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Comprehensive imaging of cortical networks

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Neural computations are implemented by activity in spatially distributed neural circuits. Cellular imaging fills a unique niche in linking activity of specific types of neurons to behavior, over spatial scales spanning single neurons to entire brain regions, and temporal scales from milliseconds to months. Imaging may soon make it possible to track activity of all neurons in a brain region, such as a cortical column. We review recent methodological advances that facilitate optical imaging of neuronal populations *in vivo*, with an emphasis on calcium imaging using protein indicators in mice. We point out areas that are particularly ripe for future developments.

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Introduction

A fundamental question in neuroscience is how information relevant to behavior is processed in neural circuits. Even the simplest perceptual behaviors engage thousands of neurons across multiple regions of cortex [1,2]. In contrast, typical electrophysiological studies sample only a handful of neurons in a single brain area [3]. Moreover, the type of neuron recorded and its position within the neural circuit are typically unknown [4] and long-term recording from the same neurons is inefficient [5]. As a result, the dynamics of neural circuits during behavior and learning are poorly understood.

Over the last decade, cellular calcium imaging has become widely used to image activity in neuronal populations [6]. In most neurons, action potentials (APs) are tightly coupled to large (20-fold) and rapid (rise time, 1 millisecond) increases in intracellular free calcium concentration, which can be used to read out neural activity [7–9]. Calcium imaging samples activity of all neurons in an imaging volume [10] and can readily be combined with visualization of cell type markers to analyze activity in specific nodes of neural circuits [11–15]. With genetically encoded calcium indicators, activity in the same neuronal populations has been imaged across days and weeks [16,17•,18,19••].

Calcium imaging is now routinely used to measure the spatial organization of receptive fields [10,20,21] and to provide a relatively unbiased view of behavior-related activity in populations of neurons [13,22]. Calcium imaging efficiently samples activity in relatively rare cell types [11] and measures changes in neural coding during learning [17•,18,19••,23,24].

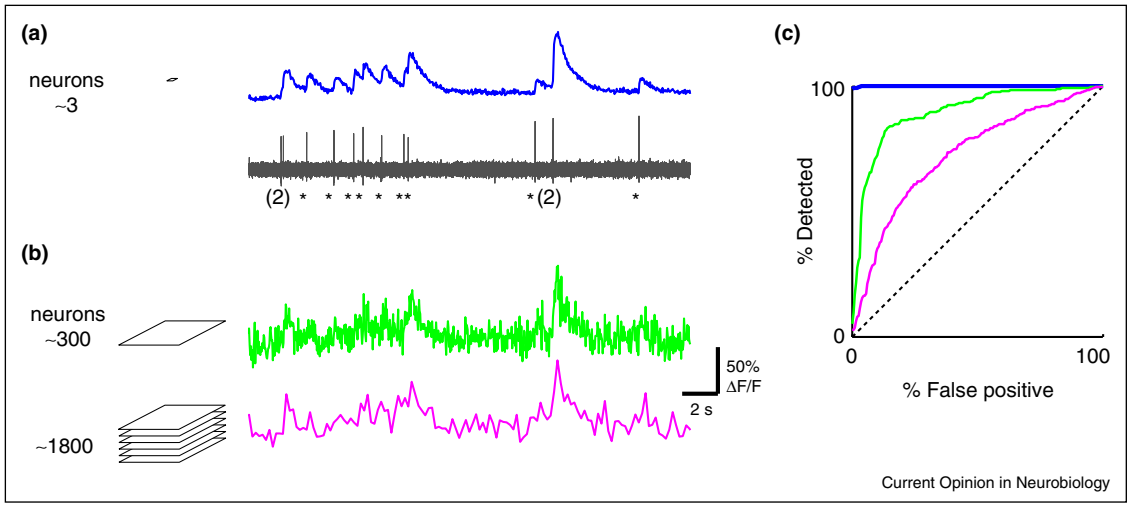
The majority of studies still image only dozens to hundreds of neurons at a time. Here we review the challenges faced by attempts to produce comprehensive activity maps based on large-scale imaging. Our focus is on studies with single cell resolution based on two-photon laser scanning microscopy (TPLSM) in behaving head-fixed mice.

