging in the intact brain in vivo. Using piezoelectric translator-mediated sinusoidal vibration of the objectives combined with coordinated movements of the galvanometric mirrors, functional imaging of brain cells on complex 3D trajectories has been performed (Gobel et al., 2007; Kerlin et al., 2010). Simultaneous imaging at different depths within the intact mouse cortex has been achieved also with spatiotemporal multiplexing (Cheng et al., 2011). In this configuration, the pulsed two-photon laser beam (80 MHz repetition rate) is divided in four beams which are delayed by 3 ns each (Fig. 1D). The four beams converge on the scanning mirror and are then projected on the objective back aperture. If the fluorescence decay time of the fluorescence indicator (typically around a few ns) is shorter than the laser repetition period (~12 ns), then the temporal multiplexing of the laser beam allows to detect fluorescence excited from spatially distinct beams. In this configuration, the number of beams that can be implemented is ultimately limited by the decay time of the indicator and the laser repetition rate. Two layer imaging in the intact mouse brain

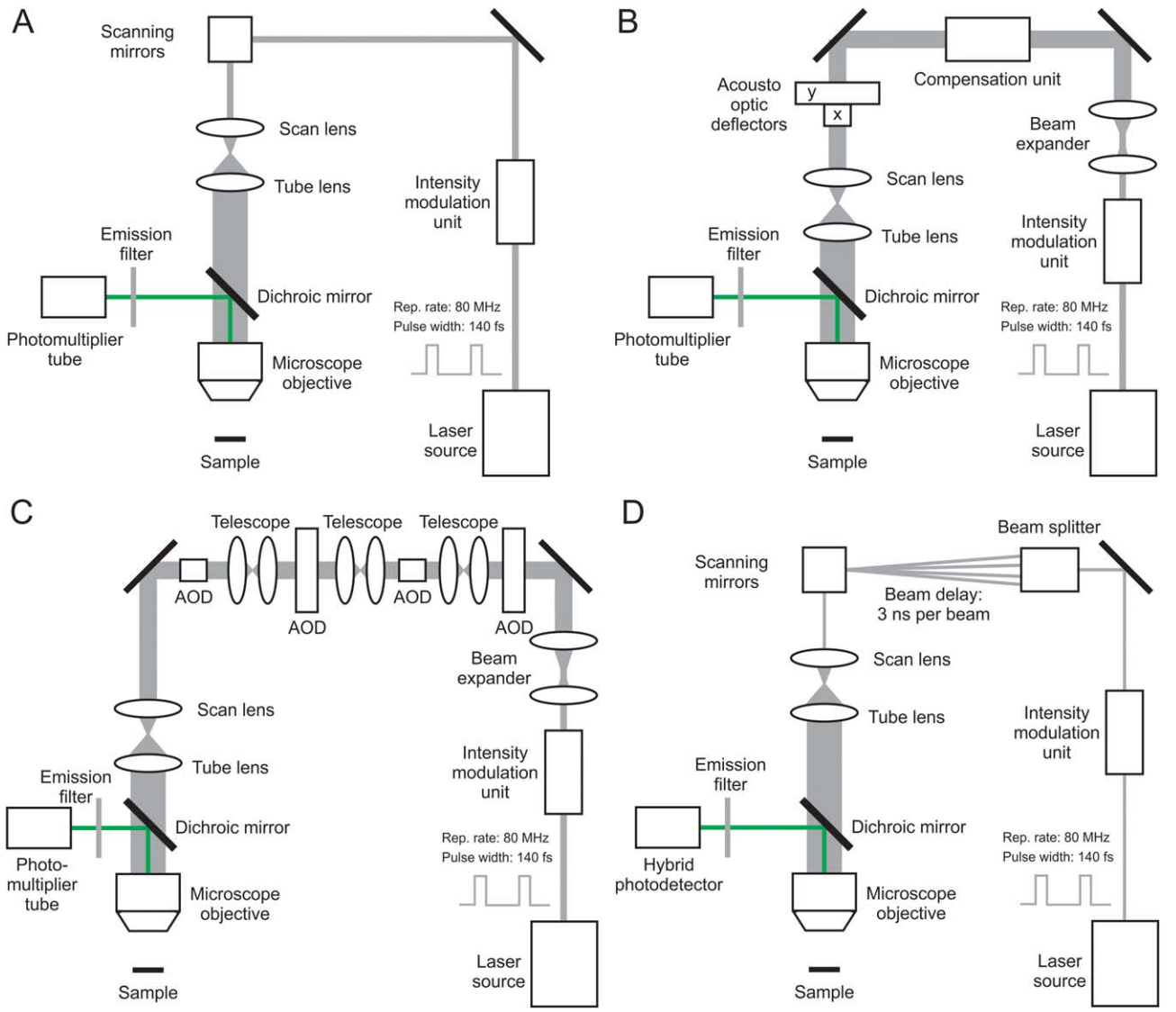

Fig. 1. Basic design of two-photon microscopes. A:, B: Schematic drawing showing the optical set-up required for two-photon fluorescence imaging with scanning mirrors (A) and acousto-optic deflectors (B). C: Optical set-up to perform 3D imaging as described in Cotton et al. (2013). For alternative optical configurations see also Katona et al. (2012). D: Layout of the optical set-up to perform simultaneous imaging at different depth with spatiotemporal multiplexing (Cheng et al., 2011).

