Short Course I

Advances in Multi-Neuronal Monitoring of Brain Activity

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Society for Neuroscience
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Introduction

Electrophysiological recording from one neuron at a time has been invaluable in developing our current understanding of the neural basis of sensation, perception, and behavior. However, in order to develop a detailed microcircuit-scale framework of brain function, direct monitoring of hundreds to thousands of neurons within and across brain regions is necessary.

Parallel advancements in biology and optoelectronics already allow for measuring the activity of entire neuronal populations at single-cell resolution. For example, genetically encoded sensors (e.g., GCaMP6), delivered virally or transgenically, permit monitoring of spiking and synaptic activity in specific neuronal cell types. Sensors can be tweaked for the optimal sensitivity, speed, and dynamic range needed for specific brain regions or imaging modalities. Optical prisms inserted into the brain allow for simultaneous two-photon functional imaging of all layers of neocortex. In addition, deep-tissue functional imaging without optical prisms is possible using newly developed red-shifted fluorescent proteins (e.g., RCaMP, R-GECO, O-GECO, and CAR-GECO). Importantly, new strategies are also being devised for imaging in the macaque monkey brain with single neuron resolution. Moreover, single-neuron responses can now be imaged simultaneously across multiple brain areas using a new objective lens design and beam multiplexing. Three-dimensional acousto-optical scanning or scanless holographic methods attain orders of magnitude higher-speed imaging compared with conventional galvanometer scanning. Finally, considering new electrode designs and spike-sorting algorithms, state-of-the-art multielectrode recordings remain a gold standard for monitoring neuronal populations in behaving animals.

This short course will provide a comprehensive evaluation of the latest genetically encoded sensors and electrodes, experimental techniques, and optoelectronic innovations that allow investigators to monitor multineuronal activity in the brain during behavior.
Engineering Fluorescent Calcium Sensor Proteins for Imaging Neural Activity

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Introduction

Understanding neural circuit function requires making measurements of neural activity over many spatial and temporal scales. Calcium indicators have been especially useful for studying neuronal activity over these scales, especially for action potential (AP) detection. APs in most neurons are tightly coupled to large (20-fold) and rapid (rise time <1 ms) increases in intracellular free-calcium concentration. These calcium transients are proxies for the underlying electrical activity. They can be measured in vitro and in vivo as fluorescence changes of calcium indicators in neuronal somata (Stosiek et al., 2003; Reiff et al., 2005), dendrites (Svoboda et al., 1997; Seelig and Jayaraman, 2013), and axons (Cox et al., 2000; Petreanu et al., 2012). Calcium indicators are also useful in nonspiking, graded potential neurons (Haag and Borst, 2000; Seelig et al., 2010), although optimal indicator parameters may differ. Additionally, excitatory synaptic transmission is typically associated with large-amplitude (20- to 100-fold) increases in calcium concentration in small synaptic compartments (e.g., dendritic spines). These synaptic calcium transients can be used to quantify synaptic transmission in vitro (Yuste and Denk, 1995) and in vivo (Jia et al., 2010; Chen et al., 2013). Calcium imaging is useful for probing neural activity over physiological and behavioral timescales of milliseconds to months, and wide spatial ranges from micrometers to millimeters.

Genetically Encoded Calcium Indicators

Small-molecule calcium indicators (e.g., Fura2, Fluo4, OGB1) have excellent properties in terms of kinetics and fractional fluorescence change (Tsien, 1989). However, introducing these molecules into tissue is invasive and nonspecific. Loading the molecules is destructive and incompatible with long-term imaging. Fluorescent protein–based indicators can overcome these problems. Genetically encoded calcium indicators (GECIs) can be expressed in specific types of neurons using minimally invasive methods over chronic timescales (Mank et al., 2008; Tian et al., 2009; Lutcke et al., 2010; Akerboom et al., 2012; Chen et al., 2013). Additionally, protein indicators can be targeted to subcellular locations to report specific dynamic events of interest (Miyawaki et al., 1997; Mao et al., 2008). GECIs occupy a niche that complements single-unit electrophysiology. Although single-unit methods have better sensitivity and time resolution, through optimization, GECIs have been engineered that achieve signal-to-noise ratios (SNRs) that rival electrophysiological methods.

Multiple GECI types have been developed and improved over several development cycles. GCaMP and related sensors are based on circularly permuted green fluorescent protein (cpGFP) (Baird et al., 1999) linked to calmodulin (CaM) and the M13 peptide from myosin light-chain kinase (Nakai et al., 2001; Ohkura et al., 2005; Tallini et al., 2006; Tian et al., 2009; Zhao et al., 2011; Akerboom et al., 2012; Chen et al., 2013; Wu et al., 2013). For most GCaMP sensors, fluorescence intensity increases with elevated calcium. Ratiometric GCaMP variants have also been reported (Zhao et al., 2011). Cameleons consist of a pair of fluorescent proteins linked by CaM/M13 (Miyawaki et al., 1997; Nagai et al., 2004; Palmer et al., 2006). Calcium elevations cause a change in Förster resonance energy transfer. TN-XXL and its derivatives are similar to cameleons, but use the calcium-binding protein troponin to sense calcium (Mank et al., 2008; Thesrput et al., 2014).

Optimizing GCaMP, a GFP-based calcium indicator

GCaMP-type GECIs are widely used to provide a unique view into nervous system function. They have been used to measure the dynamics of large populations of neurons over times of weeks, even during learning (Tian et al., 2009; Akerboom et al., 2012; Huber et al., 2012; Chen et al., 2013). They can track activity in local and long-range axonal projections and provide information about the activity of neurons that are inaccessible to electrophysiological methods (Suzuki et al., 2003). Moreover, this GFP-based indicator has a large two-photon absorption cross-section at accessible excitation wavelengths for in vivo imaging through hundreds of microns of brain tissue.

GCaMP engineering has benefited from structure-based design (Fig. 1). GCaMP relies on a calcium-dependent interaction between CaM and the CaM-interacting M13 peptide, both fused to cpGFP. With cooperative calcium binding, the CaM/M13 interaction changes sensor conformation. This conformational shift modulates the environment of the cpGFP chromophore through formation of new domain interfaces, rearrangement of protein side chains, and reduction of solvent access to the chromophore. These rearrangements cause increased absorption and brightness with increasing calcium concentrations. The GCaMP crystal structure (Wang et al., 2008; Akerboom et al., 2009; Ding et al., 2014) revealed the importance of the CaM/cpGFP proto-interface in regulating chromophore function. Functional screening of GCaMP variants at the CaM/cpGFP interface and in other domains
has produced indicators with improved fluorescence changes (Akerboom et al., 2012; Chen et al., 2013).

**Sensitive GCaMP indicators**

Recently, we developed the general-purpose GCaMP6 sensors for in vivo imaging of neuronal populations (Chen et al., 2013). We used a neuronal culture assay for GCaMP6 development (Wardill et al., 2013). The assay enabled rapid GECI characterization and optimization in the relevant cell type. Neurons have fast and small-amplitude calcium accumulations that are difficult to model in nonneuronal systems. Primary rat hippocampal cultures in multiwell plates were transduced with GECI variants and a nuclear fluorescent protein construct to control for expression level. APs were triggered using extracellular stimulation electrodes, while neurons were imaged using fluorescence microscopy. We imaged the fluorescence dynamics of multiple individual neurons responding to a variety of trains of APs. This neuronal culture assay allowed us to characterize large numbers of GECI variants under relevant conditions (Fig. 1).

The GCaMP6 variants can reliably detect a single AP stimulus, with responses that equal or exceed the fluorescence response of the synthetic calcium dye OGB1 (Fig. 1). These highly sensitive GCaMP6 indicators differ in their speed. In cultured hippocampal neurons, GCaMP6s is slowest, with a decay time of 1.8 s (τ/2 after 10 APs). GCaMP6m has faster kinetics while maintaining a high response. GCaMP6f is the fastest variant, with a 1 AP rise time-to-peak of ~50 ms and a decay time (τ/2 after 1 AP) of ~140 ms. The GCaMP6 indicators have basal fluorescence levels (F0) similar to those of GCaMP5G (Fig. 1c) (Akerboom et al., 2012).

Under favorable imaging conditions, these sensors detect single spikes in neuronal populations in vivo (Fig. 2) (Chen et al., 2013). GCaMP6s was expressed virally in the visual cortex of an anesthetized, adult mouse. Visual stimuli in the form of drifting gratings in different orientations of movement were shown to the mouse. The fraction of responding neurons using GCaMP6s exceeded the fraction detected with OGB1, likely because they detected weak responses missed by OGB1. In additional imaging experiments, responses from a layer 2/3 pyramidal neuron were simultaneously imaged using two-photon microscopy and recorded by patch electrode. The orientation-selective responses to single APs at the soma were reported with high sensitivity by GCaMP6s compared with those of previous indicators. GCaMP6f exhibited large ΔF/F0 (fluorescence change normalized by resting fluorescence) responses and a fast single-spike rise time-to-peak of ~50 ms. The high sensitivity of GCaMP6s enabled detection of even subthreshold dendritic spine activity in response to drifting gratings. Moreover, repeated imaging of the same neuron and processes over several weeks was conducted using GCaMP6s. The stability of the orientation tuning of dendritic spines was assessed with this high-sensitivity, genetically encoded sensor. GECIs with even higher sensitivity will allow larger populations of neurons to be imaged more rapidly and under more challenging conditions (e.g., imaging in awake and behaving animals).

**Figure 1.** GCaMP6 engineering. A, GCaMP structure (Akerboom et al., 2009) and mutations in different GCaMP6 variants. M13 peptide (yellow), linker 1 (gray), cpGFP (green), linker 2 (gray), CaM (blue), and mutated positions found in GCaMP6 variants relative to GCaMP5G (red text). B, Responses averaged across multiple neurons and wells for GCaMP3, 5G, 6f, 6m, 6s, and OGB1-AM. Fluorescence changes in response to 1 AP. C, Screening results, 447 GCaMPs. Top, fluorescence change in response to 1 AP (vertical bars, ΔF/F0; green bar, OGB1-AM, left; black bars, single GCaMP mutations; red bars, combinatorial mutations; blue, GCaMP6 indicators) and significance values for 1, 2, 3, 5, 10, 20, 40, 80, and 160 AP stimuli compared with GCaMP3 (color plot). Middle, half decay time after 10 APs. Bottom, resting fluorescence, F0 normalized to nuclear mCherry fluorescence. Red line, GCaMP3 level; green line, GCaMP5G level; blue line, OGB1-AM level. Figure adapted with permission from Chen et al. (2013), their Fig. 1a–c.

**Strategies for increasing GECI sensitivity**

GECI sensitivity could be increased in several ways for different applications.
Figure 2. Imaging activity in neurons and dendritic spines in the mouse visual cortex. A, Top, schematic of the experiment. Bottom, field-of-view (FOV) showing neurons color-coded according to their preferred orientation (hue) and response amplitude (brightness) for GCaMP5G (left) and GCaMP6s (right). Scale bar, 20 μm. B, Example ∆F/⟨F⟩ traces from 3 neurons expressing GCaMP6s. Single sweeps (gray) and averages of 5 sweeps (black) are overlaid. Directions of grating motion (8 directions) are shown above traces (arrows). C, The fraction of cells scored as responding to visual stimulation when loaded with different calcium indicators. Error bars correspond to SEM (n = 70, 39, 23, 21, 34 FOVs) for GCaMP3, 5G, OGB1-AM, 6f, 6m, and 6s, respectively. D, Simultaneous fluorescence dynamics and spikes in a GCaMP6s-expressing neuron. GCaMP6s-expressing neuron with the recording pipette indicated schematically. Scale bar, 10 μm. E, Median fluorescence change in response to 1 AP for different calcium indicators. Shading corresponds to SEM, n = 9 (GCaMP5K, data from Akerboom et al., 2012), 11 (GCaMP6f), 10 (GCaMP6m), and 9 (GCaMP6s) cells. F, Image of a layer 2/3 dendritic branch expressing GCaMP6s. Regions of interest (ROIs) are indicated as dashed circles (red, spines; yellow, dendrites). G, Map of fluorescence change (∆F = Fresponse − Fbaseline) in response to drifting gratings of 8 different orientations. H, Responses of dendritic spines (s1–s3) and neighboring dendritic shafts (d1–d3) to drifting gratings with different orientations (corresponding to ROIs indicated in F). I, Left, the same GCaMP6s-labeled spine imaged over weeks. Right, fluorescence responses to oriented drifting gratings. Insets, parent soma of imaged spines. J, Orientation selectivity of single spines measured over time (same as I). Figure adapted with permission from Chen et al. (2013), their Figs. 2–4.
The high sensitivity of GCaMP6s and 6f was achieved by both increasing the dynamic range (ratio of calcium-saturated fluorescence to calcium-free fluorescence; $F_{\text{sat}}/F_{\text{apo}}$) and increasing calcium affinity of the sensor compared with previous GCaMP variants. The high affinity leads to saturation of these sensors at modest activity levels, making them unsuitable for monitoring activity patterns in neurons with high spike rates (>100 Hz). The saturation of GCaMP6 variants was evident in the high-frequency motor neurons of Drosophila larvae (Fig. 3). For example, GCaMP6m accurately tracked individual spikes in motor neuron boutons at the larval neuromuscular junction when stimulated at low frequencies (1–5 Hz), but the fluorescence responses reached a plateau at higher frequencies (40–160 Hz). Engineering GECIs with lower affinity will aid in tracking high-frequency activity and graded potential neuronal activity. In addition, GCaMP is CaM-based and contains four calcium-binding domains that cooperate in binding calcium. Reducing the binding cooperativity could increase the linear range for detecting calcium changes.

Intracellular calcium is highly buffered by endogenous proteins in some neurons and can make imaging with calcium sensors challenging. Parvalbumin-positive interneurons exhibit relatively low fluorescence responses when loaded with OGB1 compared with excitatory neurons (Kerlin et al., 2010). In contrast to the above approach, engineering GECIs with very high affinity could be particular useful for increasing sensitivity in these neuronal subtypes. Ultrasensitive YC-nano indicators with very high calcium affinity ($K_d \sim 15$ nM) have been reported (Horikawa et al., 2010).

Additional strategies for increasing GECI sensitivity can be explored. The SNR of GECIs can also be improved by minimizing the resting fluorescence without reducing the peak fluorescence. Lowering the background fluorescence from inactive neurons and processes while maintaining peak fluorescence will serve to increase SNR, especially

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**Figure 3.** Fluorescence activity imaging in Drosophila larval neuromuscular junction boutons. A, Schematic of larval NMJ stimulation and imaging, epifluorescence (green arrow heads), and high magnification $\Delta F/F_0$ images of Type 1b boutons from muscle 13 (segments A3–A5). B, Average $\Delta F/F_0$ (± SEM) traces recorded after 1, 5, 10, 20, 40, and 80 Hz (green, cyan, magenta, black; $n = 10, 12, 14, 12$ FOVs, respectively) stimulus for 2 s (red) for GCaMP5G, 6m, 6s, and 6f. C, Average $\Delta F/F_0$ traces recorded after 1 and 5 Hz stimulus for 2 s. D, Average peak $\Delta F/F_0$ from 1 to 160 Hz. E, Average half decay time after 20 Hz stimulation for 2 s. Figure adapted with permission from Chen et al. (2013), their Supplementary Fig. 3.
with epifluorescence imaging of large populations of neurons. In the past, GCaMP variants with appreciable resting fluorescence were produced to enable structural imaging and identification of GECI-expressing neurons. However, transgenic animals now allow imaging in a background in which neurons, nuclei, or other structures of interest are labeled with a red fluorescent protein (RFP), obviating the need for substantial GCaMP resting fluorescence. Reducing resting fluorescence even by a factor of two while maintaining high peak fluorescence levels will have a substantial impact on SNR, increasing the yield in cellular imaging experiments by several-fold. In this manner, the scale of monitored neurons could be increased.

Fast GCaMP sensors

Existing GECIs are relatively slow compared with the speed of APs in part because of sensor protein dynamics. Calcium binds and unbinds cooperatively to the CaM EF-hand domains 100- to 1000-fold faster than the onset and offset of GCaMP fluorescence (Faas et al., 2011). GCaMP response kinetics have been improved, particularly with GCaMP6f, which has relatively fast rise (~50 ms, 1 AP $\tau_{\text{peak}}$) and decay (~140 ms, 1 AP $\tau_{1/2}$) times while maintaining OGB1-like SNR (Chen et al., 2013). GCaMP6f incorporates a mutation that is predicted to disrupt the M13/CaM interaction (A317E, Fig. 1). The M13/CaM interaction is likely a slow step downstream of calcium binding that affects the rate of fluorescence onset (Sun et al., 2013). The M13/CaM interaction affects calcium binding and unbinding, and thus, changing this interaction can also affect fluorescence offset rate.

