TechSights

Characterizing Cortex-Wide Dynamics with Wide-Field Calcium Imaging

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The brain functions through coordinated activity among distributed regions. Wide-field calcium imaging, combined with improved genetically encoded calcium indicators, allows sufficient signal-to-noise ratio and spatiotemporal resolution to afford a unique opportunity to capture cortex-wide dynamics on a moment-by-moment basis in behaving animals. Recent applications of this approach have been uncovering cortical dynamics at unprecedented scales during various cognitive processes, ranging from relatively simple sensorimotor integration to more complex decision-making tasks. In this review, we will highlight recent scientific advances enabled by wide-field calcium imaging in behaving mice. We then summarize several technical considerations and future opportunities for wide-field imaging to uncover large-scale circuit dynamics.

Key words: **decision making; large-scale cortical dynamics; learning; multimodal recordings; sensorimotor integration; wide-field calcium imaging**

Introduction

The brain is a modular structure in which communication across multiple regions functions to drive behavior and cognition. The emergent properties of such macroscopic interactions cannot be deduced simply by characterizing individual brain regions separately. Therefore, to better understand how the brain functions as a whole, it is critical to record from multiple brain regions simultaneously. Wide-field functional imaging is well suited for this purpose. In systems neuroscience, wide-field calcium imaging has been used to record activity across broad brain areas simultaneously through one-photon excitation (Cardin et al., 2020). Although this technique normally does not resolve single cells, it enables simultaneous capturing of neural dynamics across brain areas with a sufficient spatial and temporal resolution to resolve behaviorally relevant information (for comparisons of various large-scale imaging modalities, see Table 1). This review will mainly focus on macroscale wide-field calcium imaging applied to most of the dorsal cortex in mice. Similar approaches are also called "mesoscale" and "mesoscopic," often emphasizing the spatial resolution that can resolve subregions within individual brain areas but does not achieve single-cell resolution.

Wide-field functional imaging has traditionally been achieved by measuring the "intrinsic signal" or using fluorescent voltage-sensitive dyes. Intrinsic signals are changes in optical reflectance caused by changes in blood volume and oxygenation which correlate with local neural activity (Berwick et al., 2005; Ma et al., 2016b; Mateo et al., 2017). Unlike intrinsic signals, voltage-sensitive dyes serve as direct indicators of neural activity by responding to membrane potential changes; furthermore, they provide a higher temporal resolution owing to their faster kinetics (Orbach et al., 1985; Grinvald and Hildesheim, 2004). Although both approaches have been used to characterize large-scale functional properties of cortex (Blasdel and Salama, 1986; Grinvald et al., 1986; Frostig et al., 1990; Bonhoeffer and Grinvald, 1991; Prechtl et al., 1997; Mohajerani et al., 2010), their ability to capture cortical dynamics is limited because of relatively low signal-to-noise ratio (SNR). Therefore, extracting activity patterns often relies on averaging over repeated measurements, ignoring the variability in moment-by-moment interactions between cortical regions.

In recent years, the application of wide-field imaging in systems neuroscience has been revolutionized with the improvement of genetically encoded fluorescent indicators. These engineered proteins change the fluorescence intensity in response to a variety of neuronal events, including transmembrane voltage, intracellular calcium concentration, vesicle release, and changes in neurotransmitter concentration (Lin and Schnitzer, 2016; Sabatini and Tian, 2020). Among these protein sensors, genetically encoded calcium indicators, especially the GCaMP family (Tian et al., 2009; Akerboom et al., 2012; T. W. Chen et al., 2013; X. R. Sun et al., 2013; Y. Yang et al., 2018; Dana et al., 2019), have become a standard choice to visualize neural activity in both one-photon and multiphoton imaging. GCaMP fluorescence is sensitive to changes in intracellular calcium dynamics that are dominated by action potentials and thus reports neuronal spiking activity with high SNR. Genetic encoding of GCaMP also enables stable expression over time for longitudinal recordings. These advantages of GCaMP allow wide-field calcium imaging to overcome the difficulties often encountered with intrinsic signal imaging and voltage-sensitive dye imaging, making it an attractive approach to characterize large-scale cortical dynamics in behaving animals.