has also been performed using an electrically tunable lens which is placed in the excitation path of a two-photon microscope (Grewe et al., 2011). More recently, acousto-optic deflectors have been used in vivo to control the beam divergence in addition to the deflection angle, resulting in the movement of the excitation spot in the z direction (Cotton et al., 2013; Katona et al., 2012). The optical configuration for this type of experiments is shown in Figure 1C (Cotton et al., 2013). A chain 4 AODs and telescopes is used to control the collimation and angle of the expanded laser beam at the level of the objective back aperture. To achieve movement in the z direction, the AOD is driven with a linear frequency sweep which generates a beam with cylindrical phase. By pairing two AODs, each with a sound wave propagating in the opposite direction the cylindrical phase curvature is doubled. To obtain spherical phase curvature and movement of the excitation spot in the axial direction an additional orthogonal pair of AODs is used (Fig. 1C) (Cotton et al., 2013).

Application of fluorescence two-photon microscopy in combination with fluorescent calcium indicators has proven to be a successful approach to image neuronal circuits in action (Dombeck et al., 2007, 2010; Huber et al., 2012; Jia et al., 2010; Margolis et al., 2012; Ohki et al., 2006). Indeed, recent studies report simultaneous and noninvasive monitoring of hundreds of cells with high spatial and temporal resolution in three dimensions in the intact brain using fluorescence indicators, which were able to identify the cellular response to small stimuli (Grewe et al., 2010; Katona et al., 2012). In the following paragraphs, we will

