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Electrophysiology in the age of light

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Electrophysiology, the 'gold standard' for investigating neuronal signalling, is being challenged by a new generation of optical probes. Together with new forms of microscopy, these probes allow us to measure and control neuronal signals with spatial resolution and genetic specificity that already greatly surpass those of electrophysiology. We predict that the photon will progressively replace the electron for probing neuronal function, particularly for targeted stimulation and silencing of neuronal populations. Although electrophysiological characterization of channels, cells and neural circuits will remain necessary, new combinations of electrophysiology and imaging should lead to transformational discoveries in neuroscience.

More than 200 years ago, Galvani discovered that the function of the nervous system is intrinsically linked to electrical activity[1] (Fig. 1). Since then, generations of investigators have invested immense effort into building instruments capable of measuring and controlling this electrical activity. The unprecedented sensitivity and temporal resolution of modern electrophysiological tools allow us to study the properties of single ion channels and more complex phenomena extending to the activity of hundreds of cells within networks of neurons (Fig. 2). Thus, electrophysiology embraces all levels of our understanding of nervous system function, from the molecular to the behavioural.

By using metal, glass or silicon electrodes to record electrical signals associated with ion fluxes across neuronal membranes, electrophysiology allows us to listen directly to the 'language' of neurons at an extremely high signal-to-noise ratio. This is the main strength of the method, because electrical activity is recorded directly, without the need for a 'translator', that is, a probe that transforms electrical activity into a different signal. However, this is also the main weakness of electrophysiology, because electrical access to the nervous system necessitates physical contact with the tissue under investigation.

The use of light to probe neuronal function avoids the need for direct physical contact with the tissue, because light can travel through biological membranes. Furthermore — with a few exceptions such as the retina, where specific wavelengths need to be used — light does not interfere with neuronal function. Importantly, light offers numerous additional crucial advantages (Fig. 2): outstanding spatial resolution, allowing signals in even the smallest neuronal structures to be resolved[2,3]; the possibility for simultaneous measurement from a wide range of spatial locations[2,4]; and — with the use of genetically encoded and targetable probes — the critical advantage of being able to access specific cellular subtypes and subcellular domains[5].

To investigate neuronal function using light, however, a reporter is required. This typically takes the form of a molecule that converts membrane potential (or its consequences) into an optical signal. Thus, optical measurements are necessarily indirect. This means that careful calibration is required for quantitative measurements, and the properties of the molecular reporters and optical detection systems can limit the temporal resolution of functional measurements. However, owing in part to advances in our understanding of the biophysical, molecular and genetic mechanisms of excitability and synaptic transmission, the development and refinement of optical reporters is occurring at a very high pace. By harnessing these mechanisms, it is possible to develop optical reporters to monitor previously inaccessible variables with unprecedented spatial resolution and limited invasiveness (Table 1). In combination with new forms of microscopy, these reporters have been used to make a wide range of discoveries about nervous system function, including the quantitative link between presynaptic calcium and neurotransmitter release; the degree of chemical and electrical compartmentalization of dendritic spines[6]; the spatial extent and plasticity of dendritic spikes[7]; the spatial organization of cortical maps on the level of individual neighbouring neurons[8]; and functional map topography in the visual system[9]. Together, these discoveries herald a revolution in the way we investigate the function of the nervous system.

Does this revolution spell the end of electrophysiology? In this Review, we debate the respective strengths and weaknesses of electrophysiological and imaging approaches in neuroscience, and identify those applications in which optical approaches surpass, or will probably soon surpass, electrophysiology for both the monitoring and the manipulation of neuronal function. We also describe applications in which electrophysiology will remain necessary, and point towards important future developments. Finally, we highlight how, by being used in concert, these two techniques will lead to transformational discoveries in neuroscience.

