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Optical probing of neuronal ensemble activity

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Neural computations are implemented in densely interconnected networks of excitable neurons as temporal sequences of coactive neuronal ensembles. Ensemble activity is produced by the interaction of external stimuli with internal states but has been difficult to directly study in the past. Currently, high-resolution optical imaging techniques are emerging as powerful tools to investigate neuronal ensembles in living animals and to characterize their spatiotemporal properties. Here we review recent advances of two-photon calcium imaging and highlight ongoing technical improvements as well as emerging applications. Significant progress has been made in the extent and speed of imaging and in the adaptation of imaging techniques to awake animals. These advances facilitate studies of the functional organization of local neural networks, their experience-dependent reconfiguration, and their functional impairment in diseases. Optical probing of neuronal ensemble dynamics *in vivo* thus promises to reveal fundamental principles of neural circuit function and dysfunction.

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Introduction

Animal behavior emerges from neural computations implemented across spatial scales from the microscopic level of synapses to the macrosopic level of interconnected brain areas. At the intermediate 'mesoscopic' level neural information processing occurs in complex microcircuits containing thousands to tens of thousands of excitatory and inhibitory neurons [1]. The dynamic organization of a local population with n neurons can be described by the temporal evolution of the n-dimensional 'state vector', which contains 'ones' for all active, action potential-generating cells, and 'zeros' for all inactive cells [2]. For distinct sensory inputs or behaviors the trajectory of the state vector passes through particular subvolumes of the high-dimensional state space, corresponding to specific sequences of coactive ensembles of neurons that are engaged during particular computational tasks. Additional 'hidden states' (reflecting for example subthreshold membrane potential or second messenger concentrations) dynamically change as well and may significantly influence network dynamics [2]. To understand the principles of microcircuit operation we need to identify coactive ensembles within local neuronal populations and reveal their dynamic properties when they are performing real tasks.

Ideally, one would like to record activity in large neuronal populations with high temporal resolution and during behavior. While large-scale electrical recordings can measure population spiking activity in behaving animals [3] they sample local networks only sparsely and are limited in revealing cell types or spatial relationships. As alternative approach optical imaging techniques are rapidly developing [4,5]. In particular, two-photon microscopy of fluorescent indicators provides new opportunities for measuring the spatiotemporal dynamics of well-identified neuronal populations with single-cell resolution in living animals.

Here we review progress in the field of *in vivo* neuronal population imaging over the past three years. For *in vivo* imaging of glial function we refer to another recent review [6]. Using primarily examples from the mammalian brain we report on advances regarding imaging speed, measurements from 3D volumes, and imaging in awake animals. We highlight recent applications that demonstrate the newly emerging opportunities and discuss remaining future challenges.