GCaMP6f reports AP timing in neurons better than other GECIs. For example, GCaMP6f accurately tracked spiking at moderate frequencies (20 Hz) better than GCaMP6s at the Drosophila neuromuscular junction (NMJ) (Fig. 3). The decay time was inversely related to calcium $K_c$. GCaMP6f ($K_c\sim375$ nM) is also faster than GCaMP6s ($K_c\sim144$ nM). Faster variants can be engineered without altering affinity (Sun et al., 2013). In the future, fast GECIs with appropriate affinity, high SNR, and broad dynamic range will be most useful.

Optimizing red GECIs, RCaMP, and R-GECO

GECIs based on red-shifted fluorescent proteins have been made, including RCaMP, R-GECO, O-GECO, and CAR-GECO (Fig. 4) (Zhao et al., 2011; Akerboom et al., 2013; Wu et al., 2013). Red indicators are of particular interest for several reasons. First, longer wavelength excitation light penetrates deeper into tissue. In addition, red fluorescence is absorbed much less than green fluorescence, especially in mammalian tissue, giving red fluorophores a competitive advantage for in vivo imaging. Red GECIs therefore enable deeper and less invasive imaging than green GECIs. Second, combinations of red and green GECIs promise simultaneous imaging of multiple distinct neural structures (Li et al., 2013). Third, red GECIs are more easily combined with channelrhodopsin-2 (ChR2)–based manipulations.
of neural circuits because their excitation spectra are separated. Finally, many transgenic animals with green fluorescence have been created; red GECIs can be used in these animals. Optimized red GECIs will thus be useful individually or in combination with green GECIs.

Multicolor neuronal imaging ideally employs sensors with matched performance in each channel. The first-generation red GECIs, RCaMP1h and R-GECO1.0, underperformed green GECIs, such as GCaMP6s and GCaMP6f, in several ways (Figs. 1, 4). Both had inferior maximal dynamic range in solution measurements and responses after 160 AP stimulation at 83 Hz (Table 1). The red GECIs also had lower apparent calcium affinity compared with the green GECIs. The 1 AP fluorescence response was worse than that of GCaMP sensors. The red sensors were also slower than GCaMP6f. In general, RCaMP1h and R-GECO1.0 were less sensitive than GCaMP6.

Newer red GECIs are improved and have begun to approach GCaMP6 sensitivity levels (Ohkura et al., 2012; Wu et al., 2013). R-GECO1.2 has improved dynamic range \( \left( \frac{F_{\text{calcium-saturated}}}{F_{\text{calcium-free}}} = 33 \right) \) compared with R-GECO1.0 but has lower affinity (K \(_d\)~1200 nM) than GCaMP6 variants (Wu et al., 2013). The orange GECI, O-GECO1 (based on mOrange) has excellent dynamic range \( \left( \frac{F_{\text{calcium-saturated}}}{F_{\text{calcium-free}}} = 14 \right) \) but lower affinity (K \(_d\)~1500 nM). Further optimization of these and other red GECIs will enable multicolor imaging with GCaMP6. Improved red GECIs will also enhance single red-channel imaging.

Red GECIs have been more challenging to engineer because of photophysical limitations of RFPs related to brightness and bleaching. Ideally, the brightness of the calcium-bound state would be maximized. Dim GECIs have lower SNR and require more laser power. Among red GECIs, RCaMP is the brightest molecule (extinction coefficient \( \times \) quantum yield = 33 mM\(^{-1}\) cm\(^{-1}\)) relative to GCaMP6s (42 mM\(^{-1}\) cm\(^{-1}\)) (Table 2). The calcium-saturated brightnesses of R-GECO1.0, R-GECO1.2, O-GECO1, and CAR-GECO1 are all less than half that of RCaMP1h.

Under two-photon excitation, RFPs and red GECIs tend to bleach rapidly and/or show reversible dark states. The peak brightness per molecule (an experimental quantity proportional to quantum efficiency and inversely related to bleaching rate) (Mutze et al., 2012) is substantially smaller for R-GECO1.0 and RCaMP1h compared with GCaMP (~33% and ~50%, respectively) (Akerboom et al., 2013). In addition, R-GECO1.0 is transiently induced to become red fluorescent after exposure to blue light, which makes it incompatible with the action spectrum of ChR2 (Akerboom et al., 2013; Wu et al., 2013). Engineering to increase chromophore brightness, stability, and spectral purity could improve red GECIs substantially.

**Conclusion**

For more than 15 years, fluorescent proteins have been engineered to report neural activity. Although GECIs are now routinely used to image neuronal activity in vivo, they still have limitations. Quantitative testing of GECIs in neurons has accelerated calcium-sensor optimization in terms of sensitivity, speed, and dynamic range. GECI photostability and expression systems for in vivo imaging await further improvement. Better indicators will enable the dissection of neural circuits through the correlation of specific neuronal activity with behavior at higher resolution and over wider populations of neurons. Activity indicators for other neuronal state variables

Table 1. GECI sensitivity and kinetic parameters (Akerboom et al., 2012; Chen et al., 2013)

<table>
<thead>
<tr>
<th>GECI</th>
<th>( \frac{F_{\text{calcium-saturated}}}{F_{\text{calcium-free}}} )</th>
<th>( \frac{\Delta F/F_0}{160 \text{ AP}} )</th>
<th>( K_d ) (nM)</th>
<th>( \frac{\Delta F/F_0}{1 \text{ AP}} )</th>
<th>Decay ( \tau_{1/2} ) (10 AP) (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCaMP1h</td>
<td>11</td>
<td>3</td>
<td>1300</td>
<td>0.02</td>
<td>1.8</td>
</tr>
<tr>
<td>R-GECO1.0</td>
<td>16</td>
<td>8</td>
<td>449</td>
<td>0.08</td>
<td>0.8</td>
</tr>
<tr>
<td>GCaMP6s</td>
<td>63</td>
<td>17</td>
<td>144</td>
<td>0.23</td>
<td>1.8</td>
</tr>
<tr>
<td>GCaMP6f</td>
<td>52</td>
<td>13</td>
<td>375</td>
<td>0.19</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 2. GECI brightness measured in solution (Akerboom et al., 2012; Chen et al., 2013)

<table>
<thead>
<tr>
<th>GECI</th>
<th>Brightness (extinction coefficient ( \times ) quantum yield, mM(^{-1}) cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCaMP6s</td>
<td>42</td>
</tr>
<tr>
<td>RCaMP1h</td>
<td>33</td>
</tr>
<tr>
<td>R-GECO1.0</td>
<td>11</td>
</tr>
<tr>
<td>R-GECO1.2</td>
<td>15</td>
</tr>
<tr>
<td>O-GECO1</td>
<td>14</td>
</tr>
<tr>
<td>CAR-GECO1</td>
<td>8</td>
</tr>
</tbody>
</table>
(e.g., membrane voltage) and light-gated effectors that alter activity will also be critical for in vivo, light-based neurophysiology.

Acknowledgments
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References


NOTES


Simultaneous Two-Photon Calcium Imaging of Entire Cortical Columns

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Introduction

Two parallel approaches have dominated the physiological study of cortical columns. The first approach involves electrophysiological recordings in living coronal brain slices (typically ~400 µm thick). Even though many long-range axonal inputs to cortical columns within each slice are severed, this reductionist approach has provided a wealth of insight into layer-specific physiological properties of neurons and interlaminar flow of neural impulses. This approach makes use of multiple intracellular and extracellular recordings (Sanchez-Vives and McCormick, 2000; Adesnik and Scanziani, 2010; Thomson, 2010), two-photon calcium imaging (MacLean et al., 2006), and voltage-sensitive dye imaging (Petersen and Sakmann, 2001).

The second approach involves making neuronal recordings from the intact brain, where it is possible to correlate neural activity with sensory perception and behavior. As in recordings in brain slices, simultaneous neuronal recordings across cortical layers have been achievable in vivo, typically via extracellular recordings that use laminar electrode arrays (Niell and Stryker, 2008; Sakata and Harris, 2009; Adesnik and Scanziani, 2010; Berenyi et al., 2014). However, these methods are typically limited in their ability to achieve a combination of fine spatial resolution, extended recording durations, and elaborate identification of cell types.

As an alternative, two-photon calcium imaging has provided a complementary assay for monitoring activity in awake animals (albeit at lower time resolution) in large numbers of identified neurons across days and weeks (Trachtenberg et al., 2002; Dombeck et al., 2007; Mank et al., 2008; Andermann et al., 2010). However, for all its strengths, this method suffers from two important limitations: (1) high-speed imaging is often confined to a single focal plane parallel to the cortical surface, and (2) light scattering makes it difficult to image deep cortical layers, such as layer 6, which plays a major role both in regulating response amplitudes in superficial layers (Olsen et al., 2012) and in distributing information to a variety of cortical and subcortical targets (Thomson, 2010).

In this chapter, we review the pros and cons of recent methods for deep-layer two-photon imaging. We then describe our recent efforts, in collaboration with the laboratory of Dr. Michael Levene, to employ chronic two-photon calcium imaging through a microprism. This novel strategy combines the optical access of ex vivo brain slice preparations with in vivo behavioral context and long-range connectivity that is relatively intact.

Previous Methods for Deep-Layer Two-Photon Imaging in Cortex

Optical scattering degrades image quality at increasing imaging depths within brain tissue. Regenerative amplifiers (Theer et al., 2003; Mittmann et al., 2011) and long-wavelength (1300–1700 nm) Ti:Sapphire lasers (Horton et al., 2013) have both been used to extend the imaging depth of multiphoton microscopy, but practical limitations have restricted their use. Blunt-ended gradient index (GRIN) lenses have been used as implantable micro-optical probes for deep imaging (Jung et al., 2004; Levene et al., 2004; Barretto et al., 2011) but suffer from limited fields of view and significant optical aberrations. Because of the damage incurred above the imaging plane, such probes are more effective when positioned directly above intact structures, such as hippocampus (Barretto et al., 2011), than when placed directly above deep layers of cortex. Traditional multiphoton imaging in certain thinner cortical areas in mice (e.g., mouse primary visual cortex [V1]) (Glickfeld et al., 2013) can reach layers 4 and 5, but this solution often requires high average laser power and/or sparse labeling of neurons. Half-millimeter prisms have been used with one-photon excitation to measure the net fluorescence emission from layer 5 apical dendrites in superficial cortical layers in rats (Murayama et al., 2007), but these fluorescence images lack cellular or subcellular resolution. Current methods allow two-photon imaging of small volumes typically spanning 50–250 µm in depth within a cortical layer, using piezoelectric scanners (Gobel et al., 2007; Kerlin et al., 2010), tunable lenses (Grewe et al., 2011), or multibeam systems (Amir et al., 2007; Cheng et al., 2011). Faster in vivo volume imaging methods using single-photon excitation are also emerging, but are currently limited to weakly scattering or sparsely labeled brain tissue (Quirin et al., 2014).

Microprism Imaging: A Synthesis of in Vivo and ex Vivo Approaches

To rapidly obtain two-photon imaging data from a larger range of depths, Levene and colleagues have previously shown that inserting a sharp 1 mm glass microprism into the neocortex of an anesthetized mouse can be used for acute, single-session two-photon imaging of anatomical structures across all six cortical layers, including the soma and dendrites of cortical layer 5 pyramidal cells, in a single field of view (Chia and Levene, 2009a, b, 2010). The hypotenuse of the microprism is coated with aluminum and thus serves as a right-angled mirror or “micro-periscope,” with a vertical field of view parallel to the prism face (Fig. 1A). The use of a reflective glass microprism minimizes the distance that excitation photons must travel through scattering tissue to reach deep cortical...
tissue, allowing high spatial resolution across layers while requiring only moderate laser power.

Dr. Levene’s lab and our lab each developed versions of this approach that are suitable for chronic, large-scale and simultaneous two-photon imaging in vivo (Andermann et al., 2013). Here, we focus on our lab’s efforts using microprisms to achieve chronic two-photon calcium imaging across all layers of visual cortex in awake mice. Acutely implanted microprisms have been used for wide-field epifluorescence imaging of bulk calcium activity of the apical dendrites of layer 5 neurons (Murayama et al., 2007), following acute insertion into superficial cortical layers. However, the use of epifluorescence imaging precluded visualization of individual neurons. While our current microprism approach provides a means for chronic monitoring of activity in individual neurons and processes using two-photon calcium imaging, it is also compatible with chronic epifluorescence imaging simultaneously across a large, 1 × 1 mm field of view, providing a useful means for rapid mapping of bulk calcium or autofluorescence signals across all cortical layers.

Overview of microprism implant procedure for chronic two-photon calcium imaging

The procedure we use for implanting a chronic microprism into cortex for the purpose of awake functional imaging has been described elsewhere in great detail (Andermann et al., 2013; Goldey et al., 2014) and is summarized below (Fig. 1B). A critical first surgery involves the implant of a two-armed headpost and a large (3–5 mm diameter) cranial window and imaging well (Fig. 1C), which we have used in previous studies for stable imaging of posterior cortex of anesthetized (Kerlin et al., 2010; Bock et al., 2011; Bonin et al., 2011) or awake (Andermann et al., 2010, 2011, 2013; Glickfeld et al., 2013) mice with high-NA objectives across weeks or months. Following recovery from surgery, mice are trained to tolerate several hours of head restraint (Andermann et al., 2011). Cortical areas are then delineated using wide-field intrinsic autofluorescence imaging in awake mice (Andermann et al., 2011; Glickfeld et al., 2013). During a subsequent surgery, the window is removed and replaced following viral injection of a genetically encoded calcium indicator (GECI, e.g.,

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Chronic functional imaging across cortical layers using a micro prism. A, Illustration of laser beam path through a prism implanted into neocortex. The reflective hypotenuse of the prism converts the horizontal imaging plane into a vertical plane. B, Flowchart of surgical procedures. Surgeries are separated by ≥7–10 d recovery. C, Initial cranial window and head post implant. Headpost and imaging well are designed to permit an unobstructed visual field, access to high-NA water-immersion objectives, and effective light shielding. D, Micro prism and cranial window assembly and positioning using vacuum line (red arrow). E, Left, Wide-field epifluorescence image of a 1 mm prism implanted in visual cortex, focused 1 mm below the cortical surface. Fluorescent expression of AAV-GCaMP3 can be seen through the cranial window (green arrow) and through the prism. Scale bar, 1 mm. Right, Two-photon image taken through the same prism (maximum intensity projection across 15 min of recording) at 205 μm away from the microprism imaging face, 21 d after prism implant. Images spanned 600 × 900 μm² of cortex (1 Hz acquisition, 960 nm laser excitation, 45 mW power at sample). Scale bar, 100 μm. This figure was adapted with permission from Goldey et al. (2014) and Andermann et al. (2013), their Fig. 1B.
no edema at time of prism implant, thus decreasing bleeding and making insertion more precise.

Effects of microprism insertion on anatomical and functional properties of nearby neurons

The implantation of a 1 mm prism for chronic imaging involves severing some horizontal cortical connections. Thus, as with live imaging studies of coronal brain sections, it is important to carefully assess whether the local cortical circuitry near the prism imaging face is sufficiently preserved to provide meaningful anatomical and functional data. We therefore undertook a multitiered approach, using functional and anatomical imaging, microelectrode recordings, and histological staining (Andermann et al., 2013). Histological evaluation with staining for hematoxylin and eosin (H&E), Nissl, and DAPI indicated that the imaged regions were comparable with more distant brain tissue 400–500 µm away, and to neurons from nonimplanted mice. Small but significant increases in cell density were observed within the first 50 µm from the prism face, followed by small but significant decreases at 50–100 µm and 100–150 µm away, and a return to normal density beyond 150 µm from the prism. Several weeks after surgery, staining for astrocytes (anti-GFAP) and microglia (anti-CD11b), both indicators of brain trauma, did not show evidence of chronic tissue scarring.

Perhaps the most direct evidence that the functional properties of neurons remain relatively intact following prism insertion came from an assessment of the visual response properties of the same nearby neurons, before and after prism implant (Andermann et al., 2013; Fig. 2). We first measured the visual response properties of neurons through a standard cranial window and then assessed the response properties of the same neurons following insertion of a microprism at a distance of 350 µm away (Fig. 2A, white dashed squares). We used an identical approach across sessions: two-photon imaging of visual responses in a 3D volume of GCaMP3-expressing layer 2/3 neurons through the cranial window in an awake, head-fixed mouse that was free to run on a linear trackball. We found that orientation and direction tuning in the same neurons were mostly stable across days: 2 d before, and 1, 4, and 5 d following prism implant (Andermann et al., 2013; Fig. 2B). These data were also consistent with other multiunit electrophysiological recordings from mouse barrel cortex, which showed that cortical neurons close to the prism face were endogenously active and responsive to whisker stimulation at 10 min, 3 d, and 120 d following prism implant.
Epifluorescence and two-photon imaging demonstrated blood perfusion in intact radial and surface vessels at distances >50 μm from the prism face (Andermann et al., 2013; Fig. 2A), which likely helped maintain the functional integrity of local circuits. The insertion of the microprism resulted in the accumulation of some blood at the brain surface and prism surface, which cleared up over the course of several days (Andermann et al., 2013). Thus, the basic cell health and sensory response properties of neurons at >100–150 μm lateral to the prism face (a distance that exceeds the lateral dendritic reach of most pyramidal neurons) were qualitatively similar to what we and others have observed in experiments not involving chronically implanted prisms.