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Table 1. Comparison of several large-scale imaging modalities in mice based on the parameters typically used in recent studies[a]

	Wide-field calcium imaging	Other wide-field functional imaging	Large-scale two-photon calcium imaging	3D volumetric two-photon calcium imaging	BOLD fMRI
FOV	~10 × 10 mm^2 to cover most of the dorsal cortex	~10 × 10 mm^2 to cover most of the dorsal cortex	~5 × 5 mm^2	Varying across different designs and studies, typically ~0.16-1 mm2 × 100-600 μm axial range	Whole brain
Spatial resolution	~10–100 μm/pixel	~10-100 μm/pixel	Cellular or subcellular resolution	Cellular or subcellular resolution	~0.2–0.4 × 0.2-0.4 × 0.5–1.2 mm^3/voxel in recent studies
Temporal resolution/sampling frequency	~30 Hz, actual temporal resolution may be lower (~50-100 ms) because of slow kinetics of some indicators	~30 Hz for intrinsic signal imaging, actual temporal resolution may be lower (~100 ms) because of slow kinetics of intrinsic signals; ~0.1-1 kHz for voltage-sensitive dye imaging	Varying across different designs and studies, ranging from 0.1-7.5 Hz to scan the whole FOV with cellular resolution	Varying across different designs and studies, usually ~10-50 Hz	~1 s
Recording depth	Superficial layers, < ~200 μm	Superficial layers, < ~200 μm	Up to ~600 μm	Up to ~600 μm	Whole brain
Optics/camera/lens requirement	Custom-built or commercial fluorescence stereo microscopes and CCD or CMOS cameras	Custom-built or commercial fluorescence stereo microscopes and CCD or CMOS cameras	Custom-built or commercial two-photon microscopes with wide FOV and random-access scanning	Custom-built or commercial two-photon microscopes with multidepth scanning, using, e.g., deformable mirror, spatial light modulator, Bessel optical module, and variable-focus lens	Commercial systems
Selected references	Makino et al., 2017; Musall et al., 2019; Pinto et al., 2019; Peters et al., 2021	Bauer et al., 2014; Kyriakatos et al., 2017; Kura et al., 2018; Karimi Abadchi et al., 2020	Sofroniew et al., 2016; Stirman et al., 2016; Ota, 2020; Yu et al., 2020	Ji et al., 2016; Nöbauer et al., 2017; Song et al., 2017; W. Yang et al., 2018; Weisenburger et al., 2019; Lu et al., 2020	Schwalm et al., 2017; Schlegel et al., 2018; Jung et al., 2019; Lake et al., 2020

[a]Within each modality, higher sampling frequency can be achieved at the sacrifice of spatial resolution and vice versa.

Several studies have conducted one-photon calcium imaging with GCaMP at a mesoscale level with the field of view (FOV) covering several adjacent cortical regions in adult animals (Vanni and Murphy, 2014; Niethard et al., 2016; Wekselblatt et al., 2016; T. W. Chen et al., 2017; Zhuang et al., 2017). This approach has also been used to investigate the developing circuits in both cortex and subcortical regions (Ackman et al., 2012; Burbridge et al., 2014; Gribizis et al., 2019). Meanwhile, a growing list of studies use wide-field calcium imaging to characterize cortical activity at a macroscopic level with an FOV encompassing most of the mouse dorsal cortex (Fig. 1). Such studies have deepened our understanding of cortex-wide dynamics in various cognitive processes, ranging from relatively simple sensorimotor integration to more complex decision-making tasks (Allen et al., 2017; Makino et al., 2017; Gilad et al., 2018; Musall et al., 2019; Pinto et al., 2019; Shimaoka et al., 2019; Gilad and Helmchen, 2020; Salkoff et al., 2020). In this review, we first focus on recent studies performing wide-field calcium imaging in behaving mice. Using these example studies, we highlight the versatility of wide-field calcium imaging for revealing novel insights into various questions. We then discuss several technical considerations for wide-field calcium imaging. Finally, we discuss future opportunities for the development and application of wide-field imaging to uncover large-scale circuit dynamics.