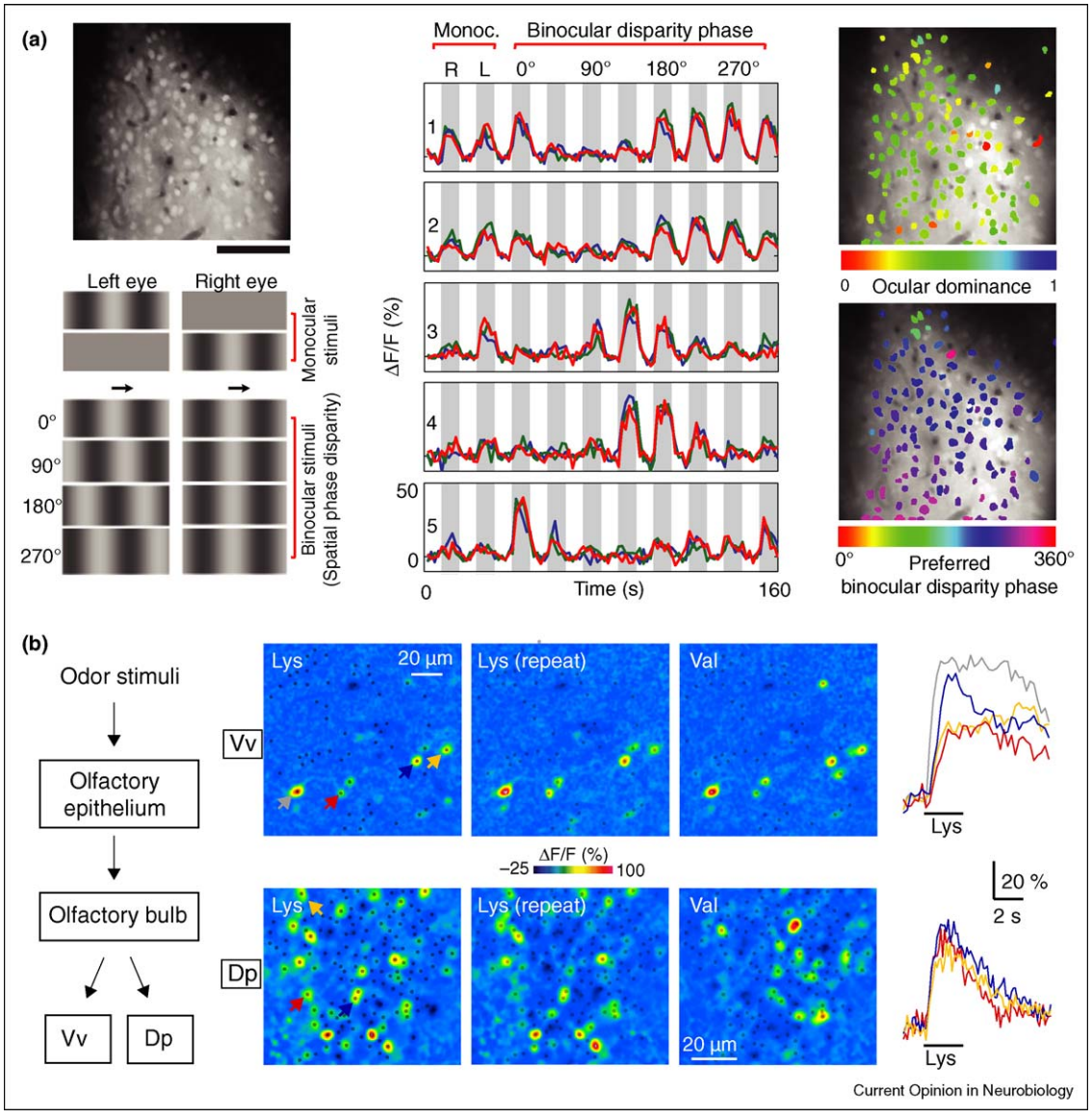
Visualizing neuronal ensembles with calcium indicators

Calcium imaging is the currently prevailing optical method for probing neuronal ensembles *in vivo* [7–9]. Fluorescent calcium indicators report intracellular calcium concentration changes evoked by action potentials and thus infer neuronal spiking indirectly; nonetheless they are advantageous over voltage-sensitive dyes owing to their high dynamic range, good signal-to-noise ratio (SNR), versatile labeling options, and low phototoxicity. A tight correlation between spiking activity and somatic calcium transients has been confirmed by many studies and single action potentials are detectable under favorable conditions [10–13]. During trains of action potentials, individual calcium signals summate so that fluorescence transients reflect changes in spike frequency [14,15].

New *in vivo* labeling techniques have triggered numerous calcium imaging studies, mostly employing two-photon

microscopy owing to its superior depth penetration [16]. A highly successful approach has been bolus loading of cell populations in specific brain areas with traditional synthetic indicators, for example, Oregon Green BAPTA-1 (OGB-1), Fluo-4, or Rhod-2 [17,18]. Recent work includes studies on the zebrafish olfactory system [19,20,21••], on the optic tectum in zebrafish larvae [22•,23], on visual cortex of cats [24••], ferrets [25], and rodents [12,26•,27], and on rodent somatosensory cortex [11,13,28•], motor cortex [29••] and cerebellum [30–32,33••]. Figure 1 illustrates two examples of how population calcium imaging is utilized to reveal sensory coding by neuronal ensembles. The first example shows visually evoked neuronal calcium transients in the binocular region of cat visual cortex [24••]. Responses showed variable tuning across the population with respect to both ocular dominance (OD) and binocular disparity (BD) and the spatial maps for OD and BD tuning were found to have orthogonal orientation (Figure 1a).

Figure 1



Examples of *in vivo* two-photon calcium imaging of neuronal ensembles from **(a)** cat visual cortex [24••] and **(b)** the zebrafish olfactory system [21••]. (a) A cell population in cat visual cortex about 200 μm below the pia labeled with the calcium indicator OGB-1 (upper left, scale bar 100 μm). Example calcium transients in response to monocular and binocular stimuli (lower left) are shown for five cells in the middle column (three trials superimposed). Colored maps of preferred ocular dominance and binocular disparity phase are shown on the right. (b) Odor responses in neuronal populations of two target areas (Vv and Dp) of zebrafish olfactory bulb, bulk-loaded with Rhod-2 as calcium indicator. Color-coded response map for Vv (upper row) and Dp (lower row) upon odor stimulation with lysine (Lys) or valine (Val). Dots in the left images indicate positions of somata. Response maps are reproducible (Lys versus Lys repeat). Overlap between response patterns evoked by different stimuli is high in Vv but low in Dp. Traces show the time course of calcium signals in the somata depicted by arrows. (a) and (b) adapted with permission from [24••] and [21••], respectively.