Fluorescent probes for neuronal function

The rapid development of protein sensors for neuronal function has been a major driver of new applications for imaging *in vivo*. In the past, experimenters had to choose between sensitive small-molecule sensors, which need to be loaded into brain tissue using invasive chemical methods [25], and less sensitive protein sensors, which can be delivered using the versatile tools of molecular genetics [26]. Recent efforts in protein engineering [16,27••,28•,29,30••] have boosted the sensitivity of GECIs, allowing them to largely supplant synthetic indicators for imaging neuronal populations. Under favorable conditions, new GECIs detect single action potentials (APs) in the intact brain [27••] (Figure 1a).

Despite these advances, compared to electrophysiology calcium imaging still has several drawbacks. Existing GECIs are not sufficiently sensitive to detect low levels of activity in some cell types, such as parvalbumin-positive interneurons [27••]. The performance degrades as the per-neuron sampling time declines (Figure 1b,c). Furthermore, calcium sensors are slow compared to the precision in neural spike trains [27••,29], limiting the insights that can be drawn about spike timing in neural coding [31,32] and connectivity in neural circuits [33]. Imaging is confined to optically accessible structures, typically less than one millimeter from the surface of the brain or an optical implant (e.g. endoscope). In contrast, electrophysiology has excellent signal-to-noise ratio, dynamic range, time resolution, and spatial reach.

Figure 1



Action potential detectability in imaging experiments. **(a)** GCaMP6s signal (blue, top) and extracellular spikes (black, bottom; single spikes correspond to asterisks) recorded from a single neuron (data from Ref. [27••]). The size of the field of view (FOV, 30 μm square) corresponded to approximately three cortical neurons, imaged at 30 Hz. **(b)** Simulations of different imaging conditions based on the data from panel A assuming identical laser power. Green, approximately 300 neurons imaged at 30 Hz. The signal-to-noise ratio is smaller compared to (a) because the signal is distributed across a larger number of neurons. Magenta, approximately 1800 neurons imaged at 5 Hz. **(c)** Single action potential detection probability as a function of false positive rate for isolate single spike events (ROC curve; colors corresponding to a, b). The ability to detect single action potentials deteriorates as the number of simultaneously imaged neurons increases. In experiments in behaving animals many other factors further degrade detectability, including movement noise, increased fluorescence baseline at higher spike rates, and uncertainty about spike timing.

For these reasons calcium imaging currently occupies a niche complementary to electrophysiology.

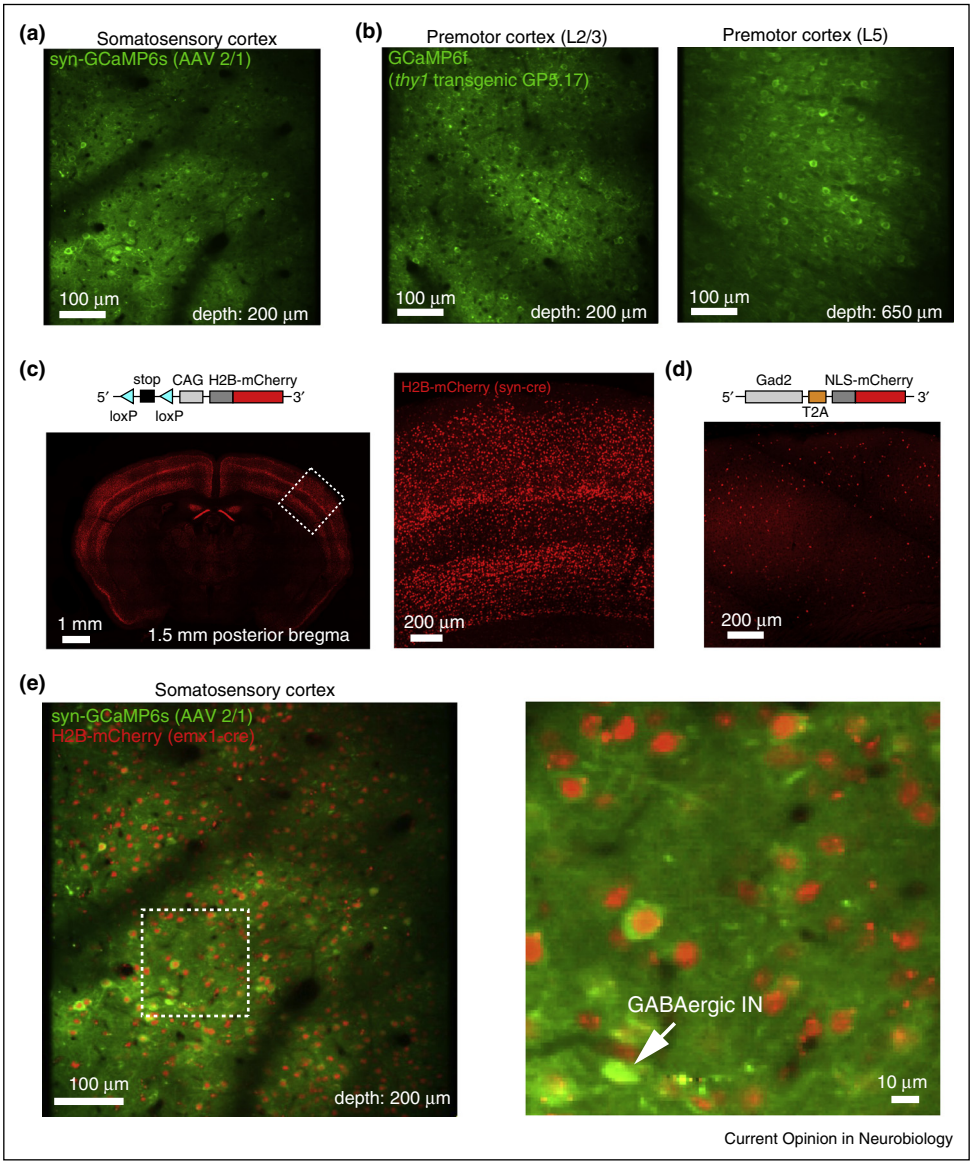
Improvements in proteins sensors will continue to drive advances in population imaging. For example, even more sensitive GECIs would expand the range of cell types amenable to calcium imaging and increase the number of neurons that can be imaged simultaneously. Given that the brightness of state-of-the art GECIs is already high [27••], improvements in signal-to-noise ratio will mainly be driven by increasing the fluorescence change upon calcium binding [34,35]. The most obvious path to better detectability is thus to engineer GECIs with zero resting fluorescence and higher affinity for calcium. Red-shifted GECIs will enable imaging deeper in tissue [36•].