The fundamentals

A fundamental consideration when comparing electrophysiological and optical approaches is the signal-to-noise ratio that can be achieved for a particular measurement. In electrophysiology, the noise sources are primarily instrumental, and the signal-to-noise ratio has been maximized by developing low-noise headstages and amplifiers, as well as by the gigaohm seal of the patch-clamp technique[10], which provides sufficient signal-to-noise ratio to allow resolution of the opening of single ion channels. In imaging, the measured signal — photon flux — results from conversion of the electrical (or chemical) signal into photons by means of a reporter, and is normally observed as a change in fluorescence (although some indicators use a change in reflectance, absorbance or even the ratio of two different fluorescence processes). By contrast to electrophysiology, in which electrons associated with the signal are typically plentiful, only a small number of photons are usually detected in optical measurements from small structures. If this number becomes sufficiently low, fluctuations around the mean become relatively large, a phenomenon called shot noise, and the signal-to-noise ratio of the measurement drops. To illustrate the contribution of shot noise, we will use the most common case, namely a change in fluorescence, which is

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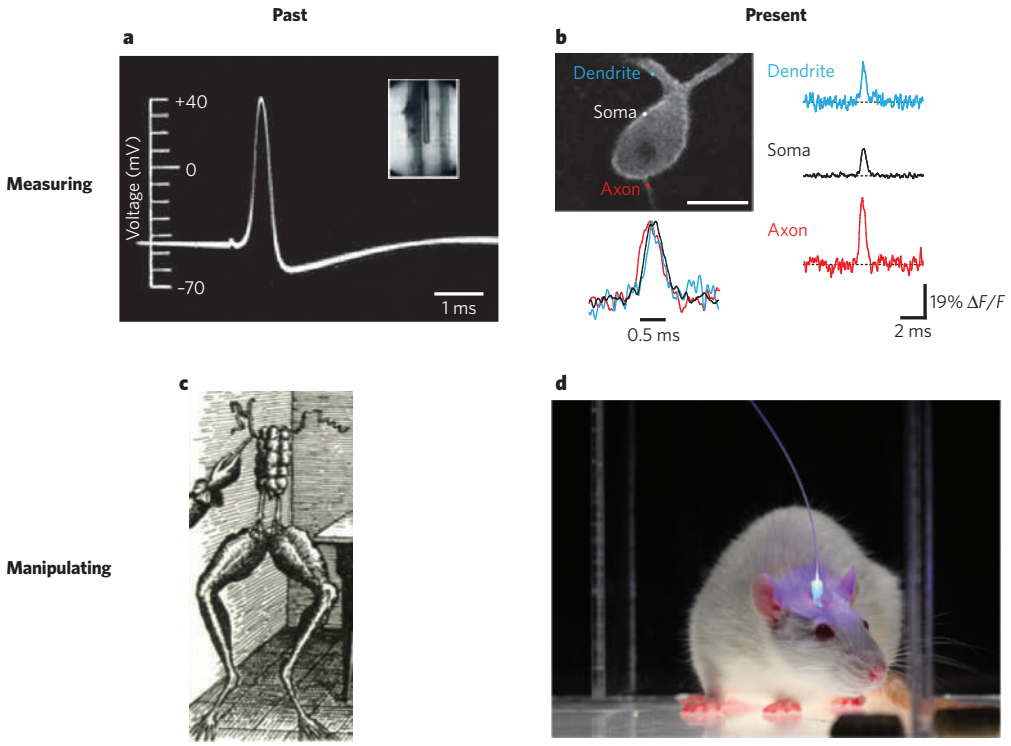


Figure 1 | Recording and stimulation: past and present. a, The first action potential recorded intracellularly from a neuron, by Hodgkin and Huxley[91]; inset, the electrode inserted into a giant squid axon. **b**, The multisite optical recording of action potentials in a cerebellar Purkinje neuron by using voltage-sensitive dyes[22]. **c**, The electrical stimulation of a frog nerve as illustrated by Galvani[1]. **d**, Optical deep-brain stimulation of neurons expressing microbial opsin genes. (Courtesy of V. Gradinaru, M. Mogri, J. Carnett and K. Deisseroth, Stanford University, California.)

measured as $\Delta F = F_{peak} - F_0$, where F_0 is the baseline fluorescence and F_{peak} is the above-baseline peak of the fluorescence transient associated with the electrical (or chemical) transient. The signal-to-noise ratio (SNR) of the measurement is thus proportional to the ratio of the signal change, ΔF, and the baseline shot noise ($F_0/\sqrt{N}$, where N is the number of photons detected): SNR $\propto (\Delta F/F_0)\sqrt{N}$.