The second example shows odor-evoked activation of neuronal ensembles in two target areas of the olfactory bulb in zebrafish, revealing distinct transformations of odor representation in these downstream brain areas [21••] (Figure 1b).

Other methods for functional labeling include retrograde uptake of dextran-conjugated dyes, which has been extensively used to study spinal cord circuits [34–36], electroporation [34,37,38], and particularly the use of genetically encoded calcium indicators (GECIs), such as members of the GCaMP, yellow cameleon, or troponin C-based indicator families [39]. *In vivo* application of GECIs has commenced in insects [40], lower vertebrates [35,41], and mice [42,43•,44•] and can be expected to greatly expand in the future.

Gaining speed with fast imaging techniques

In spite of the success of *in vivo* calcium imaging for visualizing neuronal ensemble activity, a number of challenges remain. One crucial issue is the limited temporal resolution. A first strategy to improve imaging speed is to simultaneously excite fluorescence at multiple spots. For example, wide-field illumination, spinning-disk confocal microscopy, or light-sheet illumination techniques [45] together with readout by fast cameras or photodiode arrays nowadays support frame rates of several hundred hertz (for review see [9]). Multi-spot excitation has also been implemented in laser-scanning systems either by splitting the laser beam in multiple beamlets, creating an array of laser foci [46,47], or by creating arbitrary excitation patterns in a 'scanless' approach using a diffractive spatial light modulator [48]. Disadvantages of multi-spot excitation are the reduced laser power available per spot and strong sensitivity to light scattering leading to cross talk between imaged pixels and reduced image resolution. Consequently, depth penetration is limited (<150 μm) and applications so far have been mainly restricted to extracted tissues and slice preparations (but see [46]).

Improving imaging speed with single-focus laser-scanning techniques is more difficult because a tradeoff between speed and the spatial extent of imaging is necessary (the latter determining the maximum number of simultaneously sampled neurons). While line scans enable recordings from a few neurons at kilohertz rate, this rate reduces to a few hertz or less when 2D movies of larger groups of cells are taken (see Figure 1). In this case, a useful strategy to increase acquisition speed is to restrict fluorescence excitation to the structures of interest and minimize background scanning. For instance, standard laser scanning with galvanometric mirrors has been adapted to scan arbitrary free line scans on pre-selected subpopulations of cells [30,49,50•]. In the extreme case galvanometers can be driven hard, near their maximum acceleration, to rapidly move the laser focus from one area to another remote area where it is slowed down again to scan a few cells [50•] (Figure 2). This approach, used *in vitro* so far, should be easily adaptable for *in vivo* measurements.