Propagation of cortical activity in sensorimotor integration

Generating appropriate actions requires integrating sensory information from the environment, and such sensorimotor processing often recruits distributed brain regions to achieve precise sensory perception, action selection, and movement execution. The spatiotemporal dynamics of large-scale cortical activity during sensorimotor transformation have been studied extensively in the rodent whisker system (Ferezou et al., 2007; Matyas et al., 2010; Kyriakatos et al., 2017; Sreenivasan et al., 2017; Gilad et al., 2018). A series of studies using wide-field voltage-sensitive dye imaging has revealed that a single whisker deflection evokes a highly distributed cortical sensory response, starting in barrel cortex and then propagating to primary motor cortex, to drive whisker movements (Ferezou et al., 2007; Matyas et al., 2010; Kyriakatos et al., 2017). The spread of the sensory response is attenuated during active whisking, when the animal's ability to detect weak stimuli is impaired, suggesting that the distributed sensory response is dynamically modulated by ongoing behavior (Ferezou et al., 2007; Kyriakatos et al., 2017). With wide-field calcium imaging, Gilad et al. (2018) further investigated the macroscopic cortical dynamics under different behavior strategies in a whisker-based texture discrimination task with delayed actions to report the choice (lick or no lick). During the delay period between the texture sensation and the chosen action, the activation of different cortical regions, especially the secondary motor cortex (M2) and a posterior cortical region area P, was contingent on the behavioral strategies animals deployed to solve the task. When mice took an active strategy (engaging their body toward the approaching texture), M2 showed sustained activity during the delay period, holding information about the future action. In contrast, in mice using a passive strategy in which they quietly awaited the texture touch, area P displayed enhanced activity during the delay period, holding information about the stimulus identity. Furthermore, optogenetic inactivation of M2 and area P during the delay period led to impairment in behavioral performance during active and passive strategies, respectively. These results support the model that cortical activity can be dynamically routed to different regions to hold the task-relevant information before converging to similar chosen actions (Gilad et al., 2018). It is worth noting that the unbiased observation with wide-field calcium imaging revealed a novel role of area P in texture discrimination. Area P has been mainly implicated in visual processing in previous literature (Garrett et al., 2014; Zhuang et al., 2017), and its function in tactile texture discrimination suggests that it may be generally involved in processing information related to object identity (Gilad et al., 2018). With wide-field imaging, these studies provide the first glimpse of the macroscopic activity pattern during sensorimotor

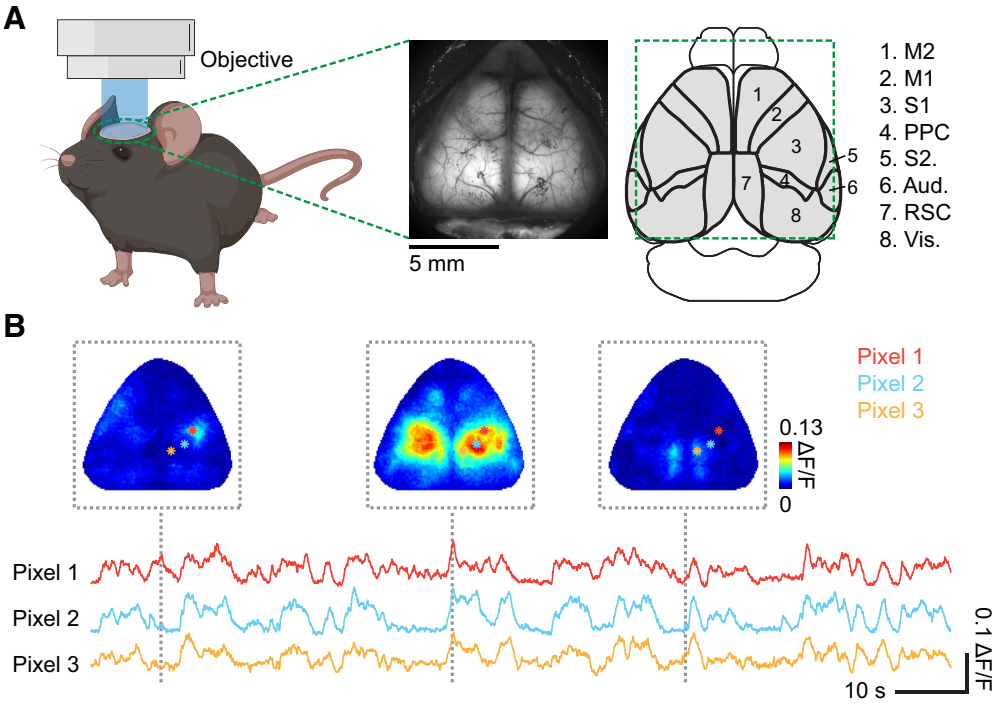

Figure 1. Wide-field calcium imaging of cortex-wide activity. ***A***, Left, Imaging setup. Middle, An FOV of wide-field calcium imaging in a mouse cortex expressing GCaMP6s in cortical excitatory neurons. Right, Cortical regions (based on the mouse brain atlas from the Allen Institute) simultaneously recorded by wide-field calcium imaging. Green dashed box represents the area within the FOV. M2, Secondary motor cortex; M1, primary motor cortex; S1, primary somatosensory cortex; PPC, posterior parietal cortex; S2, secondary somatosensory cortex; Aud., auditory cortex; RSC, retrosplenial cortex; Vis., visual cortex. ***B***, Example cortex-wide image frames and fluorescence traces of individual pixels in a behaving mouse. Gray dashed lines indicate the time of image frames in fluorescence traces of example pixels.

integration and demonstrate its fundamental flexibility even in simple sensorimotor processing.