Protein sensors for state variables other than calcium could overcome some of the limitations of calcium imaging for measuring population activity. In particular, protein voltage sensors can be much faster than calcium sensors [37]. But despite recent improvements, their sensitivity for detecting activity at the level of single neurons is still substantially inferior to protein calcium sensors [38–41]. The best-of-class calcium indicators change fluorescence up to 50-fold during physiological changes in intracellular calcium, whereas voltage sensors change less than 2-fold for typical voltage changes. Fundamental biological constraints provide additional challenges for voltage imaging. Calcium can be sensed by molecules distributed throughout the neuronal cytoplasm. Typical expression levels (50 μM) [17•,42] correspond to approximately 10^7 fluorescent molecules in the soma, sufficient to produce a bright signal. In contrast, voltage needs to be sensed across the lipid bilayer, and many fewer fluorescent proteins can be incorporated into the two-dimensional membrane (a typical density for membrane proteins, 10 μm^{-2}, corresponds to 10^4 molecules in the somatic membrane). Intracellular fluorescence contributed by sensors in the endoplasmic reticulum produces non-productive background. Orders of magnitude improvements will be required to make voltage sensors competitive with GECIs for imaging of neuronal populations at cellular resolution.

Gene delivery methods

In the mammalian brain, stable long-term expression of protein sensors for imaging remains challenging. Adeno-associated viruses (AAVs) and other viral vectors can produce the high intracellular GECI concentrations, typically 10–100 μM [17•,42], required for *in vivo* imaging [16,43] (Figure 2a). However, concentrations vary across neurons, within a cell type, and across cell types [19••]. Expression levels continue to rise over months until they cause aberrant cell health [16,27••], limiting the window for GECI imaging to a few weeks, depending on the promoter construct, viral titer, injection volume, type of GECI and other factors. Viral gene transfer also requires challenging surgeries. Best-practice procedures demand