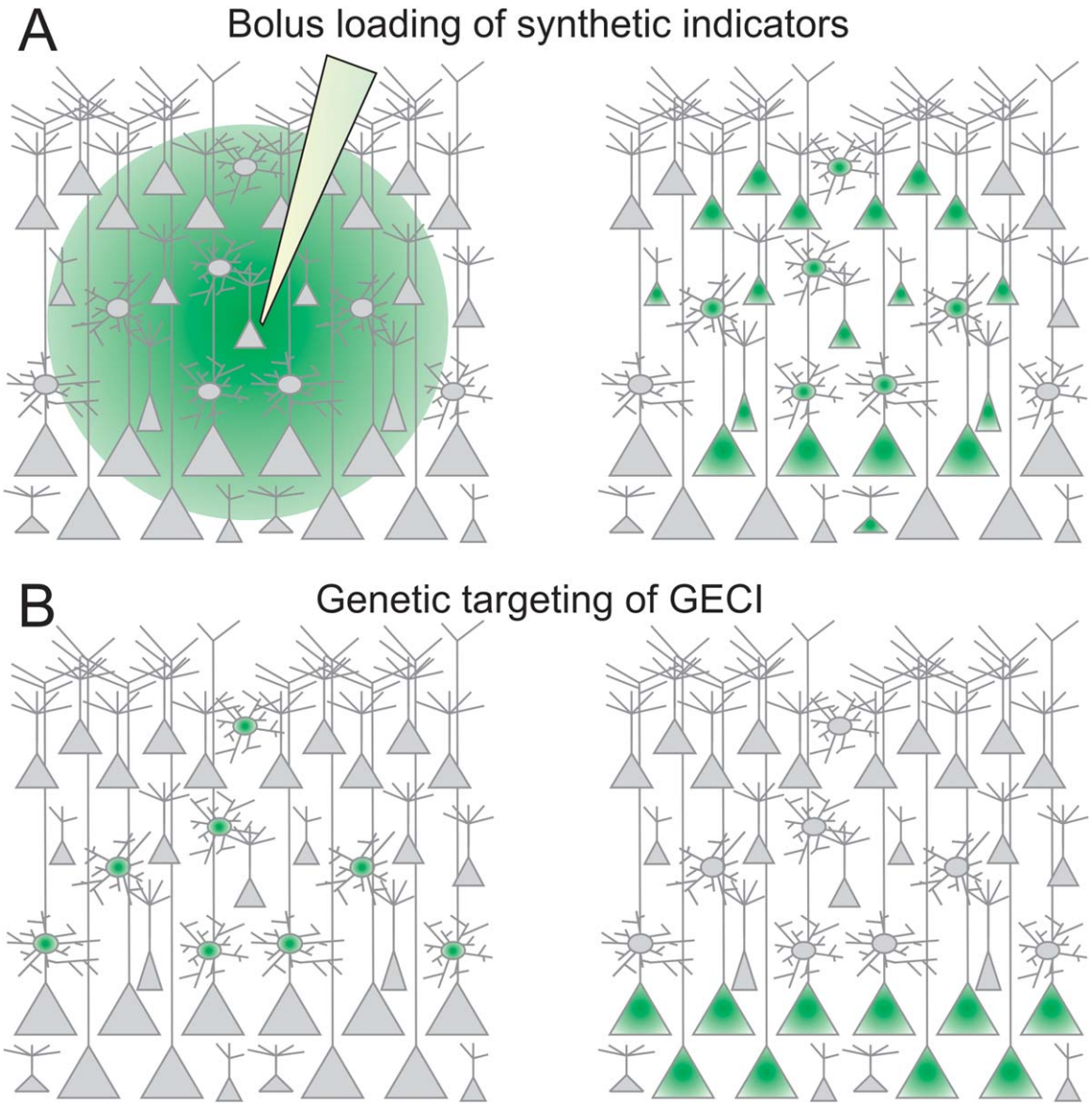

Fig. 2. Delivery of fluorescent indicators in vivo. A: Synthetic indicators are usually loaded into cells via a pressure injection within the brain tissue. Indicators are injected into the extracellular space (left panel) and then diffuse inside all cells that are in the proximity of the injection site, with no cell-type specificity (right panel). B: The DNA coding for GECI can be targeted to all cells. However, with cell-type specific DNA sequences, the indicator is expressed only in specific cellular subtypes (left and right panels).

introduce calcium indicators as reporters of neuronal activity and will focus on recent advances in our understanding of brain function using fluorescence two-photon microscopy in combination with genetically encoded GCaMP indicators.

The Calcium Ion as an Indirect Reporter of Neuronal Activity

In neurons, action potential firing and synaptic inputs generate a transient rise in the intracellular calcium concentration that returns to baseline via the activity of calcium extrusion mechanisms and internal buffering molecules. Non-neuronal glial cells also display increases in their intracellular calcium concentration in response to neuronal activity. Thus, imaging calcium in both neurons and glia is an effective way to monitor their activity. One way to measure free cytosolic calcium variations optically is using molecules that change their fluorescence or absorbance properties upon calcium binding (Grienberger and Konnerth, 2012; Terai and Nagano, 2013). This response strictly depends on the affinity, dynamic range, selectivity, and kinetic properties of the indicator (Perez and Nagai, 2013). Early insights into the study of cellular calcium dynamics were obtained using bioluminescent calcium-sensitive photo-proteins, such as aequorin (Shimomura et al., 1962), followed by the development of synthetic small molecule calcium indicators obtained from the hybridization of highly sensitive