Distributed encoding of different types of information in cortex

The distributed activation of many brain areas has been observed in various sensorimotor tasks (Goard et al., 2016; Allen et al., 2017; Kyriakatos et al., 2017; Makino et al., 2017; Gilad et al., 2018; Hattori et al., 2019; Musall et al., 2019; Pinto et al., 2019; Shimaoka et al., 2019; Steinmetz et al., 2019; Gilad and Helmchen, 2020; Salkoff et al., 2020); however, systematic optogenetic inactivation generally localizes behavioral effects to only a few regions (Guo et al., 2014; Goard et al., 2016; Allen et al., 2017; Pinto et al., 2019; Zatka-Haas et al., 2020). Therefore, it is important to resolve the information represented in each cortical region and its relevance to the ongoing behavior. Compared with wide-field imaging using intrinsic signals or voltage-sensitive dyes, the higher SNR of wide-field calcium imaging enables a detailed examination of information encoded in cortical activity using regression and decoding analyses on a trial-by-trial or moment-by-moment basis, without averaging out the behaviorally relevant variability (Allen et al., 2017; Gilad et al., 2018; Musall et al., 2019; Pinto et al., 2019; Salkoff et al., 2020; Zatka-Haas et al., 2020). By monitoring a variety of behavioral information and task events encoded in cortex-wide activity, researchers are able to systematically relate behavioral processes to neural activity (Musall et al., 2019; Shimaoka et al., 2019; Salkoff et al., 2020; Zatka-Haas et al., 2020).

Task-relevant information, such as sensory stimuli and choice, is represented in distributed but specific sets of cortical regions, generating distinct cortical activity patterns during task performance (Gilad et al., 2018; Musall et al., 2019; Pinto et al., 2019; Salkoff et al., 2020; Zatka-Haas et al., 2020). Furthermore, the cortical activity pattern is modulated by task demands. Tasks with complex cognitive demands evoked activity profiles that were more different across cortical regions and engaged more spatially distributed information processing in the cortex (Pinto et al., 2019). For example, the encoding of sensory and choice information was more distributed in evidence accumulation or memory-guided tasks than simple perceptual decision-making tasks (Pinto et al., 2019; Salkoff et al., 2020; Zatka-Haas et al., 2020). The widespread cortical involvement in more demanding tasks was further confirmed with optogenetic inactivation (Pinto et al., 2019). These results suggest that the representation of task-relevant information in the large-scale cortical network is dynamically modulated by the cognitive processes required in different tasks, and more complex cognitive processes engage more spatially distributed computations across the cortex.

In contrast to task-relevant information, movement is represented in widespread areas of the dorsal cortex regardless of the task complexity (Musall et al., 2019; Shimaoka et al., 2019; Salkoff et al., 2020; Zatka-Haas et al., 2020) and learning stage (Musall et al., 2019). The widespread dominance of movement-related information in cortex has also been observed in spontaneous activity recorded with two-photon calcium imaging and in electrophysiological data collected from multiple brain regions during task performance (Steinmetz et al., 2019; Stringer et al., 2019). The prevalent encoding of movement can precede movement onset, arising in the primary motor cortex and expanding to the rest of cortical regions before movement (Zatka-Haas et al., 2020), suggesting an origin from efference copy of the motor command rather than sensory feedback generated by the movement. Further investigation revealed that uninstructed movements, which were not required for the task but spontaneously

made by the animals, better explained the trial-by-trial variability in cortex-wide activity than instructed movements and task events. Meanwhile, uninstructed movements could also become correlated with instructed movements and stereotypically occurred around task events, affecting the trial-averaged neural activity (Musall et al., 2019). Although the function of such prevalent encoding of movements, if any, needs further investigation, the profound impact of movements on neural activity has raised the importance of careful behavioral monitoring in the interpretation of neural activity, especially for choices associated with asymmetric motor outputs (e.g., Go/NoGo tasks).

Learning-related dynamics in macroscopic cortical activity

Learning-induced plasticity has been under intense scrutiny with electrophysiological recordings and two-photon calcium imaging (Costa et al., 2004; Peters et al., 2014; Makino and Komiyama, 2015; Grewe et al., 2017). Most of these investigations have focused on the plasticity of local circuits in only one or a few brain regions, omitting one important piece of the puzzle: the interaction across many brain regions during learning. Taking advantage of the stable expression of genetically encoded calcium indicators over time, several recent studies have performed longitudinal wide-field calcium imaging to investigate learning-related macroscopic dynamics (Makino et al., 2017; Musall et al., 2019; Gilad and Helmchen, 2020). Makino et al. (2017) systematically characterized the reconfiguration of cortex-wide activity during motor learning. Consistent with what we have discussed in previous sections, motor learning evoked distributed activation of most of the cortex, forming a macroscopic sequential activity. With learning, this macroscopic sequence of activity during movement execution became more temporally compressed and reproducible from trial to trial, suggesting that more efficient and reliable signal transmission across cortical regions evolves as a function of learning. At the same time, learning rerouted the cortical activity flow. With learning, a novel activity stream originated from M2 and flowed to the rest of the cortex, and the activity of M2 became predictive of the activity of other cortical regions on a moment-by-moment basis. The novel function acquired by M2 during learning was further confirmed with perturbation experiments. Bilateral M2 inactivation with muscimol in expert animals reversed both the cortical dynamics and behavioral performance toward the naive stage of learning, suggesting an indispensable role of M2 in coordinating cortex-wide dynamics for learned behavior (Makino et al., 2017).