Chronic functional imaging of cortical neurons through a microprism
Having established that superficial layer neurons located >150 μm from the prism face were relatively unaffected by prism insertion, we then recorded visual responses simultaneously from large numbers of neuronal cell bodies across all layers of mouse V1 using the prism method (Andermann et al., 2013; Fig. 3A). To visualize the density of responsive neurons, we plotted a maximum intensity projection of increases in GCaMP3 calcium indicator fluorescence across average response maps for each of the 96 visual stimulus conditions. Neurons in all cortical layers demonstrated orientation tuning curves generally similar to previous electrophysiological reports (Niell and Stryker, 2008; Fig. 3B). Orientation tuning curves imaged through the prism were stable across multiple days (Fig. 3B), consistent with previous studies in layer 2/3 of mouse V1 (Mank et al., 2008; Andermann et al., 2010). Figure 3C illustrates the orientation preference of simultaneously recorded, orientation-tuned neurons throughout the depth of mouse V1 (Andermann et al., 2013). Consistent with previous reports in superficial cortical layers, we did not find any evidence of columnar clustering of orientation preference in any cortical layer (Andermann et al., 2013).

Microprism imaging of long-range axonal boutons
A unique advantage of two-photon imaging is the ability to monitor subcellular structures, such as axonal boutons. Recently, we and others have described functional imaging of long-range projection axons using GCaMP3 in awake mice (Petreanu et al., 2012; Glickfeld et al., 2013). Because of the small size of individual axons and synaptic boutons, functional imaging of axons has been restricted to superficial depths in cortex (~0–150 μm deep). However, many classes of projection neurons selectively innervate deep cortical layers (Petreanu et al., 2009).

To determine whether the use of a microprism could enable monitoring of long-range axonal activity deep within the cortex, we made a small injection of GCaMP3 into area V1 (Glickfeld et al., 2013) and inserted the prism into the postmedian secondary visual cortical area (PM), an area densely innervated by V1 axons, with the prism oriented to face area V1 (Fig. 3D). We observed robust single-trial visual responses of individual boutons at depths of 480–510 μm below the cortical surface (putative layer 5) during presentation of stimuli at multiple temporal frequencies and spatial frequencies at 1 d postimplant (Fig. 3E). Thus, this method extends in vivo functional imaging of axonal arbors (Petreanu et al., 2012; Glickfeld et al., 2013) to arbors that innervate deeper cortical layers (Petreanu et al., 2009). Further, the study of long-range projection axons via a microprism represents a less invasive application of this method with fewer caveats than for imaging of cell bodies near the prism face. That is,

Figure 2. Comparison of cortical neuron visual response properties, before and following prism implant. A, Bright-field image through a cranial window, before and 4 d after prism implant. Scale bar, 300 μm. B, Comparison of functional properties of neurons using two-photon GCaMP3 calcium imaging through the surface cranial window, both before and following insertion of a prism at a nearby location. Polar plots of normalized visual responses to 12 directions of motion (arrows in top left polar plot) in 3 example neurons at varying distances from the prism face, across 4 sessions (2 d before, and 1, 4, and 5 d after prism implant; Peak response amplitudes (% ΔF/F) are noted at bottom right. This figure was adapted with permission from Andermann et al. (2013), their Fig. 2.
although damage to long-range axonal boutons near the prism face cannot be ruled out, these boutons report the activity of neurons whose dendrites are safely located millimeters from the prism implant.

High-speed cellular imaging of endogenous activity across all cortical depths

In addition to functional mapping of average evoked responses, simultaneous imaging of all cortical layers enabled direct examination of interlaminar neural dynamics, on a trial-by-trial basis, in awake mice that stood or ran on a linear trackball (Fig. 4). Using a fast resonance scanning two-photon microscope (~32 Hz frame rate, 720 µm × 720 µm, 256 × 240 pixels/frame) (Bonin et al., 2011; Andermann et al., 2013), we were able to measure endogenous neural activity simultaneously in 208 neurons spanning all 6 cortical layers of V1 (Fig. 4A). Data were collected in near complete darkness, 38 d after prism implant, ~140 µm from the prism face. This dataset provided a proof-of-principle demonstration of the capacity of chronic

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activity across individual running onsets (Fig. 4B). While such activity changes typically had the same sign for each neuron, different neurons demonstrated different degrees of variability among trials, and most pairs of neurons had relatively low trial-to-trial covariability both within and across layers (Andermann et al., 2013). These data illustrate the rich repertoire of interlaminar and intralaminar neural dynamics accessible using microprism-based columnar recordings in behaving animals.

Challenges of the prism approach
We were able to routinely record from the same large set of cell bodies across all cortical layers across days and weeks in awake mice using calcium imaging through a microprism (Andermann et al., 2013). Although images could be obtained on the day of surgery (Chia and Levene, 2009b), they were sometimes less clear and required somewhat higher laser power than images obtained later on. In these cases, imaging clarity improved over subsequent days. This initial clouding could have been caused in part by gliosis (Barretto et al., 2011). However, the timecourse of immediate clouding and subsequent improvement in clarity was more consistent with clearing of blood at the prism and cortical surfaces (Chia and Levene, 2009b; Andermann et al., 2013).

As discussed in detail elsewhere (Andermann et al., 2013), the chronic insertion of a 1 mm microprism into the cortex is clearly accompanied by damage to local neural processes and to the neurovascular unit. Therefore, care must be taken when interpreting neural data and validating results against known benchmarks (Andermann et al., 2013). Importantly, concerns regarding damage to local circuitry should decrease with increasing distance from the prism face to the focal plane. This distance (currently 100–350 µm using our approach) can likely be further extended by implementing adaptive optical correction of aberrations (Ji et al., 2012). Despite the aforementioned challenges, microprism imaging is a relatively simple and straightforward tool that can be used on any conventional multiphoton microscope at a cost of approximately $50 per prism.

**Technical Outlook and Applications**
Advances in a variety of optical techniques for neurophysiology hold promise for rapid advances in systems neuroscience research. The chronic two-photon calcium microprism imaging technique presented here can expand the capabilities of two-photon functional imaging by allowing simultaneous access to multiple genetically, chemically, and...
anatomically defined neuronal populations throughout the depth of cortex. Also, the large field of view available using microprisms enables high-throughput functional imaging of hundreds of neurons within local circuits of mammalian cortex. Placing the prism face at the cortical surface of extremely medial or lateral cortical regions (Fig. 5) should also prove useful for noninvasive imaging of superficial cortical activity in hard-to-reach brain regions. Prism implants should also be easily adaptable for reaching deeper cortical layers in carnivores and primates. Finally, efforts to combine prisms with GRIN lenses hold promise for two-photon imaging in deep subcortical brain structures with decreased tissue damage at the imaging plane (Kim et al., 2013).

Optogenetic methods for one-photon and two-photon (Fenno et al., 2011; Prakash et al., 2012; Begue et al., 2013) excitation or suppression of individual neurons or local neural populations are also improving rapidly. Such methods stand to benefit greatly from the technique presented here, which should enable repeated photo-stimulation of neurons across cortical layers, in combination with concurrent monitoring of local neural activity. Ultimately, continued integration of microprism imaging with the above methods should provide a powerful yet relatively simple strategy for understanding interlaminar flow of information through cortical circuits in behaving animals.

In the future, the use of higher-speed two-photon imaging, coupled with expression of recent, promising genetically encoded voltage indicators (Han et al., 2013; St-Pierre et al., 2014), may allow observation of the electrical flow of neural signals at cellular resolution in a behaving animal. In addition, future analyses of trial-to-trial covariability in activity of neurons across cortical layers in vivo may help identify interlaminar assemblies within a cortical column.

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References


Two-Photon Calcium Imaging in the Macaque Monkey

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Introduction
The nonhuman primate has proven to be an invaluable animal model for neuroscience research. Nonhuman primates such as the Rhesus macaque have a complex brain that is organized similarly to the human brain (Sereno and Tootell, 2005). In addition, monkeys can be trained on the complex tasks necessary to study the neural underpinnings of such complicated processes as perception, cognition, and decision making. Because these are the types of behaviors affected by mental disorders, the monkey will remain a crucial animal model for investigating brain function. To date, a wealth of research has established which areas in the monkey brain are likely to be involved in a particular task, as well as the tuning properties of neurons in these areas. Going forward, we will have to build on this knowledge to pursue questions such as: How are the neural circuits within and between these areas organized? What is the function of their individual elements, and how do they interact? Only by solving these puzzles will we be able to address what goes awry in mental disorders.

Investigating these issues is complicated by the fact that neural circuits are formed with a high degree of specificity and on very fine scales. Thus, we will need tools that operate at a resolution appropriate to appreciate the fine-scale organization of neural circuits. Two-photon microscopy (Goeppert-Mayer, 1931; Denk et al., 1990) fills an important technological gap in this respect. At its coarsest resolution, two-photon calcium imaging allows the simultaneous recording of activity of tens to hundreds of neighboring neurons while maintaining single-neuron resolution. At the finest resolution, responses of individual spines on a single dendrite can be investigated. (General reviews of two-photon microscopy can be found in So et al., 2000; Helmchen and Denk, 2005; Svoboda and Yasuda, 2006; and Kerr and Denk, 2008.) No other technique currently is able to operate at these resolutions in vivo.

Two-photon calcium imaging is by now routinely used in anesthetized and even awake mice. In contrast, only few studies have applied two-photon imaging to investigate brain functions in larger animals, in particular, monkeys (Nauhaus et al., 2012a; Ikezoe et al., 2013). This chapter will describe the necessary steps to perform two-photon microscopy in monkeys, including some of the commonly encountered complications, and discuss how to interpret the data. Most of the text will focus on an established imaging paradigm using an injectable calcium dye, Oregon Green BAPTA-1AM (OGB), in acute experiments in anesthetized animals. Genetically encoded calcium indicators (GECIs) and experiments in awake animals are discussed in the last section. This text does not include more general details on how to perform acute experiments in anesthetized monkeys, such as the choice of anesthesia, paralysis, or vital-sign monitoring. For more information on these issues, refer, for example, to Nauhaus et al. (2012a).

Setup
Choice of two-photon microscope
A large number of two-photon microscopes are commercially available, and are used to perform calcium imaging in mice. However, the size and curvature of the monkey brain place specific demands on the two-photon microscope. In the monkey, most brain areas are located laterally or posteriorly enough that their surface is at a significant angle relative to the horizontal plane in which imaging is typically performed. In some cases, it may be feasible to rotate the animal’s head to bring the brain area of interest into the horizontal plane. Often, this is not possible, either because the angle difference is too large (e.g., posterior V1 is tilted approximately 50° relative to the horizontal) or because rotating the animal’s head would interfere with stimulus presentation (e.g., rotating the head downward poses problems for visual stimulus presentation). In these cases, the only solution is to rotate the microscope and to image in a plane tilted relative to the horizontal plane. Increasingly more commercial two-photon microscopes offer this capability. Another important consideration in selecting a two-photon microscope for imaging in larger animals, such as the monkey, is the amount of space available under the objective. This space has to be large enough to fit the animal’s head, or (as in our typical setup) the animal and a stereotaxic frame.

Specific imaging equipment
In addition to the usual equipment necessary for acute experiments in anesthetized animals, we use two specially designed pieces of hardware: an imaging well and a simplified stereotaxic frame. The imaging well serves the following purpose: The objectives typically used for two-photon microscopy are water-immersion objectives, and therefore require water or artificial CSF (ACSF) between imaging region and objective. This can be relatively easily achieved when imaging in the horizontal plane. However, once the microscope has been rotated and imaging is performed at an angle, a specially designed imaging well becomes necessary to hold water between imaging region and objective. Our imaging well consists of a large metal plate with a hole in the center (Fig. 1A). This metal plate is attached to the skull using dental cement. During imaging, a plastic
cylinder is fitted into the well, and the interface of cylinder and well is sealed with bone wax to prevent water leakage. The cylinder can then be filled with enough ACSF to cover the imaging region. The cylinder can be removed for easy access to the skull for surgical and other procedures. We also use the imaging well to stabilize the brain during imaging (see Minimizing Movement Artifacts, below).

The second specialized piece of equipment in the imaging setup is a simplified version of a stereotaxic frame (Fig. 1B). The stereotaxic frame is designed to rigidly hold the animal's head while being small enough not to interfere with the two-photon microscope. It consists of a metal base plate, large enough to support the animal's body, and two posts at one end. These posts initially hold the animal's head via attachments for ear bars, eye bars, and a palate bar. This setup provides sufficient stability to perform initial surgeries. To further increase stability during imaging, we cement a headpost to the skull at the beginning of each acute experiment. For the remainder of the experiment, the head is then rigidly held in place by connecting the headpost to the stereotax posts.

**Dye Loading**

Multiple calcium dyes are available, of which OGB has been most commonly used. OGB has been the calcium dye of choice because of a number of advantages over other dyes: Labeling neurons with OGB is feasible via pressure injection into the brain (Stosiek et al., 2003). Furthermore, because of its acetoxymethyl (AM) ester group, OGB is not fluorescent until it is transferred into cells and the AM group is cleaved, which helps to reduce background fluorescence (Tsien, 1981). OGB is stable in the cells for a relatively long period of time (in some cases, up to 12 h) and has a signal-to-noise ratio (SNR) sufficient to detect even single angle potentials (Kerr et al., 2005; Chen et al., 2013). The basic protocol for OGB injections has been described in depth previously (Stosiek et al., 2003; Garaschuk et al., 2006) and widely applies to monkeys. Listed below are the most important steps of OGB injections in the monkey:

1. **OGB and SR101 solution**: OGB labels both neurons and astrocytes. Sulforhodamine 101 (SR101), a red fluorescent dye that selectively labels astrocytes (Nimmerjahn et al., 2004), is therefore often added to the OGB injection solution. For the monkey, our typical injection solution consists of 2 mM OGB with 10% DMSO, 2% Pluronic F-127, and 25% SR101 (all from Life Technologies, Grand Island, NY).

2. **Craniotomy**: The hole in the imaging well allows access to a large skull region. We usually make small craniotomies (~5 x 5 mm) within its perimeter. Each craniotomy is used once to attempt dye loading. If the dye loading is unsuccessful, or after completion of data collection, the next craniotomy is made. Craniotomies are kept relatively small to help stabilize the brain (see Minimizing Movement Artifacts, below). It is important to thin the skull around the perimeter of each craniotomy to be...
able to place the objective close enough (within its working distance [WD]) from the brain.

(3) Durotomy: Monkey dura is opaque and needs to be removed before imaging. Usually, one durotomy of approximately $3 \times 3$ mm is made per craniotomy, trying to avoid major blood vessels in the dura. Care needs to be taken to avoid bleeding while cutting through the last layer of dura: Blood touching the brain seems to interfere with dye loading.

(4) Dye injection: Dyes are pressure-injected into the brain using a Picospritzer (Parker Hannifin, Hollis, NH) and glass pipettes with a relatively blunt taper and a tip size of $\sim 5 \mu$m. We have found that the blunt taper helps with preventing backflow of the dye along the track of the pipette and onto the surface of the brain. Tip sizes smaller than $\sim 4 \mu$m tend to clog, whereas larger tip sizes tend to lead to backflow. Pipettes are inserted into the brain at an angle of $35^\circ$–$45^\circ$ relative to the surface of the brain, and the dye is injected at a depth of $\sim 300$–$400 \mu$m. The progress of each injection is monitored by observing the flow of SR101 into the brain. This is a crucial step of the procedure, as it ensures that (a) the pipette is not clogged, and (b) dye is not flowing back onto the surface of the brain. The latter needs to be avoided, as staining the surface of the brain with SR101 drastically degrades imaging quality. Unlike OGB, SR101 is fluorescent before entering a cell, and monitoring the dye spread during injection is thus feasible by observing SR101 fluorescence using the two-photon microscope. Alternatively, most two-photon microscopes have an epifluorescence path that will allow observation of the dye using low power, dry objectives. Multiple injections can be made within the same durotomy, as long as they are done within a short amount of time ($\sim 1$ h) after each other and after making the durotomy. Reloading OGB into a previously labeled, "old" durotomy appears to not be feasible.