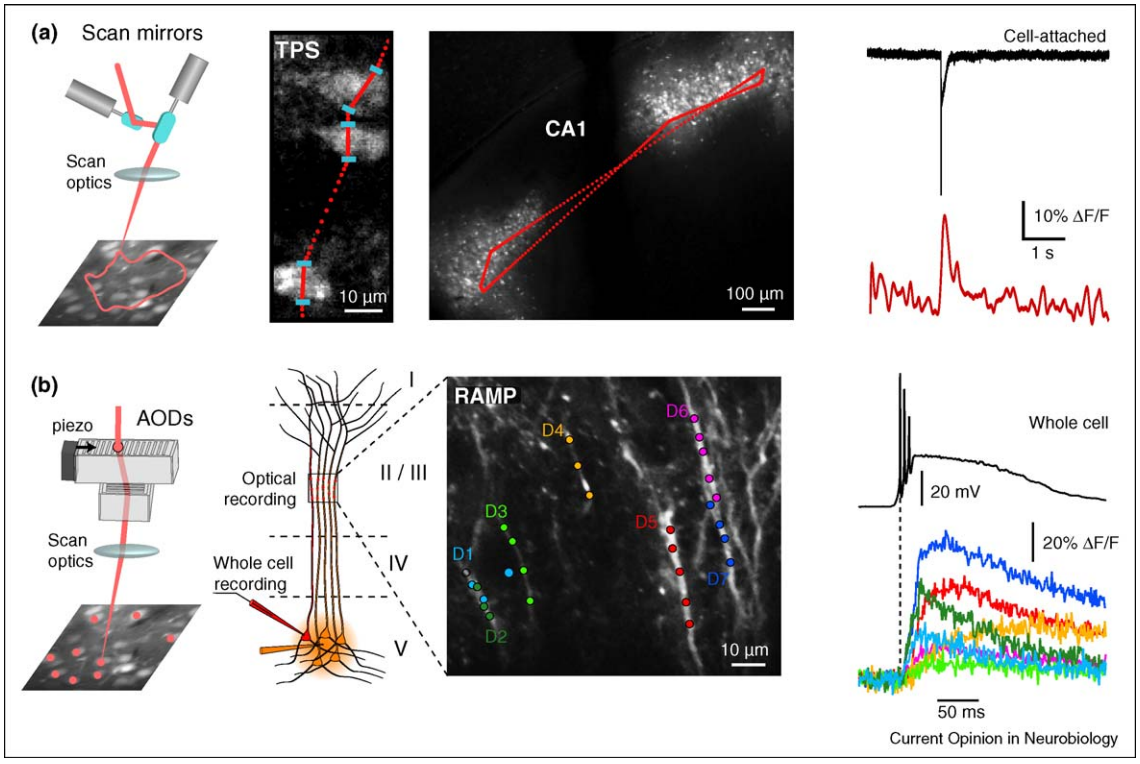
Full and deliberate restriction of scanning to the structures of interest is possible with acousto-optic deflectors (AODs) [51]. Employing acoustic waves in two crossed AOD crystals a laser beam can be deflected with controllable angles in 2D. Owing to the rapid (a few microseconds) AOD transition time between focus positions more than 100 000 points can be addressed per second, enabling kilohertz scan rates for arbitrary sets of preselected positions [52•]. So far AOD scanning has been applied *in vitro*, for example to measure action-potential-evoked calcium transients at multiple dendritic sites in individual cells [52•,53•,54] and in groups of neuronal dendrites [52•] (Figure 2b). Recently, we achieved AOD-based calcium imaging *in vivo*, with single action potential-evoked calcium transients resolved in groups of neocortical neurons with up to 500 Hz sampling rate per cell (Grewe *et al.*, abstract in *Soc Neurosci Abstr* 2009, 484.1).

Towards 3D imaging of large neuronal ensembles

Another goal is to expand neuronal population sampling to three dimensions. Of course, using reproducible stimuli, relatively slow signals can be reconstructed throughout a volume from sequential recordings at different focal depths [19,21••,55]. Eventually, however, comprehensive fast measurements in 3D will be required to obtain a complete picture of local network dynamics on a single-trial basis. Adding a third scan dimension does, however, exacerbate the difficulties of imaging large populations with high temporal resolution. Recently, we introduced a mechanical 3D-scanning approach that combines *x*/*y*-scan mirrors with a piezoelectric *z*-focusing device [56•]. Custom 3D line-scan modes enabled *in vivo* calcium measurements from several hundred neurons at 10 Hz sampling rate within a cubic volume of about 250 μm side length (Figure 3a and b). Even though mechanical scanning is limited by the inertia of the movable components, video-rate recordings seem possible for small volumes. Addition of an extra imaging stage, leaving the front objective stationary and shifting *z*-scanning to a small lightweight mirror in the intermediate optical path [57], might facilitate even faster volume scanning (Figure 3c).

A promising alternative are special arrangements of multiple AODs that allow high-speed inertia-free 3D scanning [53•,58] (Figure 3d). The basic idea is to employ chirped acoustic waves in the AODs to control beam divergence in addition to deflection angle, resulting in a movement of the excitation spot along the *z*-axis. This approach enables random access scanning in a circumscribed volume but is limited to octahedron-shaped