The reorganization of cortex-wide activity is not unique to motor learning. Gilad and Helmchen (2020) reported a spatiotemporal refinement of cortex-wide activity flow in an associative learning task, where mice learned to report different textures through licking. At the early stage of learning, task engagement induced a general suppression in association cortices in the interval between the auditory cue signaling the trial start and the whisker-texture touch (the "pre-period"). As learning proceeded, activation increased in rostro-lateral cortex (part of the posterior parietal cortex) and the barrel cortex during the pre-period, building up an anticipatory activity stream arising in rostro-lateral cortex and flowing to the barrel cortex immediately. The specific enhancement of task-related cortical activation emerged in parallel with improved task performance and could contribute to the improved discrimination between different textures (Gilad and Helmchen, 2020). The cortex-wide dynamics observed in different learning tasks demonstrate that the learning-induced plasticity is not only confined to individual cortical regions separately, but also involves cortex-wide changes in the interaction between regions. Such reconfiguration of the large-scale cortical network during learning often involves association cortices and eventually produces more efficient processing of relevant information and more stable representations of learned behaviors.

Multimodal recordings with wide-field calcium imaging

Combining wide-field calcium imaging with complementary imaging modalities

Although wide-field calcium imaging has revealed many novel features of macroscopic cortical dynamics, its current applications are still restricted by two major factors: the lack of single-cell resolution and limited recording depth in brain tissue. These limitations can be mitigated by combining wide-field calcium imaging with other imaging modalities, such as two-photon calcium imaging and fMRI (Barson et al., 2020; Lake et al., 2020). Barson et al. (2020) successfully performed simultaneous wide-field and two-photon calcium imaging in awake mice. To avoid interference between the two imaging modalities, the light path of two-photon calcium imaging was redirected through a microprism mounted on the cortical surface. This multimodal setup is particularly advantageous for investigating the relationships between individual neurons and the entire cortex. For example, Barson et al. (2020) found that the activity of individual neurons in the same cortical region coincided with diverse cortex-wide activity patterns, such that different neurons correlated with different cortex-wide activity patterns. The activity of neighboring neurons can couple with distinct cortical activity patterns, which may arise from different anatomic connectivity. Furthermore, the association between the activity of individual neurons and cortex-wide activity can be modulated by behavioral states (Barson et al., 2020). These results suggest diverse and dynamic associations between local and global neural networks, where information can be dynamically routed depending on behavioral contexts and cognitive processes.

To complement the limited accessibility in the recording depth of wide-field calcium imaging, Lake et al. (2020) combined wide-field calcium imaging and fMRI, which allows simultaneous recording of large-scale cortical and subcortical activity. They found that calcium signals from excitatory neurons partially explained the variance in fMRI BOLD signals. Since the fMRI BOLD signal is an indiscriminate representation of integrated brain activity while wide-field calcium imaging can achieve cell type specificity, this multimodal recording setup could be instrumental in quantifying the contributions of different cell types to the fMRI BOLD signal (Lake et al., 2020).

Combining wide-field calcium imaging with electrophysiological recordings

The relatively simple surgical preparation and imaging setup make wide-field calcium imaging a feasible platform to be combined with electrophysiological recordings. To minimize obstruction of the FOV in wide-field imaging, this combination can be achieved by either inserting a traditional probe (e.g., glass pipette or silicon probe) at an angle (Xiao et al., 2017; Clancy et al., 2019; Peters et al., 2021) or using a flexible transparent probe (Liu et al., 2021). This multimodal recoding setup has been effective in investigating the relationships between cortical or subcortical single-neuron activity and large-scale cortical activity (Xiao et al., 2017; Clancy et al., 2019), as well as the communication between the cortex and subcortical regions (Liu et al., 2021; Peters et al., 2021). Consistent with observations from simultaneous wide-field and two-photon calcium imaging (Barson et al., 2020), multimodal recordings combining wide-field calcium

imaging with electrophysiological recordings revealed that the cortex-wide activity patterns associated with single cortical or subcortical neurons were variable from neuron to neuron and modulated by behavior states (Xiao et al., 2017; Clancy et al., 2019).