The biggest challenge with OGB loading in monkeys is the observation that, in many cases, OGB is taken up not only by neurons, but also by the neuropil that makes up the extracellular space. This results in uniform OGB fluorescence throughout the entire imaging region (Fig. 2). As a consequence, neurons can no longer be discriminated from the surrounding neuropil. It appears that the likelihood of excessive OGB label in the neuropil strongly increases with animal age, making younger animals better suited for two-photon microscopy. Similar observations have been made in mice (Eichhoff et al., 2008). The reason for this increased label in the neuropil is unclear, but one possible explanation is an increase in acetylcholinesterase activity in the neuropil with age. It remains to be seen whether coinjection of esterase blockers such as eserine, which has been used to improve loading of OGB in the retina (Kurth-Nelson et al., 2009; Srienc et al., 2012), could prevent the neuropil loading of OGB without interfering with brain function.

**Minimizing Movement Artifacts**

Besides complications with OGB loading, the largest challenge in performing two-photon microscopy in monkeys is posed by artifacts caused by brain movements. In the anesthetized monkey, the largest sources of movement are breathing and pulse. Breathing usually causes vertical displacements of the brain, whereas the pulse tends to be correlated with horizontal displacements. Of these two artifacts, the vertical movements are particularly disruptive for imaging because they cause cells to move in and out of the imaging plane. Because data cannot be collected from a cell outside the imaging plane, vertical artifacts
In our experience, vertical brain movements can be minimized using the following approach: First, the brain region to be imaged is covered with a thin layer of 1.5% agarose (Type-III agarose, Sigma-Aldrich) in ACSF, followed by a small coverslip. It is important to avoid using too much agarose, as it interferes with imaging. The coverslip can be placed either on top of the bone, or between bone and dura. Second, the coverslip is held in place by small clamps attached to the imaging well (Fig. 1), which should be set up to provide gentle pressure on the brain. Care needs to be taken that neither coverslip nor clamps block the objective’s access to the brain.

The goal of the mechanical stabilization is to eliminate vertical movements. Remaining horizontal movements can then be corrected offline after data collection. A successful strategy for offline motion correction has been to use a two-stage process described in detail in Nauhaus et al. (2012a): First, slow drifts are corrected by computing the average image in every trial and aligning these average images to the average of the first trial using two-dimensional (2D) cross-correlation. Second, fast movements are corrected using an optic flow-based method. Other approaches are also possible and have been described in the literature (Greenberg and Kerr, 2009).

Offline motion correction is feasible only if data are acquired in raster scan format. In this acquisition mode, the laser beam is systematically swept over a 2D region. As a consequence, the collected data consist of a series of frames that show cell bodies and other structures, and horizontal displacements can be corrected by spatial transformations of the collected frames. Other acquisition modes increase acquisition speed by no longer sampling the entire 2D region. In random access scanning, for example, the laser beam “jumps” from one preselected point in the imaging region to the next (Reddy et al., 2008; Grewe et al., 2010). Because no data are recorded outside of the preselected points, data collected in this way cannot be corrected offline. Thus, these acquisition modes place more stringent requirements on brain stability.

**Data Analysis**

The acquisition system of the two-photon microscope records raw fluorescence time courses $F(t)$ for every pixel. Usually, raw fluorescence time courses are first transformed into $ΔF(t) = (F(t) - F_0)/F_0$, where $F_0$ is the average fluorescence during some baseline period, e.g., a time period preceding stimulus onset. In a second step, $ΔF(t)$ is then averaged across all pixels falling within a neuron. Any further analyses are then based on the $ΔF(t)$ time courses for every cell. In these analyses, it is important to take into account how the measured changes in OGB fluorescence relate to the underlying changes in neural firing rates. The spike-to-fluorescence transfer function of OGB can be modeled as a linear–nonlinear cascade (Nauhaus et al., 2012b; Fig. 4): Each spike causes a transient influx in calcium, whose time course is characterized by a sharp rise followed by a slow exponential decay with a time constant of ~0.5–1 s (Helmchen et al., 1996; Kerr et al., 2005; Sasaki et al., 2008; Smith and Häusser, 2010; Chen et al., 2013). This signal is then transformed into the observed fluorescence signal via a saturating nonlinearity (Yasuda et al., 2004; Yaksi and Friedrich, 2006; Hendel et al., 2008).

The first important consequence of the spike-to-fluorescence transfer function of OGB is the temporal summation of action potentials caused by the slow exponential decay of the linear stage. As long as firing rates are low enough, individual spikes lead to clearly visible calcium transients, and spike times can be identified. However, from firing rates >5 Hz on, the calcium transients of individual spikes can no longer be resolved because of the slow decay kinetics of the OGB signal (Sasaki et al., 2008). Thus, for firing rates >5 Hz, spike times cannot be computed from the OGB data. In most areas of the monkey brain, firing rates will be well beyond this rate even under anesthesia.

The second important consequence of the spike-to-fluorescence transfer function of OGB is the distortion in signal caused by the saturating nonlinearity. We have demonstrated that this saturation can have a significant impact on measurements of tuning functions by clipping the fluorescence signal at higher firing rates (Nauhaus et al., 2012b). Distortions caused by the saturating nonlinearity were observed not only in ferret visual cortex, which has neurons with high firing rates, but also in mouse visual cortex, where firing rates are significantly lower. It is critical to keep these distortions in mind when interpreting OGB data. While some measurements (e.g., the tuning curve maximum) may not be affected by a monotonic nonlinearity, other measurements (e.g., tuning curve width) can be strongly distorted. As a consequence, differences among neuron groups or conditions with different firing rates could be artificially enhanced or diminished.
Future Developments

So far, we have focused on how to perform two-photon calcium imaging in anesthetized animals using an injectable calcium dye. Although extremely valuable data can be collected under these conditions, it will be important for future research to be able to perform two-photon experiments in awake monkeys performing a task. A number of technical hurdles need to be overcome to make these experiments feasible. First, injectable calcium dyes will likely no longer be useful in an awake animal, in which repeated measurements will need to be carried out. As described earlier, injectable dyes can be used only in young animals, once per durotomy, and only in “fresh” durotomies. Instead, GECIs appear to be a better choice because they can be stably expressed in cells. The most recent generation of these indicators (GCaMP6s, 6m, and 6f) show great promise for future experiments because of their relatively fast response kinetics and good SNR (Chen et al., 2013).

GECIs require the injection of a virus into the brain to be expressed. Thus, going forward, we will need to identify which virus (e.g., different serotypes of AAV, lentivirus) will lead to sufficient expression in terms of spatial spread, expression density, as well as expression level within each cell. In addition, an appropriate promoter will need to be chosen. Another issue with respect to the use of GECIs is their impact on cell health. All GECIs are calcium buffers. Because calcium is important for many “housekeeping” functions in a cell (Berridge et al., 2000, 2003), excessive calcium buffering leads to cell death. How long cells remain viable after virus infection will depend on the particular indicator used and the rate at which it is expressed, which will in part depend on the virus chosen for expression. In the mouse, stable expression of GCaMP6 over several months has been demonstrated (Chen et al., 2013). Whether similar durations are possible in the monkey will need to be determined.

The switch from OGB to GECIs is one important issue that needs to be solved to be able to perform two-photon microscopy in awake monkeys. Other hurdles are the development of an imaging well, and the development of strategies to deal with brain movement. The largest engineering challenge with respect to the imaging well is the fact that most two-photon objectives are very large (diameters ≤ 3 cm) and need to be placed quite close to the brain (typical WD ~3 mm). Thus, large implants are required. In addition, because monkey dura is opaque, it will need to be removed and replaced with an artificial dura. So far, one two-photon implant has been developed (Stettler et al., 2006); implant designs similar to the ones used for optical imaging may be another possibility (Roe, 2007). Because of the necessary size of the well, and the removal of the dura, motion artifacts will be further exacerbated beyond the inevitable increase caused by using an awake animal. Thus, imaging wells likely will have to include provisions to stabilize the brain. Additionally, it may be necessary to develop ways to track brain movements online and to move the microscope in synchrony with the brain.

To summarize, two-photon microscopy is a promising addition to the toolkit available for research in the macaque monkey. Because of its unique capabilities—high resolution and high sampling density—it will open important novel avenues to study local circuit function. Two-photon calcium imaging in the anesthetized monkey is feasible today, but experiments in the awake monkey will require further technical developments.

References


Mesoscale Two-Photon Microscopy: Engineering a Wide Field of View with Cellular Resolution

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Introduction

Information is widely distributed in neuronal networks. Patterns of activity across multiple brain areas act in concert to support sensory processing and behavior. Mapping this activity with cellular resolution can reveal principles of neural function but presents technical challenges.

Electrodes offer unsurpassed temporal resolution and can easily record from spatially separate populations. However, this approach is invasive, cannot be genetically targeted to record from specific cell types, and only sparsely samples local populations. By contrast, two-photon imaging of free calcium is less invasive, can be genetically targeted, and offers dense sampling of local populations. Two-photon imaging (Denk et al., 1990) is particularly suited for imaging activity in highly scattering neuropil, such as mammalian cortex, and the latest generation of genetically encoded calcium indicators (GECIs) can provide a high-fidelity optical readout of spiking (Chen et al., 2013).

However, conventional implementations of two-photon microscopy are often limited in their primary field of view (FOV) to approximately 700 µm. Outside of this region, limiting apertures and aberrations decreases the effectiveness of two-photon excitation, leading to dim regions (“vignetting”) and reduced resolution. Brain regions can be distributed over areas larger than this FOV. For example, cortical areas in mice are distributed over millimeters of cortex (Fig. 1). Thus, engineering improvements are needed to enable two-photon microscopy of FOVs large enough to encompass multiple brain regions. We refer to this wide FOV imaging as “mesoscale two-photon imaging.”

In this chapter, we discuss the main factors that limit the FOV of two-photon imaging and how to engineer optimized implementations to retain cellular resolution across a large FOV. We will also discuss imaging speed, which is crucial to ensure temporal resolution sufficient to map neural activity on timescales that are relevant for coding and behavior.

Optomechanical Considerations

There are three main optomechanical determinants of the FOV for a laser scanning microscope: the scan angles (SAs) of the beam scanning engine (SE) (e.g., galvanometer mirrors); the magnification of the beam expansion relay (the ratio of the focal lengths of the scan lens and tube lens); and the focal length of the objective used (Fig. 2). A simple formula for the full FOV is:

$$\text{FOV} = 2 \times FL_{\text{OBJ}} \times \tan (SA \times \frac{FL_{\text{SL}}}{FL_{\text{TL}}})$$

In this equation, SA is measured from the central optical axis, and is thus half of the full scan arc. Beam expansion is crucial because at the SE, smaller beam apertures can support increased scan speeds (higher temporal resolution), but large beam diameters are needed to overfill the back focal plane of the objective, which is critical for ensuring cellular resolution along the Z-axis (Zipfel et al., 2003; Kerr and Denk, 2008).

Next, we will discuss how the practical implementation of optics to support a large FOV involves careful engineering optimizations. Many of these essentially come down to accommodating high SAs using optical elements with short focal lengths (i.e., high numerical aperture [NA]) to ensure that the beam does not get clipped at any point in the system. In a representative two-photon microscope, the SA is ± 3.5°, the $FL_{\text{SL}} = 50$ mm, the $FL_{\text{TL}} = 200$ mm, and the $FL_{\text{OBJ}} = 12.5$ mm (Nikon CFI75, 16×, 0.8 NA). This would yield a total FOV of 380 µm.

Scanning system

A conventional scanning system consists of two galvanometer mirrors placed in proximity to one another, on orthogonal axes (to scan in X and Y). The deflection of the first mirror will cause displacement of the beam from the center of the second mirror. This
Deflection is referred to as “beam walk.” At low SAs (and when the $F_{SL} \gg$ the distance between the two mirrors), this can be negligible (Tsai and Kleinfeld, 2009). However, at the relatively large SAs necessary for wide FOV imaging, beam walk can lead to a few millimeters of beam displacement on the second mirror, which can lead to beam clipping on either the scan lens, tube lens, or at the back focal plane of the objective. Any of these distortions would lead to vignetting and a reduction in the usable FOV. To combat this, an afocal relay can be placed between the X and Y scanning mirrors (Fig. 3). The afocal relay can be refractive (Kim et al., 1999; Vojnovic and Tullis, 2011) or reflective (Sharafutdinova et al., 2010a). In either case, high NA optics must be used to accept large SAs.

Scan lens
To increase the FOV, relatively high SAs are used. The scan lens must have a small enough $f$-number (i.e., sufficiently high NA) to accept these deflections. For $SA = 10^\circ$, a scan lens of $f$-number $\sim 2$ (NA $\sim 0.25$) would be required. For a 1” diameter lens system, this translates to an effective focal length of 50 mm, to permit a 6 mm diameter beam to be scanned at $\pm 10^\circ$.

Beam expansion
The ratio of the focal lengths of the scan lens and the tube lens determine the beam expansion ratio (beam magnification = $FL_{TL}/FL_{SL}$). This expansion is needed to completely fill the back aperture of the objective; therefore, decreasing the beam expansion to increase the final entrance beam angle is not always practical. Once the scan-lens focal length is chosen, it will determine the focal length of the tube lens. For example, the back aperture on the widely used Nikon CFI75 16×/0.8 NA objective is $\sim 22$ mm. If the input beam is 6 mm (limited by the clear aperture of the scanning system), then a 4× beam expansion would overfill the back aperture of the objective.

Tube lens
Since the focal length of the scan lens and the beam expansion have been chosen, the focal length of the tube lens has been determined: In this case, it is 200 mm. Because the beam is undergoing expansion, when it reaches the tube lens, if the tube lens is not sufficiently large, the beam will be clipped. The center of the beam is displaced by $(50 \text{ mm}) \times \tan(10^\circ) \approx 9$ mm. At the point it reaches the tube lens, the radius of the beam is 12 mm, and thus the minimum radius of the tube lens must be $9 \text{ mm} + 12 \text{ mm} = 21$ mm; otherwise, significant vignetting will occur, limiting the usable FOV. In this case, 2” optics for the tube lens are sufficient. Often optomechanical components are placed between the tube lens and the objective (e.g., a 45° dichroic mirror to split excitation light and emitted fluorescence). All these elements must be large enough to prevent clipping of the beam.
even at high SA. Some long-focal-length custom objectives and high NA macroscope objectives have back apertures ~50 mm in diameter—more than twice what we considered in the present analysis. Although theoretically these objectives can provide a wide FOV, implementing them in a two-photon microscope (e.g., overfilling their back apertures) can present significant challenges.

**Wavefront Considerations**

In two-photon excitation, the scanning focal spot must be of high optical quality and possess sufficient photon density to achieve two-photon excitation. Although high NA objectives are important, they are not always the limiting parameter when imaging over a large FOV. Optical aberrations, which can be introduced anywhere in the optical system, can degrade the wavefront and decrease the efficiency of two-photon excitation of a microscope. When scanning at small angles, aberrations can be minimal. However, aberrations become increasingly important at high scan angles, and these can ultimately limit the FOV. An extensive optimization study of scan and tube lens designs found that when using a single achromat for the scan lens, only approximately the central third of the theoretical FOV was diffraction-limited (Negrean and Mansvelder, 2014). This limitation is mainly the result of the significant astigmatism introduced at large off-axis angles. The usable fraction of FOV is increased with compound lens design (e.g., a Plossl lens) or completely custom scan lens systems. This study also showed that single achromats used for tube lenses can limit performance, whereas custom lens designs can again increase performance across the entire FOV (Negrean and Mansvelder, 2014).

The aberration-induced wavefront distortions must also be considered when using a relay between the scanning mirrors. Optimized relay lenses can be designed similarly to the scan lenses. The relay lenses must match the NA of the scan lens, or they will limit the usable angles. Another option is to use off-axis parabolic mirrors (Sharafutdinova et al., 2010a; Negrean and Mansvelder, 2014). Parabolic mirror–based relays can offer diffraction-limited performance across large SAs, and potentially provide lower wavefront errors compared with refractive designs or spherical mirror relays (Sharafutdinova et al., 2009, 2010a, b).

Once careful optical design has minimized most aberrations, adaptive optics can further improve performance by correcting residual aberrations, including those introduced by the preparation itself. In vivo, wavefronts cannot typically be measured directly; thus, various methods have been developed to determine patterns than can cancel aberrations during in vivo imaging and increase fluorescence yield (Debarre et al., 2009; Aviles-Espinosa et al., 2011; Ji et al., 2012).
Imaging Speed

As the FOV increases in area, if the number of pixels per neuron is preserved, the quantity of effective pixels per image is greatly increased. For example, ≥100 neurons can be imaged in a 250 × 250 µm square FOV, and raster scanning requires at least 128 × 128 pixels (214 pixels). Thus, for a 3 mm2 image, ~4800 neurons can be imaged (though in practice, vasculature obscures a significant fraction of these), and >219 pixels can appear in a raster scan. For conventional galvanometer scanning, the scan speed is inertia-limited to ~1 kHz (1 ms/line). Since ~219 lines/frame are needed for a small region, this ultimately leads to a frame rate limit of ~10–15 frames per second for a 250 µm wide FOV. This rate limit can be sufficient for many experiments, as the dynamics for current GECIs are >100 ms, and even studies of spike-count correlations often use counting windows of 0.1–1.0 s. However, some experiments require (or can be enhanced by) higher scanning speeds. Moreover, scanning entire large FOVs can take several seconds per frame. Here we briefly survey some alternative approaches that can offer higher scan speeds.