Figure 2



Methods for transgene expression for *in vivo* imaging. **(a)** *In vivo* TPLSM image from a mouse infected with AAV2/1-syn-GCaMP6s in the somatosensory cortex. **(b)** *In vivo* TPLSM images of transgenic expression of GCaMP6f in a thy-1 transgenic mouse (GP5.17, JAX 025393). Left, L2/3; right, L5. **(c)** Transgenic nuclear expression of mCherry. The Cre reporter mouse (R26-LSL-H2B-mCherry; [19], JAX 023139) was crossed to a synapsin-Cre transgenic mouse. Left, coronal section, showing dense expression of mCherry-H2B in the neocortex. Right, blow-up of the somatosensory cortex. (**d**) Expression of nuclear localized mCherry in GABAergic neurons in Gad2 knock-in mice (Gad2-t2A-NLS-mCherry; [19], JAX 023140). **(e)** *In vivo* TPLSM image in an LSL-CAG-H2B-mcherry X emx1-Cre mouse, with AAV2/1-syn-GCaMP6s infection (green). The mCherry expression is restricted to excitatory neurons in cortex, whereas GCaMP6s expression is pan-neuronal. The green GCaMP6 fluorescence in many neurons is low and indistinct with respect to the fluorescent neuropil. The high-contrast images of the red nuclei aid in movement correction, image segmentation, and cell-type specific analysis (white arrow points to a GABAergic interneuron).

tiny injection volumes (5–50 nl) [17•,19••], which can result in variable numbers of infected cells and inhomogeneous expression levels.

In transgenic mice, GECIs can be expressed at constant levels over many months, without any signs of cytotoxicity [42,44•,45,46] (Figure 2b). Expression is reproducible across neurons and individual mice, without invasive surgeries. However, established schemes for flexible reporter expression (e.g. GCaMP3 driven by the CAG promoter targeted to the *Rosa26* gene locus) [47] fail to provide adequate expression levels for cellular *in vivo* imaging [42]. Expression under the *Thy1* promoter [44•] is sufficiently high in some mouse lines. But the labeling patterns depend on transgene cassette integration site and differ in an unpredictable manner across lines.

Selected individual mouse lines typically serve specific narrow purposes. In addition, expression is limited to excitatory neurons.

A promising new strategy is based on a docking site in the transcriptionally permissive TIGRE locus, in combination with tTA-based transcriptional amplification. In this scheme, transgene expression is driven from the tetracycline response element in a cre-dependent manner. Expression of GECIs and other probes can be substantially higher compared to Rosa26-CAG based reporters, sufficient for *in vivo* imaging [48••].

Because the fluorescence change upon calcium binding is a key factor in determining the sensitivity of molecular calcium sensors [34,35], the most sensitive GECIs have low resting fluorescence [27••]. This can make neurons difficult to visualize and segment in the absence of activity (Figure 2a,b). Co-expression of a fluorescent protein with non-overlapping fluorescence emission can address this problem [19••]. Transgenic mice with mCherry [49] targeted to the nuclei of specific cell types can be combined with GECI expression for *in vivo* imaging (Figure 2c–e) [19••]. The red nuclear protein aids segmentation of neurons, movement correction, image alignment across days, and cell-type specific analysis, greatly facilitating *in vivo* imaging.

Microscopy methods

The vast majority of cellular *in vivo* imaging studies have been performed using 2-photon laser scanning microscopy [50]. 2-Photon excitation provides localization of excitation in scattering tissue, which in turn produces three-dimensional contrast and resolution. As a result of localization of excitation, scattered and non-scattered photons both contribute to signal. This greatly boosts the image contrast and signal-to-noise ratio compared to wide-field microscopy, particularly when imaging in scattering media [51].

The principles of 2-photon excitation microscopy demand that individual excitation volumes (on the order of 1 μm^3) are sampled sequentially. The sampling rate is ultimately limited by the fluorescence lifetime of typical fluorophores (3 ns for GFP) [52], which is comparable to the inter-pulse interval for commonly used pulsed light sources (10 ns) [53]. It is therefore in principle possible to sample a cortical column (300 $\mu m \times 300\ \mu m \times 1000\ \mu m$) with its 10^4 neurons at 10 Hz (100 imaging planes, separated by 10 μm, 10 ns/voxel). Two factors currently conspire to make this target unachievable by a factor of ten or more. First, scanning methods (e.g. resonant scanners) are too slow and inefficient. Second, under acceptable illumination conditions and expression levels, best-of-class GECIs are not sufficiently sensitive. Improvements in laser scanning technology (e.g. [54,55]) and GECI performance should make imaging of entire cortical columns with single cell resolution achievable.