A more systematic characterization of the functional mapping between cortex and subcortical regions was recently achieved with the Neuropixel probe, which significantly boosted the sampling power of electrophysiological recordings. By simultaneously recording in the cortex with wide-field calcium imaging and in the striatum with Neuropixel probes, Peters et al. (2021) revealed a topographical mapping between cortical and striatal activity. This functional mapping was consistent with the anatomic corticostriatal projections and independent of the animal's behavior states, suggesting that corticostriatal projections reliably propagate cortical activity to the associated striatal regions regardless of the behavioral state (Peters et al., 2021).

In addition to functional mapping, pairing wide-field calcium imaging with electrophysiological recordings can capture real-time interactions between cortex and subcortical regions. Liu et al., 2021 characterized the coordination between the cortex and the hippocampus during awake hippocampal sharp-wave ripples using a newly developed flexible transparent probe (Neuro-FITM). They found that diverse patterns of cortex-wide activity accompanied sharp-wave ripples. In contrast to the conventional view that cortical activity is mainly triggered by hippocampal sharp-wave ripples, the cortical activation preceded hippocampal sharp-wave ripples in a majority of cases. Furthermore, the ongoing cortical patterns could be decoded from the spiking activity of hippocampal neuron populations, indicating a predictable relationship between cortical and hippocampal activity patterns. These results support the model that the hippocampus and the cortex interact during sharp-wave ripples in a selective and diverse manner at the macroscale (Liu et al., 2021).

Combining wide-field calcium imaging and other recording modalities extends the application of wide-field calcium imaging in at least two aspects. First, it bridges the gap between neural activity at different spatial scales and helps study how local circuits relate to larger neural networks (Xiao et al., 2017; Clancy et al., 2019; Barson et al., 2020). As typical two-photon calcium imaging and electrophysiological recordings often focus on a single brain area, investigations of the relationship between individual neurons and the larger brain network will contribute to a more comprehensive interpretation of local neural dynamics. Second, it compensates for the limited accessibility in the recording depth of wide-field calcium imaging and offers an attractive platform to investigate the dynamics of large-scale neural networks spanning the cortex and subcortical regions during various cognitive processes (Lake et al., 2020; Liu et al., 2021; Peters et al., 2021).

Technical considerations of wide-field calcium imaging

Although wide-field calcium imaging is a powerful tool for monitoring large-scale cortical dynamics and the technique per se is relatively simple to set up using conventional wide-field microscopes, several considerations should be kept in mind in the implementation of wide-field calcium imaging. First, wide-field calcium signals are likely dominated by activity from superficial cortical layers because of the strong scattering of both excitation and emission light in brain tissue. In one-photon excitation, the intensity of excitation light (~480 nm) of GCaMP already drops to ~10% at a depth of 200 μm (Yizhar et al., 2011), suggesting that most signals come from cortical layer 1 and layer 2/3. Second, as wide-field calcium imaging does not possess single-cell resolution, the signal in each pixel is an integration of both somatic and neuropil activity. The latter mainly consists of activity from the dense neuropils in layer 1, including dendrites from local neurons whose somata reside in layers 2/3 and 5, as well as axons innervating these layer 1 dendrites. Although the majority of wide-field calcium signals reflect local activity (Makino et al., 2017), the contributions of long-range axonal projections are not negligible. Soma-targeting of GCaMP would ensure a cleaner representation of local neural activity in future studies (Y. Chen et al., 2020; Shemesh et al., 2020).

In addition, the raw fluorescence signal of wide-field calcium images is contaminated by hemodynamic changes. The excitation and emission wavelengths of GCaMP reside in the absorption spectrum of oxyhemoglobin and deoxyhemoglobin, so changes in blood oxygenation can contaminate measures of GCaMP fluorescence signals. Currently, there are several methods available to correct hemodynamic contamination. For example, using a secondary wavelength of light allows the estimation of reflectance changes caused by hemodynamics, which can then be used for a regression-based subtraction of hemodynamic signals (Ma et al., 2016a; Wekselblatt et al., 2016; Valley et al., 2020). Low-pass filtering of wide-field signals has also been used to reduce hemodynamic contamination, as hemodynamic artifacts are the strongest in the frequency range corresponding to the heartbeat (Vanni and Murphy, 2014; Xiao et al., 2017). Another analytical correction for hemodynamic signals is to extract hemodynamic components using principal component analysis followed by independent component analysis, and reconstructing the corrected wide-field signals from the remaining components that reflect neural activity (Makino et al., 2017). Alternatively or in addition, repeating the same experiments in animals expressing activity-insensitive GFP can be used as a control to test whether the observed wide-field signals are mainly attributable to neural activity instead of hemodynamic artifacts (Vanni and Murphy, 2014; Wekselblatt et al., 2016).