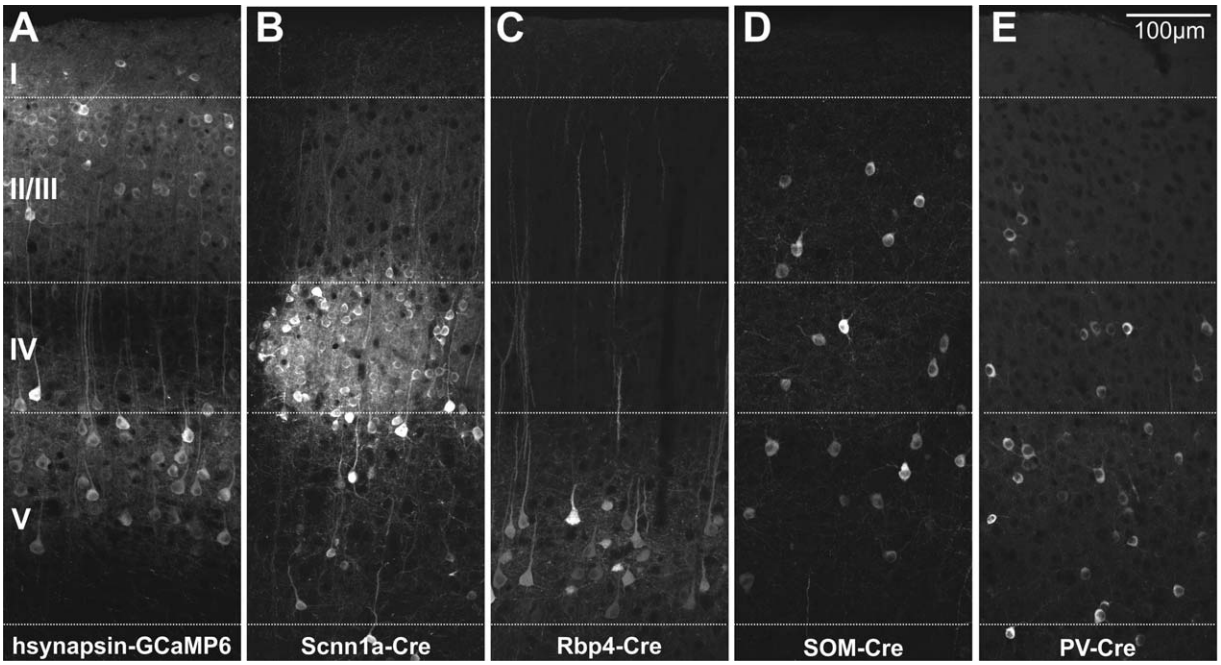

Fig. 3. Cell type-specific expression of genetically encoded calcium indicators. A: Confocal image showing GCaMP6 expression in cortical neurons of the mouse somatosensory cortex. Indicator expression was achieved through viral injection in wild-type mice using an adeno-associated virus carrying the GCaMP6 sequence under the human synapsin promoter. B–E: Confocal images showing GCaMP6 expression confined primarily to layer 4 excitatory neurons (B), layer V pyramidal cells (C), somatostatin-(D), and parvalbumin-(E)-positive interneurons. Cell type-specific expression of the fluorescence indicator is achieved using Cre-lox technology and injecting Scnn1a-Cre (B), Rbp4-Cre (C), SOM-Cre (D), and PV-Cre (E) mouse lines with adeno-associated viruses carrying a double floxed GCaMP6 sequence. Image courtesy of S. Bovetti and C. Moretti. The mouse strain Rbp4-cre was obtained from the Mutant Mouse Regional Resource Center, a NCRR-NIH funded strain repository, and was donated to the MMRRC by the NINDS-funded GENSAT BAC transgenic project. GCaMP6 plasmids were received from Vivek Jayaraman, Ph.D., Rex A. Kerr, Ph.D., Douglas S. Kim, Ph.D., Loren L. Looger, Ph.D., and Karel Svoboda, Ph.D. from the GENIE Project, Janelia Farm Research Campus, Howard Hughes Medical Institute.