Figure 2



Fast scanning techniques for neuronal population imaging. **(a)** Imaging of extended neural networks in acute hippocampal brain slices using targeted path scanning (TPS) [50•]. Cell populations were bulk-loaded with Calcium Green-1 and scanned with standard galvanometric scan mirrors (left). The scan path was predefined by selection of pairs of points outlining segments of interest (blue markers). Each segment was sampled at a constant velocity, while the intervals between segments were traversed using maximal acceleration and deceleration (middle). Scan rates of ~100 Hz could be achieved in the CA1 region over extended fields of up to 1.1 mm (20× objective). A combined cell-attached voltage clamp recording from one cell (right) shows a single action potential-evoked calcium transient during pharmacologically induced epileptiform activity (fluorescence trace low-pass filtered at 10 Hz). **(b)** Optical monitoring of pyramidal cell network using random access multi-photon (RAMP) microscopy [52•]. Relatively long laser pulses (700 fs) were used on purpose to minimize focus distortions caused by dispersion of the pair of AODs (left). Layer 5 pyramidal cells in cortical slices were bolus-loaded with Fluo-5F and Calcein orange. Seven distal dendrites in layer 2/3 were selected for measurement, colored points indicate dendritic recording sites (middle; point scan rate ~1.8 kHz, 40× objective). A whole-cell recording was performed from one of the loaded cells (right). Optically recorded dendritic calcium transients in different cells synchronized with the electrical response during pharmacologically induced epileptiform activity in the millisecond range. (a) and (b) adapted with permission from [50•] and [52•], respectively.

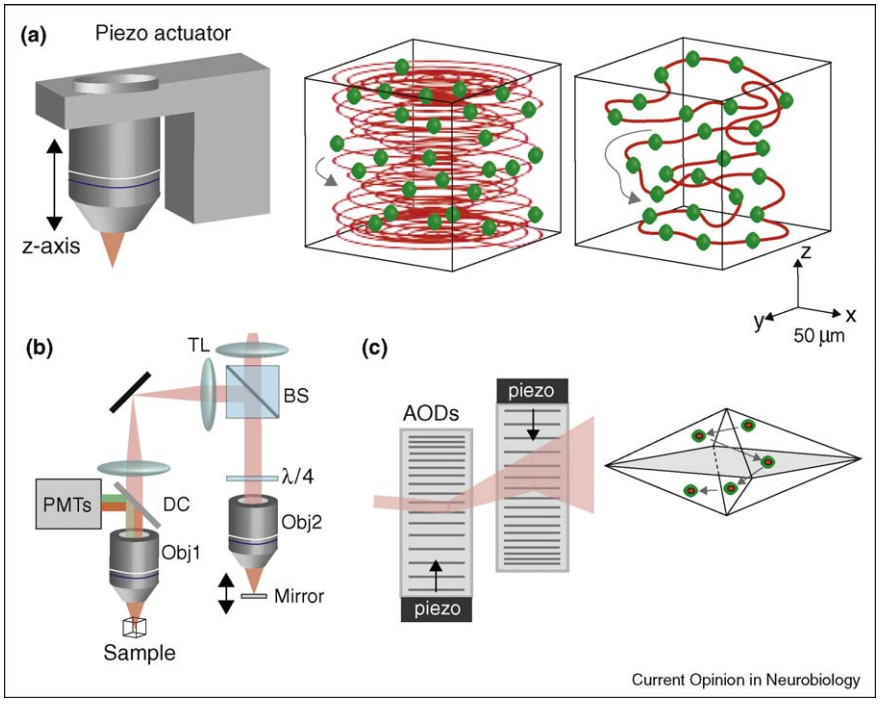
volumes [53•]. Drawbacks of AODs are dispersive effects that need to be compensated and their relatively low diffraction efficiency. Optimizing dispersion compensation and laser beam transmission should make 3D AOD imaging suitable for *in vivo* application.

Cellular imaging in behaving animals

Because neuronal ensemble activity is altered in anesthetized animals it is desirable to perform population calcium imaging in awake, behaving animals. Two main approaches have been further explored (Figure 4). The first strategy is to immobilize the animal or at least its head. Using zebrafish larvae immobilized in agar [59] a recent study demonstrated rhythmic activity in neuronal ensembles in the optic tectum that outlasted repetitive conditioning stimuli, correlating with post-conditioning repetitions of visuomotor behavior [22•]. Similarly, behavior-related calcium signals in neurons and glial cells were imaged in awake head-fixed mice [29••,60] (Figure 4b). Another study on immobilized rats compared calcium signals in the same neurons during wakefulness and anesthesia [12]. The major advantage of the head-restraint approach is that microscopes optimized for *in vivo* imaging can be employed. Despite difficulties such as motion artifacts, time-consuming habituation of animals, and reduced behavioral repertoires, we foresee widespread application of this approach in the near future.