Resonant scanning

Using a resonant scanner as the fast axis and a conventional galvanometer scanner as the slow axis, scan speeds of ≥30 frames/s are possible (Nguyen et al., 2001). The resonant axis oscillates at a fixed frequency (4–12 kHz), and thus the line scanning rate is fixed. Because the resonant scanner scans a sinusoidal path, resulting images need to be corrected for the nonlinearity. This correction can be done either by clipping the edges of the image (leaving the more linear central region) or by applying a nonlinear corrective procedure to the image (Haji-Saeed et al., 2007). Overall, resonant scanning systems can provide ample SAs, >20°, and thus are a good fit for wide FOV imaging.

Acousto-optical deflectors

An alternative method for achieving very high scan speeds employs acousto-optical deflectors (AODs; Fig. 4C). An AOD is a crystal whose angle of diffraction can be varied using an applied acoustic wave (typically in the radiofrequency range). An AOD can raster scan a laser beam at rates >100 Hz. Using AODs, fully three-dimensional (3D) noninertial scanning can be used to map neural activity (Duemani Reddy et al., 2008; Kirkby et al., 2010). The two drawbacks to AODs are added dispersion (which degrades two-photon excitation) and relatively small maximum SAs, typically <4°. While the former problem has been mostly solved, the latter issue remains, and demonstrated FOVs have been only ~700 µm across (Katona et al., 2012). Custom-designed AODs may offer increased SAs.

Arbitrary line scanning

When raster scanning, much of the FOV does not contain active neurons, and thus most of the pixels do not contain information that will be used by the experimenter. Thus, to increase the scan rate, one can utilize an arbitrary line scan (Fig. 4D). This can be supported by either conventional galvanometer scanners or AODs. In this method, a raster image is first acquired to obtain the position of all the cells of interest. Next, a scan path is generated in which the laser is directed to pass over each cell of interest. These scans can be optimized for speed and repeatability (Cotton et al., 2013).
Multiplexed beam scanning

Any of the above methods can be combined for multiplexed beam scanning. In this approach, pulses from multiple beams are interleaved. Each beam scans a separate area, and fluorescence is collected using a single objective and focused onto a high-bandwidth detector. Synchronization pulses are used to determine which beam caused each fluorescence event. Because each beam can scan a separate area, the imaging speed scales up by a multiple of the number of beams used (Amir et al., 2007; Cheng et al., 2011).

Alternative Approaches

There are completely different approaches for imaging activity in multiple brain regions as well. For example, one could use two different microscopes. In this approach, the main challenge would be to place two objectives near one another, along with the rest of the optical pathways. However, with flexible positioning microscopes and low-profile objectives, it should be achievable, at a cost of increased complexity and likely limits on how close two imaged areas can be. Another simple solution would be to rapidly reposition the sample or the microscope. Movements of <1 mm can be achieved with a motorized stage in <0.5 s. Although this approach can be relatively simple to implement, it would suffer from severely limited maximal frame rates and low duty cycles (fraction of time spent imaging) owing to the movement time.

Our Solution

We have recently developed a two-photon microscope that can scan a wide FOV with cellular resolution. We utilized highly corrected, low f-number optics throughout the optical pathway to enable large FOV imaging: 1.4 mm with the Nikon CFI75 16 × 0.8 NA objective, and 3.5 mm with a custom-built objective. Because conventional raster scanning over the entire FOV area (>9 mm²) can take 1–10 s per frame, we developed a version of beam multiplexing with dynamic beam-steering mirrors to support simultaneous scanning of two regions of interest (ROIs) anywhere within the overall FOV (Fig. 5). This method enables smaller ROIs to be scanned at ~5–30 frames/s yet still preserves the ability to have these regions widely separated within or across different cortical areas.

Conclusion

The FOV of two-photon imaging can be expanded to the mesoscale without sacrificing cellular resolution by minimizing optical aberrations and designing optomechanical systems that avoid limiting apertures. This technology will enable new kinds of experiments, in which dense samples of spatially separate populations of neurons are imaged nearly simultaneously. As a result, we will begin to map the distributed neural activity that underlies sensory processing and behavior in extended neural circuitry.
References


Acousto-optical Scanning–Based High-Speed 3D Two-Photon Imaging in Vivo

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Rationale for Selecting Acousto-optical Scanning for in Vivo Network Measurement in Three Dimensions

Several novel technologies have been developed for the three-dimensional (3D) readout of fast populations and dendritic activities. These include liquid lenses (Grewe et al., 2011), holographic scanning (Nikolenko et al., 2008), Roller Coaster Scanning (Katona et al., 2011), piezo scanning with sinusoidal resonance (Gobel et al., 2007), deformable mirrors, temporal and spatial multiplexing (Cheng et al., 2011), axicon or planar illumination–based imaging (Holekamp et al., 2008), fast z-scanning based on an axially moving mirror (Botcherby et al., 2012), simultaneous multiview light-sheet microscopy (Tomer et al., 2012), and optical fiber acousto-optical deflector (AOD)–based 3D scanning (Rózsa et al., 2007). However, only acousto-optical scanning can increase the product of the measurement speed and the signal collection efficiency by several orders of magnitude, as compared with classical raster scanning.

More quantitatively, random-access scanning increases measurement speed and signal collection efficiency in the following way:

\[
\frac{\text{(measurement speed)} \times \text{(signal collection)}}{\text{(total image volume)}} = \frac{\text{(volume covered by the scanning points)}}
\]

where “signal collection” is defined as the amount of the detected fluorescent photons in a given time interval from a given spatial location, and the “volume covered by the scanning points” is the convolution of the point spread function (PSF) and the preselected scanning points. In a typical in vivo measurement, when we consider using a 450 × 450 × 650 μm³ scanning volume with a 0.47–1.9 μm and 2.49–7.9 μm axial resolution (Katona et al., 2012), and simultaneously measure approximately 100 locations, random access scanning will provide a 2,106,000–46,534-fold, on average an increase of six orders of magnitude, in the product of measurement speed and the signal collection efficiency. According to the current state of the technology (which now provides an ~1 mm³ scanning volume with high-numerical-aperture [NA] objectives), this increase could be even larger: more than 1,000,000,000 per locations. No other available 3D method with high spatial resolution and deep penetration capability can provide a similar increase in the product of the measurement speed and the signal collection efficiency.

A further advantage of 3D acousto-optical microscope technology is that it is based on single-spot two-photon activation, which allows whole-field detection with high-quantum-efficiency photomultiplier tube (PMTs). Therefore, photons scattered from deep layers of the tissue can also be collected with high sensitivity, which is essential to realize imaging through the whole cortex. Moreover, the position of the PSF can be finely adjusted, with 50–100 nm precision (Katona et al., 2012), to any spatial coordinates during acousto-optical scanning. Therefore, there is no limitation in z-planes and coordinates, which enables a highly precise measurement of neuronal activity by eliminating neuropil contamination.

Principles

The phrase “acousto-optic” refers to the field of optics that studies the interaction between sound and light waves. In imaging, it means especially the diffraction of a laser beam through ultrasonic grating. The acousto-optical effect is produced by the periodic change in the refractive index of the medium (usually tellurium dioxide), which results from the sound-wave-induced pressure fluctuation in the crystal. This grating diffracts the light beam just like normal optical grating.

Acousto-optical deflection

AODs spatially control the optical beam using ultrasonic waves. During operation, the acoustic frequency is varied in a way that results in an acoustic-frequency-dependent diffraction of the laser beam. The change of the diffraction angle is given by the following formula:

\[
\Delta \theta_a = \frac{\lambda}{\nu} \Delta f
\]

where λ is the optical wavelength of the beam, ν is the velocity of the acoustic wave, and Δf is the change of the sound frequency (Fig. 1).

In practice, AODs or acousto-optical modulators (AOMs) are used. AOMs modulate only the amplitude of the sound waves, whereas AODs are able to adjust both the amplitude and the frequency.

Acousto-optical focusing

Besides deflection, AODs can be used for fast focal-plane shifting (Kaplan et al., 2001; Vucinic and Sejnowski, 2007; Katona et al., 2012; Cotton et al., 2013; Fernandez-Alfonso et al., 2014). If the optical aperture is filled with acoustic waves whose frequency increases as a function of time (chirped wave), different portions of the optical beam are deflected...
in different directions (Fig. 2); thus, a focusing or defocusing effect occurs, depending on the frequency slope (sweep rate) of the chirped acoustic wave.

The focal length of acousto-optical lens can be calculated from the sweep rate. The focal distance (F) of the deflectors can be defined as follows:

\[ F = \frac{v^2 \cdot T_{\text{scan}}}{2 \lambda \Delta f} \]

where \( \lambda \) is the optical wavelength of the beam, \( v \) is the velocity of the acoustic wave, \( \Delta f \) is the change of the sound frequency, and \( T_{\text{scan}} \) is the modulation rate of the sound frequency (Kaplan et al., 2001).

To keep the focus stable, the frequency gradient should be preserved in the crystal. The frequency should therefore be continuously increased (or decreased) at the piezoelectric driver to preserve the focal distance. This will result in a lateral drift of the focal spot that can be easily compensated by a second AOD with a counterpropagating acousto-optical wave. The optical grid generated within the AOD is equivalent to a cylindrical lens. In 3D two-photon microscopy, it is necessary to scan points. Therefore, a combination of two perpendicularly oriented cylindrical lenses with the same focal distance is required. And, because both acousto-optical lenses (\( x, y \)) require drift compensation, 3D microscopes need at least four AO deflectors (\( x_1, y_1, x_2, y_2 \)). The four AO deflectors can be optically coupled using “afocal projections” (telescopes with two lenses where the distance between the lenses is equal to the sum of each lens’s focal length) and air in different combinations. The largest scanning volume can be reached by grouping the deflectors into two functionally distinct groups (\( z \)-focusing and lateral scanning), which are coupled by one afocal projection (Katona et al., 2012).

Controlling 3D acousto-optical scanning

To focus the excitation beam to a given \( x, y, z \)-coordinate, the four AODs should be driven synchronously with varying frequency voltage signals. Because AODs have limited electrical bandwidths, the changing frequency can be maintained only for a limited time before the frequency has to be abruptly reset. This sudden change of frequency results in an improperly formed focal spot, and thus can be considered “dead time” (Fig. 3). The length of this period is defined by the time the acoustic wave travels through the aperture of the crystal, usually ~5–30 \( \mu \)s.

The position and the movement of the focal point are determined by eight values. Four of them control the starting acoustic frequency on the four AOD drivers, while the other four define the frequency ramp speeds (chirping). All eight parameters are updated in every sweep cycle (typically 33.6 \( \mu \)s is used; the minimum is 5–10 \( \mu \)s). The starting frequencies and the chirps together define the \( xyz \) position of the focal spot and its \( xy \) movement speed (drifting). During every sweep cycle, all PMT channels are sampled multiple times. The ratio of the dead time (offset time) to the measurement time (when the focal spot has already been formed and is ready for imaging) depends on the \( z \)-level and is optimal at the nominal focal plane of the objective.

In the simplest case, the goal is to attain a steady focal spot without lateral drifting (in random-access scanning mode). In this scanning mode, PMT data are averaged, and one value is created for each measurement cycle, corresponding to a single point in \( xyz \). Thus, this mode enables random-access 3D point scanning, which is ideal for monitoring neuronal network activity. More complex measurement modes are possible when one lets the focal spot drift, such as during Multiple 3D Trajectory Scanning.
The 3D Acousto-optical Microscope
Realization of the optical pathway

In order to realize the optical pathway of a 3D microscope, one must create a 3D random-access laser scanning two-photon microscope that simultaneously satisfies two different needs in the largest possible scanning volume. The first need is to record activity across the dendritic tree of a single neuron at high spatial and temporal resolution in 3D in a central core (≈290 × 290 × 200 µm³) of the scanning volume and to do so in such a way that dendritic spines remain resolvable. The second need is to record activity in a more extensive volume (now >1100 × 1100 × 3000 µm³ in transparent samples) at high speed but at a lower resolution, in order to capture the activities of a large number of cell bodies in a neuronal population.

Following the arrangement suggested by the model, a large-aperture (15–17 mm) optical assembly was constructed (Figs. 4, 5). Here, a Ti:S laser with automated dispersion compensation provides the laser pulses. A Faraday isolator blocks the coherent backreflections. A four-prism sequence (Proctor and Wise, 1992) adds a large negative second- and third-order dispersion (−72,000 fs² and −40,000 fs³, respectively) in order to precompensate for pulse broadening caused by the optical elements of the system (dispersion compensation unit; Fig. 5).

An automated beam stabilization unit (see Laser Beam Stabilization, below) is necessary to precisely stabilize the laser beam of the long optical pathway and to cancel out subtle thermal drift errors. The beam stabilization unit is built from position sensors (quadrant detectors) and motorized mirrors, wired in a feedback loop. The beam is then magnified by a beam expander to match the large apertures (15 mm) of the first pair of AODs. These AODs form two orthogonal electric cylinder lenses filled with continuously changing frequency (“chirped”) acoustic waves (Kaplan et al., 2001), which are used for z-focusing (AO z-focusing unit).

Next, laser beams from the x and y cylindrical lenses are projected onto the x and y AO deflectors (17 mm apertures) of the 2D scanning unit, respectively, by telecentric projection. This 2D-AO scanning unit performs lateral scanning and compensates for the lateral drift of the focal spot generated by the cylindrical lenses during z-focusing, i.e., drift compensation (Kaplan et al., 2001). The angular

Figure 3. Driving functions and timing of operation of the four-deflector sequence. Top, The four upper traces show the frequency modulation of the sine wave as a function of time for the four AO deflectors (x1, y1, x2, y2) within 1.5 sweep cycles. This results in “dead” times and periods when focus is well formed (orange). Bottom, PMT data are continuously collected and transferred to the computer; however, PMT data collected during the dead time period are eliminated during the acquisition. Red dashed lines, synchronous frequency reset of all driver functions.
dispersion compensation unit optically links the 2D-AO scanning unit with the objective. Modeling helped us design and manufacture a custom lens made of high-dispersion glass with aspheric and conic surfaces which, combined with a telecentric projection of the AO scanner unit to the objective's back aperture, diminishes the angular dispersion introduced by the AO devices at off-axis positions \((x, y \neq 0)\). Then, the 3D scanning system is coupled to a 2D two-photon microscope in which fluorescent signals are collected by PMTs fixed directly onto the objective arm for high photon-collection efficiency (Fig. 5). In contrast to previous AO microscope designs, we have also taken advantage of the active optical elements to dynamically compensate for optical errors (e.g., astigmatism, field curvature, angular dispersion, chromatic aberration) that increased spatial resolution especially during AO \(z\)-focusing (at \(z \neq 0\) planes by a factor of \(~2–3\); Fig. 4).

We determined the optimal compensation parameters in advance for each point of the scanning volume and loaded them into the driver electronics accordingly. During this optimization process, we varied the parameters of the chirped sine driver function at each of the four deflectors in order to maximize the fluorescence intensity and the sharpness of the fluorescent beads used during this calibration. Spatial resolution in the whole scanning volume was also increased by the large optical apertures used throughout the system, and \(~20\%\) of this increase resulted solely from the use of large AOD apertures (Fig. 4). In contrast to the dominantly \(z\)-focusing-dependent effect of dynamic error compensation, the angular dispersion compensation unit decreased the PSF in off-axis positions when compared with a simple two-lens telecentric projection (Fig. 4b). These factors that decrease the PSF inherently increase the lateral field of view (Fig. 4a).

One difference between the system described here and previous designs is that the AODs form functionally and physically different groups. \(z\)-focusing is carried out by the first AO pair, whereas lateral scanning is performed entirely by the second pair (2D-AO scanning unit). This arrangement increased the diameter of the lateral scanning range by a factor of \(~2.7\) (Fig. 4a). Furthermore, not only electronic driver function but also deflector geometry, \(\text{TeO}_2\) crystal orientation, and bandwidth differed between deflectors of the \(z\)-focusing and the 2D-AO scanning unit. Altogether, these factors increased the diameter of the lateral scanning range by \(\leq 720\ \mu m\) using the Olympus 20× objective and \(>1100\ \mu m\) using the Nikon 16× objective.