calcium-selective chelators, such as EGTA and BAPTA, with a fluorescent chromophore (Grynkiewicz et al., 1985; Paredes et al., 2008; Tsien, 1980). Over the years, improved versions of synthetic calcium sensors have been developed. Among others, Oregon Green BAPTA (OGB) and Fluo-4 are largely used because of their fast response kinetics, good selectivity for calcium versus other cations, and large signal-to-noise ratios (Paredes et al., 2008). The major limitations of synthetic calcium indicators, when applied to the study of neuronal circuits, are the difficulty in labeling specific cell populations or subcellular compartments (unless a transgene is inserted, see for example, Tour et al., 2007) and their limited lifetime in the cells, which prevents chronic long-term in vivo recordings (Fig. 2A). The pitfalls of synthetic indicators have been partially overcome by the development of different types of indicator molecules (Miyawaki et al., 1997; Romoser et al., 1997). Because these sensors are encoded by a DNA sequence, they have been called "genetically encoded" calcium indicators (GECIs). GECIs are fusion proteins composed of a calcium binding molecule (Calmodulin or Troponin C) combined with a single fluorophore, or two fluorescent proteins when FÖrster resonance energy transfer (FRET) is used (Knopfel, 2012; Tian et al., 2012). The general mechanism of action of GECIs consists of a change in the fluorescence emitted upon calcium binding. In combination with specific cell-type promoters, subcellular targeting sequences, and transgenic technology, GECIs can be stably expressed in specific cell subpopulations (Figs. 2B, and 3) over long periods of time (weeks to months). However, despite the fact that the first published GECIs (FIP-CM_{SM} and Cameleon) date back to 1997 (Miyawaki et al., 1997; Romoser et al., 1997), it took more than 15 years to refine and develop a family of GECIs; namely, GCaMP6 (Chen et al., 2013), with biochemical properties comparable to those of synthetic calcium indicators (Table 1).

Genetically Encoded Calcium Indicators of the GCaMP Family

Of the single fluorophore calcium sensors, GCaMPs are among the most promising for detecting neuronal activity in vivo. The development of GCaMPs was preceded by the discovery that rearrangements, such as circular permutation, or insertions of unrelated sequences within the GFP structure do not prevent fluorescence. Conversely, the fluorescence of GFP can be made highly responsive to the conformation of appropriately inserted molecules (Baird et al., 1999). This property led to the development of the first single fluorophore calcium sensors (Camgaroo and Camgaroo-2) in which the complete amino acid sequence of the calcium binding protein Calmodulin (CaM) was inserted into the fluorochrome sequence, resulting in a fusion protein whose fluorescence is modulated by calcium concentration (Baird et al., 1999; Griesbeck et al., 2001). However, limited signal intensity was a

TABLE 1. Properties of synthetic (OGB-1) and genetically encoded (GCaMPs) calcium indicators

	λ for 2P excitation (nm)	λ_{em} (nm)	Rise time (ms)	Decay time (ms)
OGB-1	810–850[a,b]	520[a,b]	8[b]	$\tau_1 = 56$[b]; $\tau_1 = 777$[b]
GCaMP3	920[c]	510–520[c]	83[c]	610[c]
GCaMP6s	940[d]	510–520[d]	179[d]	550[d]
GCaMP6m	940[d]	510–520[d]	80[d]	270[d]
GCaMP6f	940[d]	510–520[d]	45[d]	142[d]

[a]Yasuda et al. (2004).
[b]Grewe et al. (2010).
[c]Tian et al. (2009).
[d]Chen et al. (2013).

significant barrier to the use of Camgaroos for imaging calcium dynamics in vivo, although some reports have been published in flies (Reiff et al., 2005; Yu et al., 2003).

Instead of using an insertion in the GFP structure, the strategy underlying the development of GCaMPs (Nakai et al., 2001), as well as that of their close relatives "Pericams" (Nagai et al., 2001), takes advantage of the properties of circular permuted GFP (cpGFP) (Baird et al., 1999). In these molecules, the original carboxyl and amino portions of GFP are interchanged and rejoined with a short spacer, and the new C- and N-terminals, now in close proximity, are fused to calmodulin (CaM) and to its interaction partner, the M13 domain of the myosin light chain kinase, respectively. These sensors were designed based on the assumption that in the absence of free calcium, CaM does not associate with the M13 domain, but upon calcium binding, CaM interacts with the M13 moiety to induce a subsequent conformational change in the cpGFP and an alteration in the emitted fluorescence (Akerboom et al., 2009; Nakai et al., 2001). The first GCaMP to be developed, however, displayed dim and temperature-sensitive fluorescence (Nakai et al., 2001), defects which were only partially improved in the following GCaMP1.6 and GCaMP2 (Ohkura et al., 2005; Tallini et al., 2006). Over the years, a number of variants of GCaMP obtained by mutations in the cpGFP moiety, linker sequences, CaM and M13 domains have been published to achieve sensors with improved properties (Akerboom et al., 2012; Chen et al., 2013; Muto et al., 2011; Tian et al., 2009). Important contributions toward improving GCaMP performance were the determination of the GCaMP2 crystal structure and the identification of the mechanism behind calcium-induced fluorescence change (Akerboom et al., 2009). As a consequence of these findings, a combination of protein structure-guided mutagenesis and semi-rational library screening led to the development of GCaMP3, the first GCaMP used for in vivo imaging of mouse cortical networks (Tian et al., 2009).