The second principal method is functional imaging in freely moving animals using fiber-optic, head-mounted miniaturized microscopes [61] (Figure 4c). In addition to fiber-optic bulk calcium measurements [62–64], *in vivo* imaging with cellular resolution is now possible using novel lightweight fiberscopes [32,33••,65]. In major

Figure 3



3D laser-scanning approaches. **(a)** Mechanical 3D scanning. A piezoelectric focusing element allows sinusoidal movements of the objective along the optical axis (*z*-axis) with a travel range of up to 400 μm and at 10 Hz rate or higher. Right panels show two options for 3D line scanning of the laser focus, one based on opening and closing spiral patterns in the *xy*-plane (middle), the other realizing a user-defined 3D trajectory through pre-selected cells within a volume [56•]. **(b)** New method of remote refocusing according to [57]. An extra aberration-free imaging stage is added to achieve *z*-focusing by displacements of a small mirror below objective 2. This arrangement should allow for higher *z*-scanning rates while mechanical interference between the objective lens and the specimen is avoided because objective 1 remains stationary. With 2-photon excitation fluorescence photons can be collected with photomultipliers (PMTs) positioned close to objective 1. The quarter-waveplate is used to turn the beam polarization on return by 90°. BS, polarizing beam splitter; DC, dichroic mirror; TL; tube lens. **(c)** 3D random access scanning with AODs. Counter-propagating chirped acoustic waves in a pair of AODs control angular deflection and laser beam divergence, which translates to an axial shift of the focus (one-dimensional case shown). With two such pairs of AODs oriented orthogonally, 3D random access scanning is possible within an octahedron-shaped volume (right).

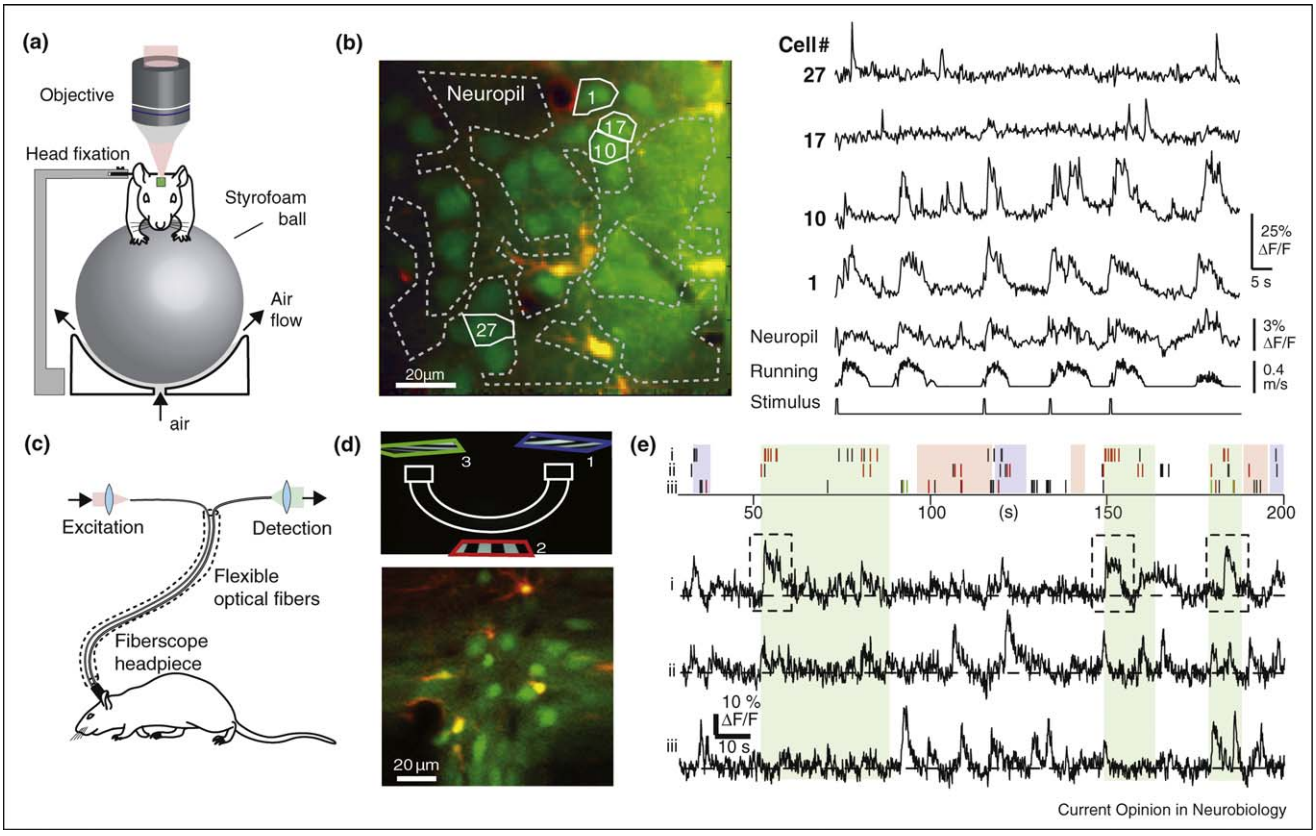
breakthroughs, two groups recently resolved calcium signals in individual cells in freely moving animals. Using a single-photon fiber-bundle fiberscope, one study showed dendritic calcium signals in cerebellar Purkinje cells in mice during locomotion [33••]. Another study succeeded in resolving calcium transients in layer 2/3 neurons of visual cortex in freely moving rats using a two-photon fiberscope [66••] (Figure 4d,e). Although fiberscopes still suffer from lower resolution, reduced SNR, lower penetration depth, and motion artifacts, further technical improvements should alleviate these problems and enable optical probing of neuronal ensemble activity during natural behaviors. In the future, long-term expression of GECIs [42,43•] will greatly facilitate measurements in behaving animals whether immobilized or freely moving.