Materials and methods

Figure 5 is a schematic block diagram of the microscope, which was realized according to detailed optical modeling (Katona et al., 2012). Laser pulses

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**Figure 4.** Characterization of the two-photon AO microscope setup. **a,** The maximal field of view (“compensated”) is shown when both deflector pairs were used for deflection (“no deflector grouping”) or when optically rotated deflectors (“no acoustic rotation”), small aperture objectives (60×), no angular dispersion compensation (“with angular dispersion”) or small aperture AODs were used (“small aperture”). **b,** The compensated PSF size along the \(x\)-axis (PSF\(_x\)) (“central”) at \((x, y, z) = (150, 150, 100)\ \mu m\) coordinates (“lateral”) or when no angular dispersion compensation (“with angular dispersion”), no electronic compensation (“no electric compensation”), or reduced AO apertures (“no large apertures”) were applied. Reprinted with permission from Katona et al. (2012), their Fig. 1b, c.
are provided by a Mai Tai DeepSee femtosecond laser (Spectra-Physics, Santa Clara, CA). The optimal wavelength range is 740–880 nm (but can also be extended ≤1064 nm). Pulse backreflection to the laser source is eliminated by a Faraday isolator (BB8-5I, Electro-Optics Technology, Traverse City, MI). Next, the beam position is stabilized using two motorized mirrors (m, Fig. 5) (Agilis Series, AG-M100N, Newport), which stabilize the position of the light transmitted by two backside custom-polished broadband mirrors (BB2-E03, Thorlabs, Newton, NJ) on the surface of two quadrant detectors (q, Fig. 5) (PDQ80A, Thorlabs). The positioning feedback loop (U12, LabJack Corporation, Lakewood, CO) is controlled by a program written in LabVIEW (National Instruments). The beam is expanded by two achromatic lenses arranged in a Galilean telescope (f = −75 mm, ACN254-075-B, Thorlabs; f = 200 mm, NT47319, Edmund Optics; distance = 125.62 mm) to match the large apertures of the first pair of AODs (15 mm). Mirrors, λ/2 wave plates and holders, are purchased from Thorlabs and Newport. AODs have been custom designed and manufactured at the Budapest University of Technology and Economics. Achromatic telecentric relay lenses were purchased from Edmund Optics (fTC = 150 mm, NT32-886). Achromatic scan and tube lenses were chosen from Edmund Optics (f = 250 mm, NT45-180) and Olympus (f = 210 mm), respectively. The AO-based 3D scanner system is attached to the top of a

Figure 5. Design and characterization of the two-photon AO microscope setup. Material-dispersion compensation is adjusted using a four-prism compressor and a Ti:S laser. A Faraday isolator eliminates coherent backreflections, while a Ti:S laser with automated dispersion compensation provides the laser pulses. Motorized mirrors (m) stabilize the position of the beam light on the surface of two quadrant detectors (q) before the beam expander. Two AODs optimized for diffraction efficiency control the z-focusing of the beam (AO z-focusing). A 2D-AO scanner unit performs x–y scanning and drift compensation during z-scanning. A spherical field lens in the second telecentric lens system (Tc3,4) provides additional angular dispersion compensation. Reprinted with permission from Katona et al. (2012), their Fig. 1a.

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galvanometer-based, upright two-photon microscope (Femto2D Alba, Femtonics, Budapest) using custom-designed rails. AO sweeps are generated by direct digital synthesizer chips (AD9910, 1 GSPS, 14-bit, Analog Devices, Norwood, MA) integrated into the modular electronics system of the microscope using FPGA elements (Xilinx).

Red and green fluorescence are separated by a dichroic filter (39 mm, 700dcxru, Chroma Technology, Bellows Falls, VT) and are collected by GaAsP PMTs custom-modified to efficiently collect scattered photons (PMT, H7422P-40-MOD, Hamamatsu Photonics, Hamamatsu City, Japan), fixed directly onto the objective arm (traveling detector system). In *in vitro* experiments, the forward-emitted fluorescence can also be collected by 2 inch aperture detectors positioned below the condenser lens (Femto2D Alba, Femtonics). Signals of the same wavelength measured at the epifluorescent and transfluorescent positions are added. The large aperture objectives, XLUMPlanFI20×/0.95 (Olympus, 20×, NA = 0.95) and CFI75 LWD 16×W (Nikon, 16×, NA = 0.8), provide the largest scanning volume (Katona et al., 2012). The maximal output laser power in front of the objective is ~400 mW (at 875 nm).

**Laser beam stabilization**

The role of the laser beam stabilization loop is to compensate for the thermal drift and small vibration of the laser resonator and optical elements. The system comprises several elements that are highly sensitive to the incidence angle. To compensate for the translation and angle deviation of the beam, a pair of two-axis piezo mirrors with two quadrant detectors act as a reference.

**Prism compressor**

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**Figure 6.** High-speed 3D calcium imaging of spontaneous neuronal network activity *in vivo*. **a,** Top, Sketch of *in vivo* experimental arrangement. Bottom, Staining by bolus loading (Oregon Green BAPTA [OGB]-1-AM and SR-101) in mouse primary visual cortex (V1). Five representative planes at different depths are imaged with 3D-AO scanning. Depths are measured relative to the pia. **b,** Example of an image plane at 200 µm depth showing neurons (green) and glial cells (magenta and white). Scale bar, 100 µm. **c,** Image and intensity profiles of a preselected bright glial cell used to establish the measurement coordinate system. Scale bar, 5 µm. **d,** Bottom, 35 µm z-projection of the region marked in b. Scale bar, 50 µm. **Top,** Neuronal somata detected with the aid of an algorithm in a subvolume (shown with projections, neurons in white and glial cells in black). **e,** Maximal intensity side- and z-projections of the entire z-stack (400 × 400 × 500 µm³) with autodetected cell locations (spheres) color-coded in relation to depth. The set detection threshold yields 532 neurons. Scale bar, 100 µm. **f,** Spontaneous neuronal network activity measured in the 532 cells in e. Left, example of a raw trace in which each line corresponds to a cell. Spatially normalized traces (middle) and corresponding Ca²⁺ transients (right). Calibration, 1 s. Reprinted with permission from Katona et al. (2012), their Fig. 4.
The material dispersion compensation problem is solved by a motorized prism compressor. The four-prism unit (Fig. 5) adds a large negative dispersion to precompensate the pulse broadening caused by the optical elements of the system. Total compensation depends on the given wavelength. Approximately 72,000 fs² second-order group delay dispersion (GDD) and ~40,000 fs³ third-order dispersion (TOD) compensation is required; the precise amount depends on the objective used and the central wavelength. We also added a two-pass, four-prism compressor (Proctor and Wise, 1992; Rózsa et al., 2007) that provides a fixed compensation amount at a given wavelength. A mechanically fixed compressor unit could be combined with the variable compensation provided by the DeepSee unit of the Mai Tai laser (Spectra-Physics) to confer dynamically adjustable dispersion compensation for an optimum fluorescence signal. Alternatively, motorizing the four-prism sequences would enable the omission of the DeepSee unit and thereby provide ~10% higher infrared transmission.

**Experiments**

AO scanning technology can be beneficial in biological experiments in which one would like to perform functional imaging in 3D from sparse regions of interest (ROIs) relatively far from each other. For example, it is beneficial when one would like to follow the activity of multiple dendritic segments of a single neuron (Chiovini et al., 2014), to measure activity from large neuronal networks (Katona et al., 2012; Cotton et al., 2013; Fernandez-Alfonso et al., 2014), or to perform simultaneous measurements of somas and dendritic spines (Katona et al., 2012; Fernandez-Alfonso et al., 2014).

**Acousto-optical scanning methods**

Multiple scanning modes have been developed for AO scanning that can also be used in different combinations as needed by the experimental protocol.

**Random-access point scanning**

Random-access point scanning is one of the most convenient applications for imaging neuronal networks in 3D. In the first step, a reference z-stack image is acquired (Fig. 6a). In the second step, points can be preselected for fast 3D measurements (Figs. 6b–e). Once a region with well-stained cells is identified, one can select one or more reference structures (typically, a brightly red-fluorescing glial cell), scan it in 3D, and define the 3D origin [(0, 0, 0) coordinate] of the recording as the center of this glial cell (Fig. 6). In order to compensate for tissue drift, one needs to rescan the “origin-glia” cell regularly during an experiment and move it back into the original position (0,0,0) by moving the microscope table and the objective. 3D Ca²⁺ responses recorded...
simultaneously in multiple points can be plotted as curves (Fig. 6f, right panel), or alternatively, responses following spatial normalization can be shown as images (Fig. 6f, middle panel; Fig. 7).

Frame scanning in 3D
One of the basic measurement modes that can be realized with a 3D-AO microscope is frame scanning. In this mode, one can freely move or rotate arbitrary areas in 3D using the real (by moving the objective) and virtual (by focusing with the AODs at a fixed objective position) \( z \) for focusing. Because frame-scanning mode is relatively slow in 3D, this mode can be used to find and preselect ROIs for the subsequent fast 3D measurements. Multiple areas situated in multiple layers can be selected and measured simultaneously.

Multiple 3D trajectory scanning
Multiple 3D trajectory scanning mode was developed for simultaneously measuring multiple neuronal processes. There are two realizations of 3D trajectory scanning. In the continuous trajectory scanning mode, instead of jumping between preselected points (as during point scanning), one lets the focal spot drift along neuronal processes (Fig. 8). In this way, information is collected with a much higher spatial discretization within the same time interval.

XYZ or volume imaging
It is possible to capture volumetric data by successively capturing \( xy \) images at different \( z \) positions. The jump between the \( z \)-planes can be obtained by using either the real or the virtual focus. The first will result in better optical quality; therefore, it can be used at the end of experiments if only volume information is acquired. However, if one would like to perform fast 3D line scanning, 3D frame scanning, or random-access scanning, the \( z \)-stack should be obtained with virtual focusing in order to precisely preserve the coordinates of the cells for the fast 3D-AO scanning because the coordinate system of 3D-AO microscopes is distorted. Alternatively, a coordinate transformation is needed to match the two types of \( z \)-stacks to each other.

Movement correction
During long in vivo measurements, sample drifting can cause problems. Because in the random-access scanning paradigm one usually measures from a single point per cell or dendritic segment, the imaging quality can be very sensitive to sample movements. Quick timescale movements can be reduced only by preparation techniques. However, slow timescale drifts can be compensated for using software tools. For this compensation, reference objects are needed, which can consists of bright, well-defined objects in

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**Figure 8.** Point-by-point and continuous 3D trajectory scanning of dendritic segments. Top right, Schema of the scanning modes (blue point-by-point scanning; green, continuous scanning). Top left, Example of Ca\(^{2+}\) responses measured by point-by-point. Scale bar, 30 \( \mu \)m. Bottom, continuous trajectory modes. Traces were spatially normalized. Scale bar, 10 \( \mu \)m; calibration, 2 ms. Reprinted with permission from Katona et al. (2012), their Fig. 3f.

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the sample (Fig. 6c). These can be either a bright nonspecific object or a bright cell from which one does not expect activity-dependent fluorescence change. During compensation, one keeps the \( xyz \) center of this object in a given point inside the volume. Using this compensation method, a slow drift of the sample can be compensated for because, in this case, all points move together along the \( xyz \) coordinates; however, torsion of the sample cannot be compensated. In practice, this means that from one set of points, it is possible to take measurements for a couple of hours without significant drop in the image quality. This compensation can be performed manually or with software tools.

**Automatic cell finding**

In the simplest cases, cells can be categorized according to their dye content as measured by fluorescence, which is possible on multiple channels. In the most straightforward case, OGB1-AM and SR-101 dyes are bolus-loaded into the animal. In this technique, neuronal cells can be detected from their elevated green fluorescence and decreased red fluorescence. We normalize green and red fluorescent channel data from each image in the stack and then scale and shift them so that its 10th and 90th percentiles match 0 and 1. Background correction is done by oversmoothing an image and subtracting it from the original. Next, we subtract the red-channel data from the green and filter each layer of the stack again. The resulting stack is then searched for local maxima with an adaptive threshold. If two selected locations are closer than a given distance threshold, only one is kept.

In the algorithmically most complex cases, cell coordinates should be found according to their response to a (visual) stimulus (e.g., when using currently available channelrhodopsin [ChR] variants). In such cases, as a first step, random-access volume scanning is done on locations evenly spread over the field of view, sampling the space at approximately every 10 \( \mu \text{m} \) so that one point is sampled from each cell. Visual stimulation is then presented to the animal to drive the normally silent cells and reveal their location. In a second step, a finer mesh is scanned around the active locations to more precisely outline the cell locations and their center.

**Simultaneous cell-attached recording**

The decay time of currently available calcium indicators is usually not fast enough in \textit{in vivo} conditions to resolve bursts of action potentials (APs); single AP resolution is also problematic in most instances. However, the amplitudes of the \( \text{Ca}^{2+} \) peaks are proportional to the number of APs. To measure this relationship, one needs to detect APs preferentially using juxtacellular recording, which is a relatively noninvasive method. Such measurements show that although the amplitude of the \( \text{Ca}^{2+} \) signal is linearly proportional to the number of APs, the proportion coefficient for the different dyes (and even for different loading levels) can be quite variable (Fig. 9). Similar variability can be detected in GCaMP6 animals.

**3D acousto-optical imaging of dendrites**

The second main field of application for AO microscopes is dendritic imaging. This method can be used to measure propagation speeds; to simultaneously

![Figure 9. Maximum \( df/d_{\text{max}} \)-dependent AP number for two different cells in two different types of OGB bolus loading. Note the difference in the slope of the responses.](image-url)
follow large-scale spontaneous dendritic events, such as sharp-wave-associated dendritic spikes in multiple dendritic segments (Chiovini et al., 2014); to study dendritic plasticity; or to measure dendritic spines in vivo. 3D fluorescence measurements and two-photon uncaging experiments in thin dendritic segments and spines are very sensitive to spatial misalignments because even displacements below the resolution limit can induce significant changes in responses. It is possible to compensate for these errors using high-resolution motion artifact compensation. Once a region for dendritic measurements has been identified, one can select two or more reference lines with high angular deviation that cross both the imaged and the neighboring bright dendritic structures. Fluorescence data are collected preferentially in the red channel. To compensate for tissue drift, one needs to repeatedly measure the spatial distribution of fluorescence along these reference lines; one should also minimize the discrepancy relative to the original distributions using cross-correlation, using either an automatic or a manual process. Alternatively, a set of reference points depicting fluorescent local maxima in the sample need to be selected at the beginning of the measurement and their x, y, z-coordinates continuously optimized during measurements in order to obtain the maximum average fluorescence level.

Measuring propagation speed along dendrites
Random-access AO scanning enables the measurement of, for example, fast-propagating calcium waves both along the dendrites (by measuring a few points relatively far from each other) and along the dendrites with fast temporal resolution. Cells need to be loaded with a high-concentration, low-affinity calcium sensor (Fluo-5F, 400 µM) in whole-cell patch–clamp arrangement to perform measurements with good signal-to-noise ratio with fast onset times of the calcium events. In this example, we selected points along the dendritic tree of one pyramidal neuron in 3D (Fig. 9). We held the cell in current-clamp mode and evoked APs by somatic current injection. At the same time, we near-simultaneously measured dendritic Ca$^{2+}$ signals associated with the backpropagating action potential (BAP) by repetitively scanning the selected 3D coordinates at several tens of kHz. We calculated the velocity of the BAP from the latency of the Ca$^{2+}$ transients and the distance from the soma. Average BAP propagation speed differed at 23°C and 33°C (164 ± 13 µm ms$^{-1}$ [$n = 9$] versus 227 ± 14 µm ms$^{-1}$ [$n = 13$]; $p = 0.006$, t-test, Fig. 10).

Looking to the Future
Although the “hardware” for the AO microscope is nearing a theoretical maximum that can be realized with a given objective lens, there is a strong untapped potential in AO scanning still to be worked out:

- Developing novel software algorithms that could realize complex measurement modes;
- Applying the technology to larger FOV objectives;
- Developing novel correction methods for eliminating movement artifacts;
- Implementing adaptive optics;
- Improving lasers and fluorescent dyes to gain access to the entire thickness of the cortex;
- Simplifying the system to lower its costs and maintenance requirements;
- Improving wavelength tunability; and
- Providing simultaneous imaging and photostimulation in multiple regions.

**Figure 10.** Measurement of propagation speed of BAP in apical dendrites. a, Experimental arrangement for signal propagation experiments. Signal propagation speed was measured by somatic whole-cell current-clamp ($V_m$, black) and 3D two-photon calcium imaging (orange, pink, green, and blue). b, AP peak triggered average of normalized dendritic Ca$^{2+}$ transients induced by bAPs (mean ± SEM; $n = 54$; top). Linear fits (red dashed lines) define onset latency times. c, Onset latency times (mean ± SEM; $n = 54$) of Ca$^{2+}$ transients in b as a function of dendritic distance. Linear fit = average propagation speed. Reprinted with permission from Katona et al. (2012), their Figs. 2c, d, g.
In addition to these improvements, 3D recordings will need to be performed in symbiosis with neuronal network and dendritic modeling; 3D recorded data must be explained by neuronal network and dendritic modeling; and, conversely, measurements must be performed according modeling predictions (Almog and Korngreen, 2014; Chiovini et al., 2014).