Penetration depth of TPLSM is limited by scattering and absorption. TPLSM imaging up to 1 mm deep has been demonstrated [51]. Deeper brain regions can be accessed by removing intervening brain tissue [22,56] or by insertion of endoscopes [57,58] or prisms [59] into the brain regions of interest.

A variety of wave front shaping methods have been proposed to overcome sample-induced aberrations, such as spherical aberrations and astigmatism [60,61,62•]. These methods can dramatically improve signal levels for small structures that are on the order of or smaller than the two-photon excitation volume, such as spines and axons, but have more modest effects on images of larger structures [62•]. Wave front engineering can also correct for strong local scattering with large improvements in signal level at depth [63•]; however, these corrections have to be recomputed for every few micrometers of sample, making this method too slow for most applications.

A promising direction for deep imaging is based on longer wavelength fluorophores. Red and near-IR fluorescence emission is absorbed less by blood [64], yielding improved signal collection when imaging *in vivo*. Furthermore, longer excitation wavelengths are scattered less on the way into the tissue [65]. The penetration depth is therefore significantly better for red and near-IR fluorophores [36•]. Red GECIs with properties comparable to the best green GECIs are on the horizon [66••,67••] and poised to boost the penetration depth of TPLSM imaging significantly.

Three-photon fluorescence excitation has been demonstrated for deep imaging [68]. However, three-photon cross-sections are tiny. As a result very high peak intensities and long pixel dwell times are required for practical image formation. Three-photon excitation microscopy is thus currently too slow for physiological imaging. The high peak intensities required to drive three-photon excitation may also cause rapid photobleaching of the fluorophores and other types of destructive photochemistry [69].

Serial sampling limits the speed of TPLSM. Spurred by new fast and sensitive solid state cameras, wide-field microscopy [70•], light-field microscopy [71], and light-sheet microscopy [72] have been used to measure neural activity in rodents. Compared to TPLSM, scattering and out-of-focus fluorescence rapidly degrade signal and contrast with imaging depth. However, many orders of magnitude more fluorescence is collected simultaneously, and computational methods can potentially overcome some of the signal degradation. In advantageous situations, such as brain areas with sparse activity patterns in space and

time, quasi two-dimensional structures, and sparse labeling, it may be possible to extract signals corresponding to activity of individual neurons [70•]. In optically clear preparations such as the zebrafish, these approaches have allowed sampling of activity across the entire brain [71,73]. An important goal is to establish the correspondence between fluorescence dynamics extracted from these data and neural activity in individual neurons, as has been done for TPLSM [25,27••,28•,74,75].

From images to spikes

Imaging experiments produce stacks of images. Extracting quantitative and robust estimates of spike trains or time-varying spike rate is a complex computational problem.

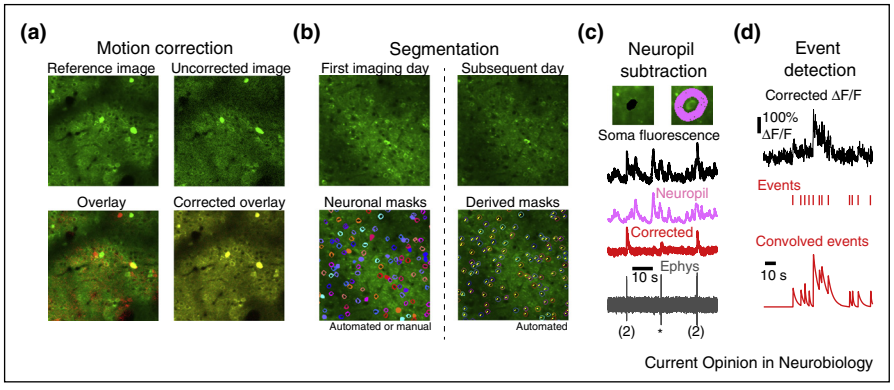
The biophysics of calcium imaging implies that no data analysis trick will provide error-free spike trains. Furthermore, the performance of any algorithm depends on many factors relating to imaging conditions (Figure 1b,c). Even the spike rate of individual neurons matters. For neurons firing at very low rates, false-positive spikes may build to become a substantial fraction of the total detected events. At high firing rates, the elevated baseline fluorescence decreases detection due to larger shot noise and reduced sensitivity if the indicator approaches saturation. We still need a better understanding of the factors underlying sensitivity and dynamic range of calcium imaging.