The temporal resolution of wide-field calcium signals is limited by the relatively slow kinetics of existing calcium indicators. For example, GCaMP6f failed to track synchronous population activity beyond 40 Hz (Li et al., 2019). Deconvolution of wide-field calcium signals can improve the temporal resolution. The heterogeneous spiking activity of many neurons contributing to wide-field calcium signals makes it difficult to generate a general deconvolution algorithm, but attempts are being made to provide the ground truth by simultaneous electrophysiological recordings in the cortex during wide-field calcium imaging (Stern et al., 2020; Peters et al., 2021).

Another issue of consideration arises from parcellation methods used to define cortical regions, as different methods can generate very different results (Barson et al., 2020; Lake et al., 2020). The most common method is to segment the cortex based on an anatomic reference atlas (Wang et al., 2020). The advantage of this approach is the consistency across different studies and research groups, making it convenient to compare results from different studies. However, anatomic reference atlases inevitably ignore individual variations in anatomic structures. Such static atlases also fail to track the dynamic organization of functional cortical modules in different sensory and cognitive processes, which may mask real activity features because of imprecise parcellation (Barson et al., 2020; Saxena et al., 2020). An alternative approach is to define cortical regions based on activity and generate a unique atlas for individual animals. Related methods include grouping pixels using clustering analyses (Barson et al., 2020; Lake et al., 2020) and extracting functional modules using

non-negative matrix factorization (Saxena et al., 2020) or independent component analysis (Makino et al., 2017). Compared with anatomic atlases, atlases derived from neural activity can more faithfully represent functional organization of the cortex in individual animals. They may also be able to detect neural dynamics localized to regions that do not correspond to standard areas in anatomic atlases. However, functional modules often vary across individual animals and different studies (Makino et al., 2017; Barson et al., 2020; Lake et al., 2020). Different research groups also use different terminologies to refer to regions in their functional atlases. All these factors make it difficult to compare and interpret results across studies. An open platform that allows researchers to register their functional atlases to a common anatomic framework based on coordinates or certain landmarks (e.g., surface blood vessels) would help comparisons across studies.

Finally, as is common in neural recording experiments, caution is warranted in interpreting cortex-wide activity patterns. Functional connectivity and information flows revealed in recent studies using wide-field calcium imaging were extracted by correlational analyses. In these analyses, whether and how cortical regions are connected and influence each other is unclear. Furthermore, cortical regions exhibiting task-related activity may not actually contribute to task performance (Goard et al., 2016; Allen et al., 2017; Pinto et al., 2019; Zatka-Haas et al., 2020). Combining wide-field calcium imaging with activity manipulations (Allen et al., 2017; Makino et al., 2017) and anatomic tracing (Oh et al., 2014) will provide additional insights into causal relationships between cortical regions and their roles in behavior.

Perspectives

The recent improvements to genetically encoded calcium sensors have resurrected broader interests in using wide-field imaging to investigate large-scale cortical dynamics in behaving animals. As we have discussed above, with wide-field calcium imaging, significant progress has been made to uncover the macroscopic properties of cortical dynamics in various cognitive processes. In the future, we see transformative opportunities for the application of wide-field imaging in the following directions.

Characterizing cell type-specific functions with genetically restricted expression of indicators

The majority of existing studies using wide-field calcium imaging focused on the dynamics of pan-cortical excitatory neurons, but cortical circuits consist of different neuronal types and each carries out distinct functions. For example, cortical excitatory neurons can be further defined by their transcriptomics and anatomic connections, and distinct subpopulations route different information from a specific set of inputs to outputs (Economo et al., 2018; Tasic et al., 2018; Harris et al., 2019). The recent expansion of transgenic mouse lines to target specific subpopulations of excitatory, inhibitory, and modulatory neurons allows genetic targeting of these distinct subpopulations (Madisen et al., 2015; Daigle et al., 2018). By restricting the expression of activity indicators, the monitoring of cell type-specific macroscopic dynamics will dissect the role of different neuronal types and help researchers understand how different components cooperate in cortical circuits at the macroscale. It will also provide valuable datasets for the development of large-scale computational models with cell-type resolution.