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**References**


Simultaneous Holographic Imaging of Neuronal Circuits in Three Dimensions

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Introduction

In this chapter, we introduce a scanless optical method for simultaneously imaging neuronal activity in three dimensions (3D). Using a spatial light modulator and a custom-designed phase mask, we collect light simultaneously (as opposed to serially) from different focal planes and perform holographic calcium imaging of neuronal activity in vitro and in vivo. This method could be used as a technological platform for brain activity mapping.

Optical imaging of neural activity has several advantages over alternative strategies for monitoring neurons, such as patch electrodes and electrode arrays. First, it is minimally invasive and allows for the monitoring of large ensembles of neurons with single-cell resolution (Yuste and Katz, 1991). Also, it is compatible with a large variety of functional and chemical sensors (e.g., voltage indicators, calcium indicator, metabolic sensors), with genetically encoded indicators, and thus can be used for chronic imaging of genetically defined cell populations (Chen et al., 2013).

However, several key weaknesses remain for functional imaging of neuronal cell activity. First, from the very first microscope designed by A. van Leeuwenhoek, image acquisition has been limited typically to a single plane, whereas nearly all interesting biological structures are 3D, requiring sequential scanning for volumetric imaging. Second, as a result of this sequential scanning, the sampling rate over the volume data is slow (normally a few Hz) relative to neuronal activity (1 kHz). Moreover, in highly scattering tissue, although two-photon excitation has afforded single-cell resolution and imaging below superficial layers (Horton et al., 2013), images are normally generated by serially scanning a single beam. More recent attempts have been made to circumvent this limitation (Gobel et al., 2007; Nikolenko et al., 2008; Cheng et al., 2011; Katona et al., 2012), but despite recent advances that increase the image acquisition speed, few proposed strategies are scalable toward meeting the challenge of fast, population voltage imaging with single-cell resolution in scattering tissue in 3D. Such a task would require milliseconds of temporal resolution across wide spatial areas (Alivisatos et al., 2012; Kralj et al., 2012).

Volume Projection Imaging

As one solution to this problem, we propose here a scanless structured illumination microscope coupled to a custom imaging system with wavefront coding. This coded imaging system creates a projection of
an extended sample volume onto a two-dimensional (2D) image plane and is therefore optimized for high-speed, 3D data acquisition. We use a phase-only spatial light modulator (SLM) to create multiple well-defined beamlets of light that illuminate many neurons in parallel (Nikolenko et al., 2008), and then use a phase mask to simultaneously acquire their fluorescence with a camera (Fig. 1a) in what we call Volume Projection Imaging (Quirin et al., 2013). In this technique, cells are not restricted to a single plane and can be freely distributed throughout the 3D sample (Fig. 1b).

While SLM-based multisite excitation has been coupled to wide-field microscopy before, it has been limited to 2D acquisition (Nikolenko et al., 2008; Packer et al., 2012). Although in some samples (transparent, or effectively sparse) single-photon excitation is sufficient, we regard two-photon capabilities as essential in turbid environments and for allowing precise modulation of activity when coupled with optogenetics or caged compounds (Packer et al., 2012).

Operationally, in our method, structural volume data are first acquired with a conventional two-photon microscope in order to identify the cells to be used for SLM targeting. We then generate a hologram that targets the regions of interest (ROIs) in the volume (Quirin et al., 2013) and image the functional activity of these ROIs simultaneously in 3D using a wavefront coded imaging system (e.g., a cubic phase mask) (Dowski and Cathey, 1995; Cathey and Dowski, 2002; Quirin et al., 2013). The deterministic illumination the SLM provides imparts a priori knowledge that can be used to extract additional information from the collected image, as explained below.

For simultaneous 3D signal acquisition, the wavefront coded imaging system enhances the standard shallow depth-of-field while maintaining the full aperture of the objective. This custom imaging system uses a phase-only optical mask in the imaging path to effectively null-defocus (Dowski and Cathey, 1995). This has the effect of greatly reducing the axial dependence of the point spread function (PSF) (i.e., the image is invariant to the 3D target position) and records an aberrated line-of-sight projection of the sample volume onto the image plane. This phase mask is described by the complex amplitude profile,

\[ p(x,y) = e^{i\alpha(x+y)} \]  

where we used values of \( \alpha = 17 \) and 12 for \( M = 20 \times 0.5 \text{NA} \) and \( 40 \times 0.8 \text{NA} \) objectives, respectively, and \( x \) and \( y \) are the normalized coordinates of the microscope pupil (Fig. 1c).

The trade-off for this defocus invariance is a decreased contrast in the focused image (Fig. 1d) (Cathey and Dowski, 2002; Quirin et al., 2013). As a result, the acquired wide-field image exhibits a characteristic blur (e.g., an aberrated Point Spread Function) that is now invariant regardless of the 3D location of the target (Fig. 1e). This blur is nonetheless spatially restricted, and reduction of the out-of-focus PSF size compared with traditional imaging allows for a much higher local spatial density of targeted ROIs (Fig. 1d). Now, 3D fluorescence signals can be simultaneously acquired without loss of contrast owing to out-of-focus collection (Fig. 1e). As an additional benefit, the data volume is projected onto a 2D image plane, thereby reducing the total data throughput required during acquisition. In principle, this image can be digitally restored to diffraction-limited resolution by use of deconvolution methods; however, these steps are not necessary here (Cathey and Dowski, 2002). Instead, we report results using simple functional signal extraction, as described in Materials and Methods.

**Demonstration of the Imaging Technique in Animals**

**Mouse models**

We first demonstrated the system capabilities using a calcium indicator in in vivo mouse hippocampus preparations. Sections of the dentate gyrus were selected for 3D imaging, because this brain structure, with its dense ensemble of cells, provides a challenging opportunity to demonstrate single-cell resolution (Fig. 2a). Recent work has demonstrated the presence and importance of functional cell-cluster activity in 2D imaging conditions of the hippocampus (Muldoon et al., 2013; Ramirez et al., 2013); however, these events have never been observed in 3D. We present here a representative result of the 3D structure, which was first acquired and then used to identify the targets (Fig. 2a). We subsequently targeted all visually identified cells with illumination beamlets by loading the associated hologram pattern on the SLM (Quirin et al., 2013).

In one demonstration, data were acquired for 107 such targets and recorded using an EM-CCD detector at 55 volume projection images per second (VPPS) (Fig. 2b). Individual fluorescence activity from a dense 3D cluster of cells demonstrated that unique signals from individual neurons can be extracted with high signal-to-noise ratio (SNR). Before and after one identified burst, independent activity was observed in each cell body regardless of 3D position, demonstrating cellular resolution. A key advantage of this method is that minute differences in the onset
timing and calcium dynamics can be resolved with high SNR, even at these high temporal sampling rates (Fig. 2C). In slices from older animals, with less dense labeling, we demonstrated this method at sampling rates up to 125 VPPS at single-cell resolution and with high SNR. Cofiring dentate gyrus cells could represent ensembles of hippocampus neurons involved in pattern separation (Muldoon et al., 2013; Ramirez et al., 2013). Our technique thus allows for the visualization of these memory patterns with unprecedented spatiotemporal precision.

Larval zebrafish
We also report on the reconstruction of neuronal activity of the larval zebrafish (P7) with GCaMP5G (Akerboom et al., 2012; Ahrens et al., 2013) to map the temporal record of brainwide calcium transients at high speed in vivo. Different spatial scales can be accessed with our optical technique by simply selecting an alternative microscope objective or modulating the phase aberration present on the wavefront coding element at the pupil (via use of \( \alpha \), in Eq. 1). As a reference illustration, we show conventional two-photon galvanometric scanning images that contain brainwide activity patterns in which both individual cells and neuropil are recruited and fluoresce (Figs. 3a, e) (Ahrens et al., 2013). In recent work, the temporal resolution of such whole-brain activity mapping has been limited by the axial scan speed and the camera frame rate. In contrast, the technique proposed here has no moving parts, and therefore exhibits no mechanical acquisition speed limitation, nor the potential of coupling motion into the sample.

As a test case, 49 total target locations within a 350 \( \times \) 350 \( \times \) 150 \( \mu \text{m} \) volume were selected. Using these targets, a custom hologram was created and loaded to the SLM (red targets, Fig. 3b). With the two-photon SLM illumination, multiple waves of calcium transients were precisely localized as they occurred at 30 VPPS. The monitored active cells in these waves were temporally sorted based on the activity in the first wave, and we observed that this ordering was preserved in subsequent waves, with subsecond precision, despite occurring many minutes later (Fig. 3c). Within the monitored cells, the spatial distribution of the activity was dispersed throughout the sample, with varying modulation strength (Fig. 3d). This is the first demonstration of simultaneous 3D calcium activity imaging in in vivo zebrafish preparations with single-cell precision at a temporal resolution sufficient to resolve the dynamics of synaptic activity patterns.

Although the larval zebrafish is transparent, many organisms are not. Our method, as implemented here, still relies on direct imaging and is adversely affected in highly scattering environments. This has been a challenge for all imaging methods in neuroscience, and the described method is not immune to it. We evaluated the effect of scattering on the PSF used here and believe that this method can be applied up to 2–3 scattering lengths deep, provided that a sparse selection of 3D points is used. However, with the active development of red-shifted indicators and light sources whose longer wavelengths penetrate more deeply through tissue, this method may allow for significant and sizable volumetric imaging with high spatial and temporal resolution. Additionally, with the development of faster SLMs, or additional modulation schemes, temporal multiplexing can be added to augment the selectivity at large imaging depths (Ducros et al., 2013). This could be a critical

![Figure 2. Simultaneous 3D imaging of hippocampal neuronal activity in vitro at 55 VPPS. A, 3D structural data acquired by two-photon scanning image stack. B, 3D imaging capability with single-cell resolution is demonstrated, where 9 of 107 total cells are selected from the 3D volume (boxed region in A) and their respective activity is given in B. Despite simultaneous burst activity in 5 of the neighboring cells, independent calcium transients were measured. The axial location of each cell is given in parenthesis behind the respective fluorescence trace. C, A fine-temporal resolution view of the burst activity highlights the variability in both the temporal and amplitude modulation of the calcium transients. Reprinted with permission from Quirin et al. (2014), their Fig. 3.](image)
improvement, since it would allow for imaging neurons whose projections fall within the same pixels of the camera.

**Materials and Methods**

**Animals**

All animal experiments were performed in accordance with the regulations and guidelines of Columbia University and were approved by the institutional review board of Columbia University. Mice experiments were performed in C57BL mice aged P11–P60 using 400-µm-thick coronal slices loaded with FURA-2AM. Zebrafish experiments were performed at ages P6–P8 and were performed in accordance with the regulations and guidelines of Columbia University. The zebrafish sample is held fixed within a bead of 2% low-melting point agarose to reduce motion artifacts.

**Setup**

The structured-illumination microscope with volume projection imaging uses one laser line for exciting fluorescence of either the calcium indicator dye or genetically encoded calcium indicator (GECI). This laser line is output from a Coherent Mira-HP (~140 fs pulses, 80 MHz) (Coherent, Santa Clara, CA), which can be manually tuned to span an excitation wavelength from 770 nm to 1100 nm. At \( \lambda = 800 \text{ nm} \), the system provides 3.8 W, and at \( \lambda = 920 \text{ nm} \), it provides 2.2 W. The output beam was steered through an electro-optic (EO) modulation device (ConOptics EO350-160, ConOptics, Danbury, CT). A broadband \( \lambda/2 \) wave plate (Thorlabs AHWP05M-980, Thorlabs, Newton, NJ) was located after the EO modulator to rotate the polarization state to be parallel with the active axis of the SLM (XY-512 Spatial Light Modulator, Boulder Nonlinear Systems, Lafayette, CO) located downstream. An LS622 shutter (Vincent Associates, Rochester, NY) was placed in the beam path in order to control illumination conditions. A 1:2 telescope \( \left(f_1 = 50 \text{ mm}, f_2 = 100 \text{ mm}; \right. \) Thorlabs plano-convex lenses) scaled the optical beam to approximately fill the active area of the SLM (XY-512, Boulder Nonlinear Systems). The optical beam was redirected by a periscope to illuminate the SLM. The SLM has a custom lookup table that was experimentally determined at both wavelengths reported for use. The angle of incidence of the illumination beam to the SLM was \( ~10^\circ \). A 4:1 telescope \( (f_3 = 300 \text{ mm}, f_4 = 75 \text{ mm}; \) Thorlabs plano-convex lenses) reduces the image of the SLM onto a set of galvanometer mirrors. The galvanometer mirrors are located conjugate to the microscope objective pupil of an Olympus BX51 microscope (Olympus America, Melville, NY) by use of an Olympus pupil transfer lens \( (f_5 = 50 \text{ mm}) \) and the mounted tube lens \( (f_{TL} = 180 \text{ mm}) \).
Two microscope objectives were tested in the manuscript: an Olympus 20×/0.5NA WI and an Olympus 40×/0.8NA WI objective. To operate in a two-photon scanning mode, Olympus Fluoview was used to control the galvos and image acquisition from a photomultiplier tube (PMT) (H7422P-40, Hamamatsu) located above the microscope. A removable silvered mirror (mounted in Thorlabs LC6W and LB4C) redirects the collected fluorescence emission into the PMT module. To operate in volume projection mode, custom software was developed for MATLAB (MathWorks, Natick, MA) to load the correct gamma map and generate a hologram with manually selected targets throughout the sample volume. When the illumination shutter is opened, the sample is illuminated with a custom pattern that is representative of where the targeted cell soma are located. For volume projection image acquisition, the silvered redirection mirror for the PMT is removed, and the fluorescence emission is passed through an optical relay system with a custom phase mask. The optical relay consists of a 1:1 telescope ($f_4 = 150$ mm, Thorlabs 2” achromatic doublets) with a custom-designed surface relief patterned onto a 1” diameter silvered redirection mirror (mounted in Thorlabs LC6W and LB4C) redirects the collected fluorescence emission into the PMT module. To operate in volume projection mode, custom software was developed for MATLAB (MathWorks, Natick, MA) to load the correct gamma map and generate a hologram with manually selected targets throughout the sample volume. When the illumination shutter is opened, the sample is illuminated with a custom pattern that is representative of where the targeted cell soma are located. For volume projection image acquisition, the silvered redirection mirror for the PMT is removed, and the fluorescence emission is passed through an optical relay system with a custom phase mask. The optical relay consists of a 1:1 telescope ($f_4 = 150$ mm, Thorlabs 2” achromatic doublets) with a custom-designed surface relief patterned onto a 1” diameter quartz plate, which is placed a distance $f_1$ behind the first lens. Note that no amplitude modulation is necessary (i.e., the mask is transparent), which is critical for high-sensitivity imaging. Also note that in the following, the imaginary component describes the surface relief of the mask where

$$h(x, y) = \left(\frac{\omega}{2\pi \text{Re}(p(x,y))} \right) \left(\frac{\lambda}{n-1}\right)$$

and where $h$ is the height, $p(x,y)$ is the complex function describing the pupil, $\lambda$ is the emission wavelength, and $n$ is the refractive index of the substrate (here, we used fused quartz with $n = 1.462$ at $\lambda = 515$ nm). The realized surface relief is representative of a discretized 8-level diffractive optic design of this profile with $\alpha = 200$ over an 18 mm diameter and with an optimal $\lambda = 505$ nm. An imaging detector (Andor iXon Ultra 897 electron-multiplying [EM] CCD, Andor Technology, Belfast, UK) is located in the imaging plane of the 1:1 relay system.

**Targeting calibration, image acquisition, and signal reconstruction**

Precise targeting of the neuron cells was facilitated by a 3D calibration of the SLM projection patterns. A calibration phantom was created using a 2% agarose mixture with a dye solution (yellow highlighter dye) at a 1:1 ratio. Standard projection patterns were input to the SLM, and the captured fluorescence image was used to load onto the SLM for precise targeting.

Images acquired in volume projection mode on the imaging detector were processed using custom analysis software written in MATLAB (MathWorks, Natick, MA). Volume projection acquisition mode consists of generating the hologram illumination pattern, setting the EO modulator for appropriate illumination power, opening the shutter, and acquiring time-lapse images, which were stored as TIFs (image acquisition used an Andor Solis environment, Andor Technology). Because the neurons were relatively large compared with the imaging detector pixel size, pixel binning of $4 \times 4$ was routinely used to improve SNR without any appreciable loss of spatial resolution performance. Using the targeting calibration matrices, the location of each target on the CCD was estimated and an experimentally measured PSF put in its place in order to form a basis set of images: one image for each target. Each frame from the time-lapse stack of images was decomposed into a linear superposition of this basis set using a least-squares fitting, plus one image for background estimation. Formally, this was accomplished by creating a matrix of $N$ images,

$$B = \begin{bmatrix} B_1(1) & \cdots & B_N(1) \\ \vdots & \ddots & \vdots \\ B_1(m \times n) & \cdots & B_N(m \times n) \end{bmatrix}$$

where each column is one $m \times n$ image, lexicographically ordered in a column, representative of the expected pattern from the target location (known from the deterministic illumination). In principle, these images can be given by simulation or found experimentally. Here, we used the experimentally determined PSF and built the library by stamping it at each target location. Given acquisition of the experimental image, $I(t)$, at time $t$ and using
\[ I(t) = B \cdot W(t) + n \quad (4) \]

to describe the image formation, where \( n \) is additive random noise, a least-squares fitting

\[ \hat{W}(t) = \min_{W(t)} \| I(t) - B \cdot W(t) \|^2 \quad (5) \]

quickly yields the individual fluorescence from each target, \( w_i(t) \).