Further guided engineering of the GCaMP3 structure was applied in the design and characterization of 12 new variants belonging to the GCaMP5 group (Akerboom et al., 2012). These sensors displayed improved characteristics and were able to detect single action potentials and bursts of 2–3 much better than GCaMP3. Although much improved as compared to their precursors, the in vivo performance of the GCaMP5s was still trailing the synthetic calcium indicator OGB in the number of responsive cells, detection of sparse spiking activity and kinetics (Akerboom et al., 2012). A breakthrough in the field occurred with the recent development of ultrasensitive protein-based calcium sensors belonging to the GCaMP6 family, which surpassed the sensitivity and reached the kinetics of synthetic calcium dyes (Chen et al., 2013), providing a new and important tool to study brain circuits at the level of genetically defined neuronal populations.

All of the single fluorochrome genetically encoded calcium sensors mentioned above are GFP-based and exploit the biochemical properties of this molecule. However, it must be mentioned that several multicolor genetically encoded calcium indicators have also been developed (Akerboom et al., 2013; Ohkura et al., 2012; Zhao et al., 2011). Among them, different red shifted molecules have been characterized, although so far none have reached the performance of the newly generated GCaMPs (Akerboom et al., 2013; Ohkura et al., 2012; Zhao et al., 2011).

Optical Mapping of Brain Circuit Function in the Intact Brain with GCaMP Indicators

Although genetically encoded indicators with properties comparable to those of the synthetic indicators have been developed only recently (Akerboom et al., 2012; Chen et al., 2013; Tian et al., 2009), their use has already provided important insights into the spatial and temporal features of neuronal circuit responses in superficial (Harvey et al., 2012; Huber et al., 2012; Akerboom et al., 2012; Chen et al., 2012; Zariwala et al., 2012; Chen et al., 2013; O'Connor et al., 2010) and deep (Cui et al., 2013; Dombeck et al., 2010) brain regions in vivo. Here, we will highlight recent results obtained using the rodent neocortex as an experimental system. In anesthetized mice, GCaMP3 was expressed in the superficial layers (II/III neurons) and two-photon imaging was performed in combination with electrophysiological juxtasomal recordings to correlate the calcium signal with the electrical signal. A linear relationship was observed between these two signals while the neuron was discharging 1–10 action potentials at 50 Hz. The single action potential detection rate was ~70%. In contrast, in awake, head-restrained mice, reliable detection of action potentials was observed only when more than three action potentials per 0.5 s were discharged (Tian et al., 2009). These results demonstrate that GCaMP3 can be effectively used to detect the activation of a small number of action potentials in the superficial neurons of awake mice. On the basis of this finding, GCaMP3 was used to map the electrical activity of networks comprising tens of cells in the somatosensory cortex of awake mice during active sensation (e.g., movement of the mystacial vibrissae to detect an object). Interestingly, neuronal activity during the sensory stimulus was highly heterogeneous, with the majority of cells displaying negligible or no response to the whisking behavior and a small subpopulation of neurons showing high calcium responses (O'Connor et al., 2010). Responsive neurons were randomly distributed within the field of view and *post hoc* immunohistochemical analysis confirmed that these neurons were excitatory cells (O'Connor et al., 2010) that release the neurotransmitter