Challenges for the analysis of calcium imaging data

The tools for fully analyzing network dynamics from calcium imaging data are still developing. A first step is to reconstruct spike trains from the fluorescence recordings, which essentially is a deconvolution of the noisy imaging data presuming elementary calcium transients. Although individual spikes in principle are detectable [10–13,44•,67], noise levels vary considerably, depending on indicator dye, imaging speed, pixel dwell time and other factors. Consequently, single-spike sensitivity is still difficult to reach routinely and has to be verified for each experimental setup. Any improvements in SNR, for example through enhanced fluorescence collection [68], will facilitate more reliable spike detection. Various spike inference techniques are currently being explored for extracting the best estimates of spike trains, in particular when high frequency spiking causes summation of overlapping calcium transients [12,19,45,67,69,70,71]. Improved imaging speed will enable determination of spike times with near-millisecond precision by fitting calcium transient onsets [52•].

Extracting spike patterns from calcium measurements is particularly challenging for awake recordings because

Figure 4



Two-photon calcium imaging with cellular resolution in awake animals. **(a)** Schematic setup for imaging in a head-restraint mouse, which moves on an air-supported styrofoam ball. **(b)** Cell population in sensory cortex labeled with Calcium Green-1 [29••]. Neurons (green) were negative for the astrocytic marker SR101 (yellow). Fluorescence traces for the neuropil and 4 out of 34 neurons after off-line motion correction (right). Running speed and air puff stimuli are also shown. **(c)** Two-photon fiberscope setup that utilizes optical fibers for two-photon excitation and fluorescence detection. Laser scanning is achieved with a miniature scanning-device inside the fiberscope headpiece. (**d**) Two photon 'fiberscope' image of a neuronal population in rat visual cortex after neurons and astrocytes were stained with OGB-1 (green) and SR101 (yellow), respectively [66••] (**d**, lower image). Fiberscope imaging was performed while animals freely explored an elevated, semi-circular ramp with three CRT monitors located at each end and at the apex of the curve that presented fixed orientation patterns (**d**, upper image). (**e**) Example fluorescence transients (bottom) and raster plots determined from an action potential detection algorithm (top) showing the activity in 3 neurons (denoted i, ii and iii) during ~ 3 min of continuous recording (black lines indicate single action potentials, red lines doubles and green lines triples). Periods where the animal gazed at one of the three monitors are indicated by blocks of different color (see monitor color coding scheme in **d**). Note large transients in neuron i in response to viewing the same monitor multiple times (dashed boxes). (**b**) and (**d**, **e**) adapted with permission from [29••] and [66••], respectively.

motion artifacts can distort cellular signals. Laser scanning leads to complicated artifacts because pixel values are separated in space and time so that image distortions cannot be reversed off-line by simple geometric transformations. The chief goal is therefore to mechanically stabilize the tissue using agar or transparent rubber pieces, at least minimizing focal plane changes [29••]. Remaining lateral movements can then be corrected offline using for example a Hidden–Markov model [29••] or a Lucas–Kanade image registration algorithm [72]. In the future online adjustment of scan signals might enable automatic stabilization of optical recordings.

Another important challenge is to discriminate different cell types, especially subnetworks of inhibitory interneurons to investigate how network activity is delicately balanced under various conditions [1]. Subtypes of cells may be identified *in vivo* using specific fluorescent markers, for example, genetically targeted GFP expression [21••,73•,74], or post mortem via histological analysis. Because calcium handling in some GABAergic neurons differs from excitatory neurons, the relationship between action potentials and evoked calcium transients will need to be assessed independently.