Extracting quantitative estimates of spike trains or time-varying spike rate from raw images involves multiple complex steps. Low-level analysis starts with movement correction (Figure 3a). Lateral motion usually dominates [76] and algorithms for correcting lateral motion are relatively mature [76,77•,78]. Axial motion correction is computationally expensive and necessitates sampling multiple planes [12]. Methods for closed-loop motion correction may ultimately become necessary.

Segmentation involves separating the signal corresponding to individual neurons from the signal of other neurons and the neuropil [12,27••,75,79•,80] (Figure 3b,c). The neuropil signal arises from fluorophore expressed in unresolved small dendrites and axons [75], as well as out-of-focus fluorescence. Regions of interest (ROIs) can be drawn using either morphological [19••,27••,78], or activity-based [79•,80] algorithms. Whereas morphological approaches are computationally light and potentially unbiased with respect to activity (e.g., when based on structural red nuclear markers [19••]), activity-based approaches can in principle yield more accurate ROI boundaries. A hybrid approach, where morphology-seeded ROIs are refined using activity-based approaches, may be optimal.

Following ROI selection, neuropil contamination must be corrected for. This problem is especially acute when GECIs are confined to the cytoplasm (a relatively thin shell around the nucleus). Because of the limited resolution of 2-photon microscopy *in vivo*, especially axially [81], the neuropil signal substantially bleeds into the somatic signal [27••,81], so that $F_{measured} = \alpha F_{neuropil} + \beta F_{soma}$. The contamination α depends on the point spread function (PSF) and neuronal geometry. Assuming an idealized PSF (Gaussian with $SD_{x,y} = 0.3$ μm and $SD_z = 3$ μm) and neuron geometry (2 μm thick shell surrounding an 8 μm diameter nucleus) implies $\alpha = 0.3$–0.5. Empirically we determined $\alpha = 0.7$–1 to be most effective for removing

Figure 3



Data processing pipeline for imaging data. **(a)** Motion correction is carried out by aligning images from one imaging session to a reference image, collected during an epoch of low motion. In the bottom panels, the reference image is shown in red, whereas the image being corrected is green. **(b)** Image segmentation involves separating the regions of interest (e.g. individual somata) from the rest of the image. Once an image has been segmented, algorithms can relocate the same neurons on subsequent imaging sessions (right). Segmentation is critical for extracting fluorescence dynamics corresponding to individual neurons. **(c)** Subtracting the local neuropil signal from the contaminated somatic signal reveals the actual somatic response. Loose-seal recordings were adapted from [27••]. **(d)** Event detection from the fluorescence dynamics of individual neurons. This provides an estimate of the timing of rapid increases in activity. Events are convolved with their respective amplitudes and decay time constants to produce a de-noised $\Delta F/F$ trace.

neuropil associated fluorescence contamination (Figure 3c; [19••,27••]). This large value for α suggests that out-of-focus fluorescence is more pronounced than expected from PSF measurements [81]. Since the neuropil signal is not spatially uniform (e.g., in mouse somatosensory cortex, neuropil pixel correlation λ is approximately 150 μm) a local neuropil estimate must be employed (Figure 3c) [19••]. The importance of neuropil correction is readily apparent in experiments combining imaging with electrophysiology ([27••]; Figure 3c).

Fluorescence change in ROIs is often reported as neural 'activity'. However, fluorescence is a non-linear function of intracellular free calcium [28•], which in turn is a non-linear function of the recent activity of the neuron [82]. This implies that calcium imaging-based 'tuning' curves of individual neurons are distorted [83] (Figure 4a,b).

The complex relationship between fluorescence dynamics and activity (Figure 4c) makes explicit spike inference desirable (Figure 3d). Most researchers use deconvolution or template matching (e.g. [14,23,84,85,86•,87]). These algorithms do not explicitly account for the non-linear coupling between spikes and fluorescence changes. Because neurons exhibit diverse spike-to-fluorescence transfer functions, one set of parameters will only be effective for a subset of cells. Future spike inference algorithms will have to employ nonlinear transfer functions and need to use neuron-specific parameters derived from fluorescence dynamics alone.

Compared to the rapid advances in experimental methods, computational analysis of imaging data remains in its infancy. Currently used methods are *ad hoc*, slow, poorly documented, and differ across labs, implying that hard-won experimental data are underutilized. A lack of standardization hinders reproducibility and comparison across studies. Moreover, with increasing imaging speeds, data sets have ballooned. A resonant scanning TPLSM produces approximately 100 GB of data per experiment-hour. Nearly complete automation and modern computational methods, such as distributed computing [88•] will have to supplant the semi-manual methods in use today to fully exploit the richness of these datasets.