Macroscopic dynamics of various neurotransmitters

The nervous system uses a large variety of neurotransmitters and modulators, each of which has unique functions. There has been a recent expansion of toolkits with genetically encoded indicators of various neurotransmitters (Lin and Schnitzer, 2016; Leopold et al., 2019; Dong et al., 2020; Jing et al., 2020; Ravotto et al., 2020; Sabatini and Tian, 2020; F. Sun et al., 2020; Wan et al., 2021; Wu et al., 2020). Wide-field imaging of indicators of specific neurotransmitters/modulators will allow direct tracking of how different molecular signaling is orchestrated at the macroscale. Several pioneering studies have started characterizing cortex-wide patterns of specific neurotransmitters/modulators in spontaneous brain activity (Xie et al., 2016; Lohani et al., 2020). Of particular interest in the future is how different neuromodulatory systems function at the macroscale, because they often project broadly to the cortex and have widespread impacts on behavior and cognition (Avery and Krichmar, 2017).

Expanding toolkits of novel genetically encoded indicators and miniaturized imaging devices

Genetically encoded indicators with enhanced brightness, sensitivity, stability, and faster kinetics will be fundamental to improving the SNR and temporal resolution of wide-field imaging in future studies. Some recently developed indicators for specific neurotransmitters hold promise for applications in *in vivo* wide-field imaging (Feng et al., 2019; Jing et al., 2020; Lohani et al., 2020; F. Sun et al., 2020). Improvements in voltage indicators could enable future wide-field voltage imaging to capture macroscopic dynamics at millisecond resolution with cell type specificity and longitudinal monitoring (Knöpfel and Song, 2019; Piatkevich et al., 2019; Pal and Tian, 2020). Furthermore, indicators targeting specific subcellular compartments (e.g., soma, axon) will help further determine the relative contributions of different sources in wide-field signals (Broussard et al., 2018; Y. Chen et al., 2020; Shemesh et al., 2020). Meanwhile, broadening the color spectrum of genetically encoded indicators could enable simultaneous imaging of different circuit components and investigations of their interactions (Inoue et al., 2019; Montagni et al., 2019). In addition, virtually all studies with wide-field imaging so far have been performed in head-fixed animals. Miniaturized devices for wide-field imaging in freely-moving animals would uncover large-scale neural dynamics in more naturalistic behavioral contexts (Scott et al., 2018; Adams et al., 2020; Rynes et al., 2021).

Activity manipulations with simultaneous wide-field imaging

Little is known about how large-scale cortical networks are influenced by individual brain regions and how they might be able to compensate for loss of individual regions. Such interactions between local and global networks can be probed by combining manipulations on certain brain regions or neuronal populations with simultaneous wide-field imaging of the cortex. This approach can dissect the functions of individual circuit components and their different contributions in various cognitive processes or at different developmental stages. For example, bilateral M2 inactivation with muscimol combined with simultaneous wide-field calcium imaging uncovered an indispensable role for M2 in orchestrating cortex-wide dynamics acquired with learning (Makino et al., 2017). Manipulating activity in specific areas will also help reveal how large-scale cortical circuits adapt to insults and neurologic disorders. Here a careful comparison between acute (e.g., optogenetics, pharmacogenetics, and pharmacology) and chronic (e.g., lesion) manipulation methods would be critical.

Analysis methods

The rich dataset obtained by wide-field imaging brings both challenges and unprecedented opportunities to gain insights into

large-scale cortical dynamics. Sophisticated data-science tools, including dimensionality reduction techniques, will help extract latent and novel patterns from such high dimensional measurements of neural activity and facilitate data-driven discoveries (Williamson et al., 2019). Meanwhile, other analysis tools and computational approaches created for other large-scale recording techniques, such as fMRI and ECoG recordings, can be transferred to wide-field imaging for analyzing network properties (Bressler and Menon, 2010; Rubinov and Sporns, 2010; Pourahmadi and Noorbaloochi, 2016; Cohen et al., 2017). We predict an exponential growth in collaborations between experimental neuroscientists and data scientists to interpret these and other high-dimensional data. For example, creating data-guided circuit models that operate similarly to biological neural networks would provide insights for understanding large-scale cortical networks and in turn guide future experiments (Chaudhuri et al., 2015; Brunton and Beyeler, 2019; Mejias and Wang, 2019).

Conclusion

Wide-field calcium imaging enables large-scale, unbiased observation of many cortical regions with a sufficient spatiotemporal resolution to capture moment-by-moment features in macroscopic neural dynamics. This technique has started to reveal how cortex-wide dynamics support various cognitive processes, including sensorimotor integration, decision-making, and learning. In addition, combining wide-field calcium imaging with complementary recording modalities provides a novel platform to examine the relationship between local and global neural networks and to characterize the interactions between the cortex and subcortical regions. Although several technical considerations still exist, future applications of wide-field imaging together with rapidly growing data science tools will advance our understanding of how different cell types, neurotransmitters, and brain regions cooperate at the macroscale to give rise to perception and behavior.

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