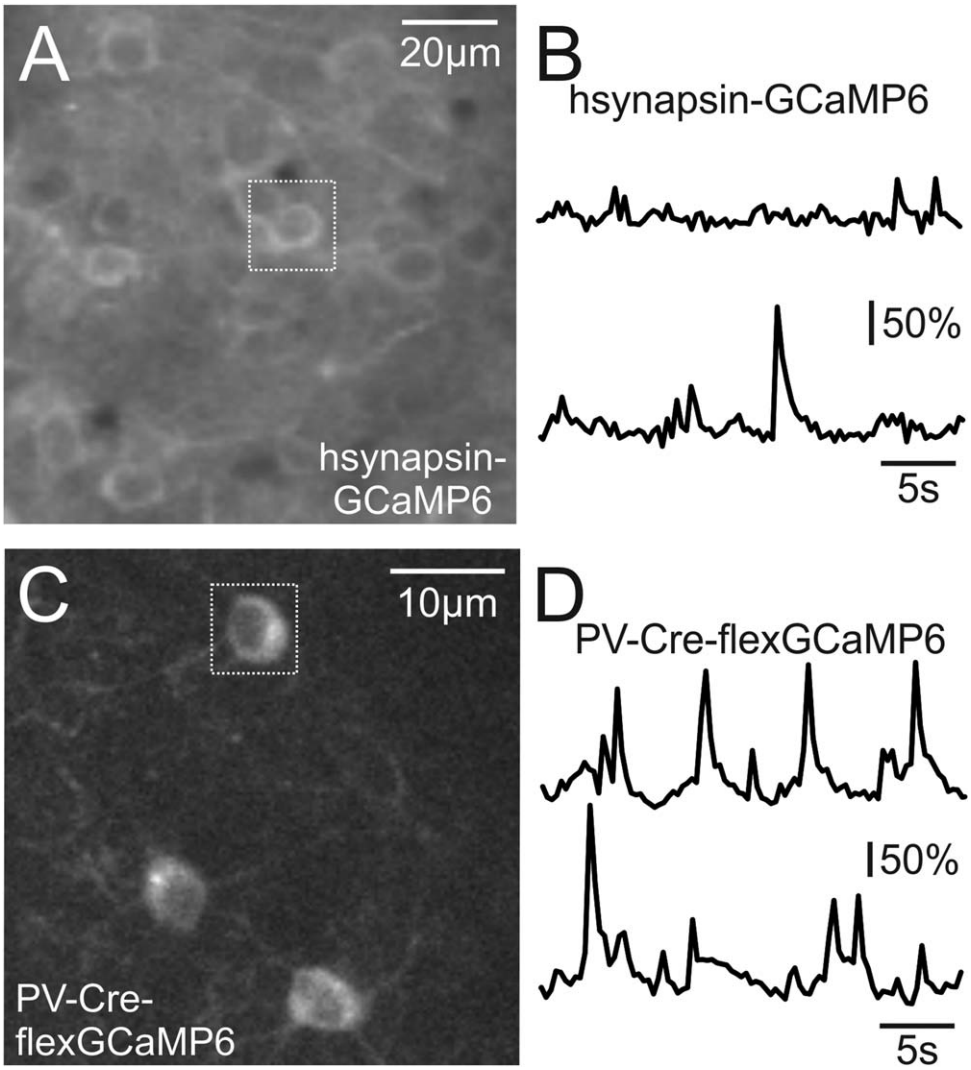

Fig. 4. Functional fluorescence imaging in identified neuronal subtypes using GCaMP in the intact brain. A:, B: A two-photon fluorescence image of a network of layer II/III cortical neurons expressing GCaMP6 in an anesthetized mouse (A). To express the calcium indicators, wild-type mice were injected with an adeno-associated virus carrying a GCaMP6 sequence under the control of the human synapsin promoter. Fluorescence changes over time for the cell highlighted in (A) are shown in (B). C:, D: GCaMP6 expression in parvalbumin-positive interneurons in the superficial cortical layers of an anesthetized mouse (C) was obtained through the injection of adeno-associated viruses carrying a flexGCaMP6 sequence in PV-Cre animals. Fluorescence changes over time for one of these cells are shown in (D). Image courtesy of S. Bovetti and C. Moretti.

glutamate. These results demonstrate that sensory information is encoded in superficial cortical layers in sparse patterns.