A principled approach for analysis of imaging data must start with quantitative comparisons of analysis algorithms. This could be achieved in challenges, where computational approaches are evaluated against datasets with ground truth, which are now publicly available (simultaneously detected spikes using electrophysiology [27••]: http://dx.doi.org/10.6080/K02R3PMN; segmentation based on structural fluorescence markers [19••], e.g. Figure 2c–e: http://dx.doi.org/10.6080/K0TB14TN). Benchmarking should be performed in an open-source environment that supports cluster and distributed computing [89]. Benchmarking would stimulate the development of new algorithms and wide adoption of best-of-class methods.

Once spike trains or spike rates have been extracted, they can be mined for insight into the neural mechanisms underlying behavior. The general goal is to relate the activity patterns of neuronal populations to animal behavior. The technical challenges of high-dimensional data analysis are shared with other types of neurophysiology data [90] and are beyond the scope of this review.

Figure 4

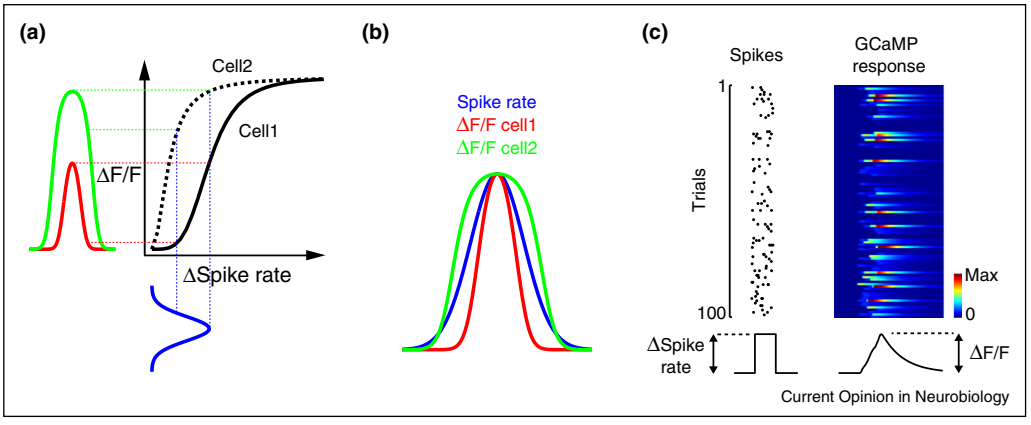


Illustration of potential biases introduced by calcium imaging. **(a)** Response curves for two hypothetical neurons experiencing an identical change in spike rate but having distinct spike-fluorescence transfer functions. At baseline Cell 2 is closer to saturation than Cell 1. This could be because of higher baseline activity or resting calcium concentration. Identical changes in spike rate (blue) would translate into different fluorescence change in Cell 1 (red) and Cell 2 (green). **(b)** The true tuning curve of both cells (spike rate, blue) produces distinct and distorted fluorescence tuning curves (Cell 1, red; Cell 2, green). **(c)** Simulated Poisson spikes (left) and fluorescence transients based on data in [27••]. Simply using $\Delta F/F$ yields a temporally distorted mean response across trials, which deconvolution or spike estimation can ameliorate.

Outlook: towards mesoscale imaging of neural networks

We are on the verge of a new kind of neurophysiology, bridging the gap between single neurons and brain areas. We recently demonstrated comprehensive measurement of behavior-related activity in the superficial layers of several cortical columns, producing a neural activity map comprising more than 10,000 neurons per animal [19••]. Microscopy schemes are in place to image multiple brain regions simultaneously [91•,92]. It will soon be possible to track activity of all neurons in a brain region and measure the interactions between populations in multiple brain areas.

We expect further improvements in proteins sensors to continue to drive advances in population imaging. More sensitive GECIs will facilitate imaging larger number of neurons simultaneously. Faster kinetics will allow estimates of spike trains over a larger range of spike rates. Red-shifted indicators will expand the range of imaging depths. Automation and standardization of image analysis pipelines will be critical to deal with the resulting big data deluge and to convert image data to neuronal state variables.

Conflict of interest statement

Nothing declared.

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