Once spike trains have been reconstructed from fluorescence recordings, they can be analyzed analogous to electrical recordings. For example, cross-correlation analysis of cellular responses can help to identify neuronal subensembles [11,12,21••]. Furthermore, it should be possible to analyze the temporal dynamics of the network state vector, in particular to what degree state vector

trajectories differ for distinct computational tasks. For visualization of high-dimensional network dynamics dimensionality reduction methods such as principal component analysis or locally linear embedding can be used [3,75]. These analysis techniques will become increasingly important with improved imaging speed and increased size of populations sampled.

Future directions

The advances summarized above create new opportunities for the investigation of neuronal ensembles *in vivo*. Experience-dependent reconfiguration of neural networks is thought to be a central mechanism of learning and plasticity. With the novel methods one can now dissect functional changes in neuronal circuits during development or following plasticity-inducing protocols. For example, in mouse visual cortex the fraction of neurons contributing to spontaneous activity was found to decrease during postnatal development [27]; in addition population calcium imaging revealed a switch from highly synchronized to more desynchronized states in mouse cortex over the first postnatal weeks [27,76]; in ferrets, early training with moving stimuli directly after eye opening accelerated the emergence of direction-selective cells in the visual cortex [25]; and monocular deprivations in mice caused changes of eye-specific responsiveness in neuronal populations [26•]. Microscopes with improved imaging speed might enable studies of plasticity effects that depend on millisecond-precise relative timing of neural spikes. Optical studies of network reconfiguration are likely to expand as soon as repeated functional imaging of the same network becomes routinely possible, for example through long-term expression of GECIs using transgenic approaches [40–42], viral delivery [44•], or *in utero* electroporation [43•]. A first study demonstrated chronic imaging of the same neurons in mouse cortex over days and weeks [43•].

A number of studies have started to use *in vivo* population calcium imaging for investigating network dysfunctions in mouse models of brain diseases. For example, following an ischemic damage in somatosensory cortex the limb selectivity of calcium signals in individual neurons was first reduced while responses became more selective for a preferred limb at later stages [28•]. In a two-photon calcium imaging study on Alzheimer's mice, a redistribution of spontaneous neuronal activity was found with hyperactive neurons appearing exclusively in the vicinity of amyloid plaques [77•]. Similarly, pathological effects on glial cells have been investigated in disease models [78,79]. This type of studies promises important novel insights into the alterations of neural network dynamics in various brain diseases.

Conclusion

In summary, emerging optical techniques are revolutionizing the study of neural dynamics on the mesoscopic scale, bridging the gap between the cellular level and the level of communicating brain areas. Our review covered only certain aspects of current developments focusing on the rapidly advancing field of *in vivo* calcium imaging from neuronal populations. In parallel, the complementary field of optical control of neural circuits using light-activated ion channels is developing at similarly rapid pace [80]. Moreover, novel techniques for high-resolution anatomical reconstructions of large tissue volumes promise to reveal detailed wiring diagrams of neural microcircuits [81,82]. With these developments coming together it no longer seems unrealistic to directly observe (and manipulate) neuronal ensemble dynamics in behaving animals and to relate it to the underlying wiring scheme. This powerful convergence of matching methods no doubt will help to uncover fundamental principles of network dynamics in the brain.

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Using two-photon calcium imaging the authors analyzed transformations of ensemble activity patterns between the zebrafish olfactory bulb and two of its telencephalic targets, Vv and Dp, to examine higher-order processing of smells. While odor responses in Vv populations were broadly tuned and showed overlapping odor representations, pattern processing in Dp was more complex and odor-specific, presumably establishing representations of odor objects.

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This two-photon imaging study reports rhythmic activity among specific neuronal ensembles in the zebrafish optic tectum, which retained the memory of repetitive sensory stimuli for a duration of up to 20 s after stimulation. Following conditioning visual stimuli the reverberating neuronal ensemble activity was reflected by motor behaviour of the zebrafish larvae in the form of post-stimulation rhythmic tail flips.

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This study investigated the effect of local brain damage on the functional properties of neuronal and glial populations in the somatosensory cortex of adult mice. Functional mapping with intrinsic optical imaging was combined with cellular imaging using two-photon microscopy. Disrupted forelimb and hindlimb representations by targeted ischemic insults altered neuronal response properties such that neurons initially showed diminished limb selectivity but after two months developed higher selectivity for a preferred limb in the center of the reorganized functional areas.

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