Genetically encoded calcium indicators can be targeted to specific classes of cells (Figs. 3 and 4). For example, GCaMP3 was expressed in deep layer V neurons and functional imaging was performed in the apical dendrites and in the cell bodies of these cells using a modified two-photon microscope containing a regenerative amplifier (Mittmann et al., 2011). Dendrites of layer V neurons in the somatosensory cortex displayed significant and reliable calcium signals following movement of the whiskers. Using conventional two-photon microscopy in combination with the expression of GCaMP3 in layer V cells, it was recently demonstrated that the apical dendrites of these cells are involved in the integration of sensory and motor information during an active sensing behavior (Xu et al., 2012).

The newly developed version of GCaMP, namely GCaMP6, very likely represents a breakthrough in the field. Indeed, GCaMP6 is the first genetically encoded calcium indicator that has biochemical properties comparable to synthetic dyes such as OGB and Fluo-4 (Chen et al., 2013). By expressing GCaMP6 in the subpopulation of layer II/III neurons in the mouse visual cortex, it was demonstrated that the detection of single action potentials and calcium transients in single dendritic spines is possible, and this detection is highly reliable. Moreover, using different genetic strategies to express the indicators in specific subpopulations of cortical cells (excitatory and inhibitory interneurons),

it was shown that the dendritic segments of inhibitory cells respond differently to the presentation of visual stimuli of different orientations, though at the level of the cell body, no orientation tuning was observed (Chen et al., 2013).

Genetically encoded indicators have allowed researchers to chronically image the same neuronal population over a period of weeks or months (Margolis et al., 2012; Tian et al., 2009). Although proper experiments need to be performed to verify that the long-term expression of the calcium indicators does not lead to alterations in cellular physiology (Chen et al., 2013; Tian et al., 2009), a chronic optical window can be opened in the mouse skull and imaging can be repeated in the same brain region multiple times. Using this experimental approach in the motor cortex of mice, it has recently been demonstrated that the neuronal representation of sensory (touch) and motor behaviors (whisker movements and licking) is a dynamic process that varies as a function of the mouse behavioral experiences (Huber et al., 2012). These studies open completely new avenues in systems neuroscience and will be crucial to reveal how neuronal networks change their properties during the slow plastic processes that occur in the brain over long periods of time, i.e., weeks and months.

CONCLUSIONS

Two-photon microscopy is increasingly recognized as a crucial tool for the functional investigation of the brain. With the development of this technical approach, the visualization of cellular activity in networks of hundreds of cells in the intact brain with unprecedented spatial resolution has become possible. This technique is providing fundamental insights into how brain circuits encode information and how these activities are transferred through different brain areas. However, technical limitations still limit the potential of fluorescence microscopy for functional investigation of brain networks in vivo. For example, two-photon fluorescence microscopy in the intact brain can be performed only in the most superficial structures (within 600–800 μm from the surface). In the last several years, much effort has been devoted to solving this problem. The use of regenerative amplifiers (Mittmann et al., 2011; Theer et al., 2003), adaptive optics (Ji et al., 2010), and three-photon excitation (Horton et al., 2013) are all exciting directions that promise to improve the depth at which fluorescence imaging can be performed. Moreover, to correlate brain activity with complex brain function and behavior, imaging in freely moving animals is needed. Although fiber optic-based probes have been developed to address this issue (Barretto et al., 2009; Helmchen et al., 2001; Wilt et al., 2009), a new generation of scopes that enable improved spatio-temporal resolution and three-dimensional imaging is needed. Finally, many improvements can be made in the sensors. For example, the development of red-shifted indicators (Akerboom et al., 2013; Zhao et al., 2011) may allow imaging in deeper structures and, potentially, through the intact skull. The development of these new spectrally shifted probes will also facilitate the combination of functional imaging with optogenetics (Akerboom et al., 2013). Moreover, new generations of indicators with better dynamic ranges and kinetics will likely be available in the future, enabling an increased signal-to-noise ratio and a more detailed description of the time evolution of the signal. In this context, imaging calcium, a slow biochemical process, is intrinsically limiting the time resolution of functional imaging. The development of new and more accurate voltage indicators (Knopfel, 2012) may represent an exciting area for future investigation. When the hardware improvements are combined with new and more accurate indicators, we envision that fluorescence microscopy will represent a unique approach for the functional investigation of brain circuits in behaving animals.

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