To control for systematic movements of the sample that could have been artificially interpreted as calcium transients, we ruled out the possibility of synchronous deflections in fluorescent transients in either direction. We also performed a positive movement test by gently tapping the sample stage, which resulted in the characteristic synchronous fluorescence signals.

**Conclusion**

In conclusion, we have presented a technique for fast, simultaneous, two-photon optical data acquisition of neuron activity that is distributed throughout three dimensions. This has been demonstrated in multiple animal preparations, both in vitro and in vivo, that are relevant for neuroscience. Both the illumination and data acquisition are simultaneous and can target multiple ROIs throughout the volume of tissue, allowing for parallel activation and imaging. Advances in lasers and SLMs may enable more complex patterns, and faster cameras could accommodate direct imaging of voltage activity in 3D. Also, although we describe two-photon SLM-based multiple beamlet excitation here, any predetermined structured illumination could be used.

The combination of holographic illumination with volume projection imaging appears to us an ideal platform for future work on brain activity mapping.

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Local Circuit Analysis with Integrated Large-Scale Recording of Neuronal Activity and Optogenetics

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Introduction
Numerous methods, such as macroscopic and microscopic imaging, molecular biological tools, and pharmacological manipulations, are available to study brain activity. However, all these indirect observations eventually should be converted back into a common currency—the format of neuronal spike trains—to understand the brain’s control of behavior. Specific behaviors emerge from the interaction of neurons and neuronal pools. Studying these self-organized processes requires simultaneous monitoring of the activity of large numbers of individual neurons in multiple brain regions at the speed of neuronal communication in the behaving animal. Therefore, a major goal is to record from statistically representative samples of identified neurons from several local areas and to interact with them to probe their contribution to circuit function and behavior.

Local circuit analysis depends on three critical components: (1) neuron–electrode interface hardware for large-scale recording of neuronal activity, (2) effective spike-sorting methods, and (3) identification and targeted perturbation of specific neuronal types (Fig. 1). This chapter will present large-scale recording and analysis methods combined with focal optogenetic perturbations that can achieve these goals.

Recording from Single and Multiple Single Neurons
Action potentials produce large transmembrane potentials in the vicinity of their somata. As a result, they can be sensed by placing a conductor (such as a saline-filled glass micropipette or the bare tip of an insulated wire) in proximity to a neuron (Fig. 2). Because neurons of the same class (e.g., pyramidal cells) generate identical action potentials, the only way to identify a given neuron from extracellularly recorded spikes is to move the electrode tip closer to its body (<20 µm in cortex) than to any other neuron.

In general, there are three problems with this spike-amplitude-based approach using “sharp” electrodes. First, moving the electrode too close to the neuron affects its biophysical and firing properties. This is a particularly difficult problem in freely moving animals because even slight movement of the head may affect the discharge pattern. The second problem is related to the first: Because of electrode instability, long-term recordings from the same neurons are not practical with single “sharp” electrodes placed close to a single neuron. The third problem is the difficulty of simultaneously recording from large number of cells. For each neuron, a separate electrode is needed, resulting in excessive tissue damage. Because most anatomical wiring in the cortex is local, the majority of neuronal interactions, and thus computation, occurs in a small volume. In the cortex, this “small volume”
corresponds to hypothetical cortical modules with mostly vertically organized layers of principal cells and numerous interneuron types. For example, in a cylinder of cortical tissue with a diameter corresponding to the extent of the dendritic tree of a pyramidal cell, hundreds to several thousand of other neurons cell bodies are located. Understanding such local computation requires improved methods that would allow for simultaneous recording of representative members of this closely spaced community but with minimal damage to neurons and wiring.

**Unit Isolation by Triangulation**

Monitoring neurons from a distance has a major advantage: stability. A disadvantage is that with only one recording site, signals from many neurons that are the same distance from the tip provide the same magnitude signal, making single-cell isolation difficult. The use of two or more recording sites allows for the triangulation of distances because the amplitude of the recorded spike is a function of the distance between the neuron and the electrode (Fig. 2). Often, this task is accomplished with four spaced wires (dubbed “tetrodes”). Ideally, the tips are separated in three-dimensional space so that unequivocal triangulation is possible in a volume. Unfortunately, in practice the wires are cut flat, providing only two-dimensional (2D) monitoring of neurons, usually perpendicular to the main dipole generated by the action potential.

Cortical pyramidal cells generate extracellular currents that flow mostly parallel to their somadendritic axis. Nevertheless, tetrodes can “hear” hippocampal CA1 pyramidal cells as far as 140 µm lateral to the cell body, although the extracellular spike amplitude decreases rapidly as a function of distance from the neuron. A cylinder with this radius contains ~1000 neurons, which is the number of theoretically recordable cells by a single tetrode. Even so, in practice, only 5–15 neurons can be reliably separated using available algorithms. The remaining neurons may be damaged by the electrode, are silent, or are too small in amplitude, thus preventing reliable separation with only four monitoring sites. Nevertheless, wire tetrodes have numerous advantages over sharp-tip single electrodes, including a larger yield of units, low-impedance recording tips, and mechanical stability.

**Recording with Silicon Probes**

An ideal recording electrode has a very small volume, so tissue injury is minimized. A wire electrode has only one useful part: the conductive tip; the rest consists of just ohmic conductor and inconvenient bulk. However, recording from large number of neurons with many tetrodes is possible only at the expense of extensive cell damage. The desire for large-scale monitoring of neurons from a small volume competes with the tissue damage inflicted by the electrodes.
Micro-Electro-Mechanical Systems (MEMS)–based recording devices can reduce the technical limitations inherent in wire electrodes because, by using the same amount of tissue displacement, the number of monitoring sites can be substantially increased. Whereas silicon probes keep the advantages of tetrode recording principles, they are substantially smaller. Further, multiple sites can be arranged over a longer distance, allowing for the simultaneous recording of neuronal activity in the various cortical layers. Six- to 8-shank probes with 8–32 sites on each probe can record from >100 well-separated neurons (Fig. 3). Because the geometrical distribution of the recording sites is known, the spatial relationship of the isolated single units can be determined. This latter feature is advantageous for studying the spatiotemporal representation and transformation of inputs by neuronal ensembles. Ideally, every part of a probe surface placed in the brain should have monitoring sites. However, this possibility is limited mainly by the width of the interconnect between the recording tips and the extracranial connector (although with the advent of signal multiplexers and on-chip active circuits, substantial volume and weight reduction can be achieved).

A further advantage of silicon probes is their ability to sample the extracellular current effectively (Fig. 4). A requirement for understanding the transformation of inputs by a neuron or neuronal assemblies is information about both their input and output. Unfortunately, no simple method is available for monitoring all inputs at the resolution of dendrites and spines of single neurons in behaving animals. However, membrane currents generated by neurons pass through the extracellular space. These currents then can be measured by electrodes placed outside the neurons. The local field potential (LFP, or more correctly, the mean local voltage) recorded at any given site reflects the linear sum of numerous overlapping fields generated by current sources (current from the intracellular space to the extracellular space) and sinks (current from the extracellular space to the intracellular space) distributed along multiple cells. LFP measurements, combined with recording of neuronal discharges, are an efficient tool for studying the influence of cytoarchitectural properties (e.g., cortical lamination, distribution, size, network connectivity) of neural elements on electrogenesis. However, a large number of observation points, combined with decreased distance between the recording sites, is required to achieve high spatial resolution and for interpreting the underlying cellular events. Recording the voltage gradients by geometrically arranged sites of silicon probes allows current densities to be calculated in order to estimate the mean input to the neuron group in the recorded volume.

Isolation and Identification of Neurons by Extracellular Signatures

An indispensable step in spike-train analysis is the isolation of single neurons on the basis of extracellular features. Spike-sorting methods fall into two broad classes. The first class attempts to separate spikes on the basis of amplitude and waveform variation; the second, triangulation method separates units on the basis of their spatial location. Unfortunately, neither method is satisfactory. When neurons are strongly activated, their amplitude, rise time, decay time, and spike width change as a function of both the membrane potential before the spike and the immediate firing history of the neurons. The spike amplitude variation is most substantial during complex spike-burst production, with as much as 80% amplitude reduction, primarily because of Na⁺ channel inactivation. Therefore, amplitude and waveform-based classification programs will separate action potentials from a single neuron as if they were emitted by many. The amplitude and waveform variability of the extracellularly recorded spike is the major cause of unit isolation errors.

Figure 3. Extracellular contribution of an action potential (“spike”) to the LFP in the vicinity of the spiking hippocampal CA1 pyramidal cell. The peak-to-peak voltage range is indicated by the color of the traces. Note that the spike amplitude decreases rapidly with distance from the soma, without a change in polarity within the pyramidal layer (approximately the boxed area), in contrast to the quadrupole (i.e., reversed polarity signals both above and below the pyramidal layers) formed in the somatodendritic axis. Reprinted with permission from Buzsáki et al. (2012), their Fig. 2d.
The second, triangulation approach is based on the tacit assumption that the extracellularly recorded spikes emanate from point sources. However, every part of the neuronal membrane is capable of generating action potentials, and what is observed in the extracellular space results from the complex geometry of neuron and spike propagation within the neuron. The extent of the somadendritic back-propagation of the action potential varies as a function of the excitatory and inhibitory inputs impinging on the neuron, as well as the state of various other conductances. The spike recorded in the extracellular space sums up the integrated signals from both soma and large proximal dendrites. Thus, the extent of somadendritic spike back-propagation can affect the estimation of the neuron’s virtual “point source” location and may place the same neuron at different locations, resulting in omission errors of unit isolation.

A further problem with the point-source assumption for action potentials is that the somatic origin is not always resolvable with distant recording sites. For example, in the rat neocortex, extracellular spikes can be recorded from the apical shaft of layer 5 pyramidal neurons as far as 500 µm from the cell body. As a consequence, electrodes placed in layer 4, for example, can equally record from layer 4 cell bodies or apical dendrites of deeper neurons. Large neurons in the primate cortex are especially prone to such misclassification errors because their spikes can be recorded from several hundred micrometers from the cell bodies.

To a large extent, these sources of unit separation errors can be reduced by recording at multiple sites parallel with the axodendritic axis of the neurons. However, most triangulation methods visually analyze 2D projected datasets simultaneously. With multiple site-recorded data, successive comparisons of the various possible projections generate cumulative errors of human judgment. As a result, a major motivation behind the development of efficient automatic clustering methods of high-dimensional data is to eliminate cumulative human errors.

The geometrically defined recording sites of silicon probes have additional advantages because, in addition to the output spikes, they can record the mesoscopic LFP signal that reflects the cooperative nature of input and synchronous activity of nearby neurons. With high enough density of recording sites, the afferents to the neurons can be precisely identified in architecturally regular structures (Fig. 5).
After isolating single neurons, the units should be classified into known cell groups of the cortex. This is a very important requirement because cortical networks consist of several neuronal classes, each having a specific computation task. Such classification will greatly improve the interpretation of hard-earned physiological data. The recent advent of optogenetics offers a solution for identifying specific, genetically defined neuronal subtypes in blind extracellular recordings by expressing light-sensitive opsins in a given neuronal population. Both activation and silencing strategies can be used for this purpose while implementing transgenic or virus infection methods. One important task for local circuit analysis is the simultaneous monitoring of all light-responsive neurons near the recording electrodes and estimating the fraction of the optogenetically responsive neurons (Royer et al., 2012; Stark et al., 2012). Integrated recording–optogenetic methods can be used to accomplish at least three goals:

1. Identifying genetically labeled neurons (optical “tagging”),
2. Characterizing and classifying neuron types physiologically, and
3. Testing the causal roles of the identified neurons on the performance of local circuits.

Because neurons are both embedded in circuits and contribute to circuit function, perturbing them can bring about secondary changes that need to be separated from the primary action of optical stimulation. A major problem with most available methods for identifying neurons and analyzing local circuits arises from the use of large-diameter optical fibers for brain surface illumination at high light power. Such technical problems can be significantly reduced by etching small-core (≤50 µm) optical fibers to a point (≤10 µm) and mounting them close (<40–50 µm) to the recording sites (Fig. 6). Hybrid devices that combine silicon probes with etched optical fibers allow the use of extremely low-power stimulation, eliminate photoelectric artifacts, and reduce artificially induced overlapping spikes. Experiments have shown that <5 µW of light (≤0.1 mW/mm²) is sufficient to activate ChR2-expressing neurons in vivo. Synchronous spiking can be minimized by using sinusoid or Gaussian waveform stimulation, as opposed to pulses. That is because neurons that express different amounts of opsins and experience different intensities at varying distances from the light source are expected to have variable thresholds for spike generation and, therefore, respond at shifting phases of the light stimulus.

The low light-intensity requirement can eliminate the need for benchtop lasers and optical cables that restrain the freedom of the animal’s movement. Instead, miniature light emitting diodes (LEDs) and/or laser diodes can be coupled to short, small-diameter (50 µm) multimode fibers and attached directly to the shanks of...
Figure 6. Diode probes for optogenetically identifying interneurons. a, Schematic of a single LED-fiber assembly. The LED is coupled to a 50 mm multimode fiber, etched to a point at the distal (brain) end. Scale bar, 10 mm. b, Left, Schematic of a drive equipped with a 6-shank diode probe with LED-fibers mounted on each shank. Etched optical fibers are attached ~40 mm above the recordings sites on the silicon probe shanks. Right, Picture of the drive depicted on the left. Scale bar, 3 mm. c and d, Local delivery of light. Magnified frontal view of the 6-shank diode probe equipped with diode-coupled optical fibers. c, Two adjacent shanks are illuminated with blue and red light. Scale bar, 1 mm. d, 3 shanks are illuminated with blue light. Scale bar, 0.5 mm. Reprinted with permission from Roux et al. (2014), their Fig. 1.

Figure 7. Unit and LFP recordings from the mouse. A, Chronic recordings from a mouse using an 8-shank 64-site silicon probe (diode probe). 100 ms epochs from each shank are shown. Inset: head stage with silicon probe, microdrive, and 64-channel signal multiplexer, surrounded by copper mesh shielding. The freely moving mouse is connected to the equipment by an ultra-flexible cable. B, Two 4-shank, 32-site probes were placed in the nucleus accumbens (top shanks 1–4) and ventral tegmental area (VTA; bottom shanks 1–4) in a TH-Cre:Ai32 mouse, expressing ChR2 in tyrosine hydroxylase-expressing neurons. One of the shanks in the VTA also contained an optical fiber for light delivery. Note VTA neuronal responses to 472 nm (bottom red trace) laser light stimulation. Reprinted with permission from Berényi et al. (2014), their Fig. 13.
a silicon probe or tetrode (Fig. 6). The small size and light weight of these integrated “diode probes” (1.0–2.5 g) enable fast, multisite, and multicolor optogenetic manipulations in freely moving animals with concurrent monitoring of the manipulated neurons (Fig. 7).

In addition to optogenetics, numerous classification schemes based on a variety of physiological criteria have been developed to assign extracellular spikes to putative interneurons and pyramidal cells and their putative subtypes. These include waveform features, firing rate statistics in different brain states, embeddedness in various population activities, firing patterns characterized by their autocorrelograms, and putative monosynaptic connections to other neurons. However, the “ground truth” of these classifying methods is essentially missing. An important goal of the optogenetic approach is to assist in the identification of distinct subtypes of neurons within individual, molecularly identified classes. However, in a single experiment, optogenetic methods can assist in the identification of only one or
two neuron types. To be able to identify more diverse components of circuits, an iterative refinement of a library of physiological parameters is needed so that, subsequently, the various neurons can be recognized reliably by using purely physiological criteria without the need for optogenetics (Fig. 8).

Dramatic reduction in light intensity, combined with dense recording of the surrounding neurons, allows one to perform single-neuron stimulation and to estimate the absolute numbers of directly driven neurons in a small illuminated volume. Being able to assess the fraction of neurons that can affect a particular physiological pattern, perception, memory, or overt behavior is an important goal of circuit analysis.

References
Extensive and excellent technical reviews, written by various experts in the field, are available for further reading, most of which are listed below.