Therefore, to optimize the signal-to-noise ratio, it is crucial to maximize both the change in fluorescence relative to the baseline and the total number of photons generated by the conversion of the electrical or chemical signal and detected by the instrumentation. Increasing the number of emitted photons can be achieved simply by increasing the number of reporter molecules (that is, the effective concentration of the reporter; however, at high concentrations there is a risk of perturbing the biological system studied and reducing $\Delta F/F_0$) and by improving reporter efficiency. Furthermore, improvements in excitation and detection efficiency — particularly with the use of two-photon microscopy[11], in which excitation is highly restricted in space and thus can be targeted to the source of the signal, and in which all collected photons can be used to form an image[12] — can also significantly improve the signal-to-noise ratio. Because the signal-to-noise ratio is linearly dependent on $\Delta F/F_0$, and only on the square root of N, optimizing reporter properties to maximize $\Delta F/F_0$ is in general a better strategy than simply increasing photon counts. In practice, additional considerations are important when choosing a reporter, such as photobleaching (which effectively reduces reporter concentration) and photodamage (which can affect physiological processes and cell health, and constrains the amount of excitation light that can be used and, thus, photon counts). Finally, electrical signals in neurons can be very rapid (<1 ms), which places further severe constraints on the kinetics of the reporter, and also on the detection system, with rapid scanning technology (together with improved collection optics and highly efficient detectors, such as those using photon counting) being required to faithfully record signals across single neurons or across networks[4]. Next we discuss how these principles and limitations apply when optical methods are used to record different kinds of electrical signal.

Measuring voltage

The primary language of neurons is electrical and involves changes in membrane voltage. The relevant fluctuations of membrane voltage associated with different electrical signals span three orders of magnitude, from ~100 μV (for quantal synaptic potentials) to ~100 mV (for action potentials), and the underlying currents can exhibit time courses of microsecond duration (Fig. 2). Being able to record these signals accurately is fundamental for our understanding of the computations performed by neurons. Developing optical approaches with the requisite sensitivity, dynamic range and speed would represent a tremendous advance over electrophysiology and would allow measurement of the spatiotemporal dynamics of membrane potential over the entire cell or across a population of neurons, information that is difficult or impossible to obtain using electrodes. Early attempts to use imaging to record activity focused on detecting changes in intrinsic optical properties of neurons, such as changes in light scattering correlated with membrane potential[13]. Although it was ultimately successful for low-resolution (~100 μm) imaging of brain function[14], this approach suffers from an inadequate signal-to-noise ratio for cellular imaging. More recently, there has been an intensive effort to develop optical reporters for recording membrane voltage, in conjunction with new developments in laser-scanning microscopy[4,15].

Let us be clear about the ultimate goal: to be able to compete with electrode-based techniques, a voltage-imaging method must be able to record subthreshold and suprathreshold events, without averaging, deep in intact tissue with submicrometre and submillisecond resolution, ideally from identified neurons. Currently, we are still far from this goal. The first milestone towards it would be to achieve reliable optical detection of single action potentials without averaging. So far, the most successful reporters have been the classic fast voltage-sensitive dyes (VSDs), such as the merocyanine dyes, which partition into one leaflet of the plasma membrane and generate fluorescence that depends on the electric field across the membrane[16]. By injecting these dyes into single neurons, it has been possible to record single-trial action potentials in small neuronal compartments such as distal dendrites[17], and such reporters have been used extensively to examine single-trial population activity *in vivo*[18,19]. Averaging of multiple trials has recently allowed the measurement of postsynaptic potentials and action potentials in dendritic spines in cortical slices[20,21], which represents a fundamental breakthrough as spines are currently inaccessible to patch-clamp recording electrodes. However, achieving single-trial resolution of subthreshold events has so far proved elusive. Averaging is an important limitation, as it prevents the measurement of spontaneous, non-stimulus-triggered subthreshold activity and response variability.

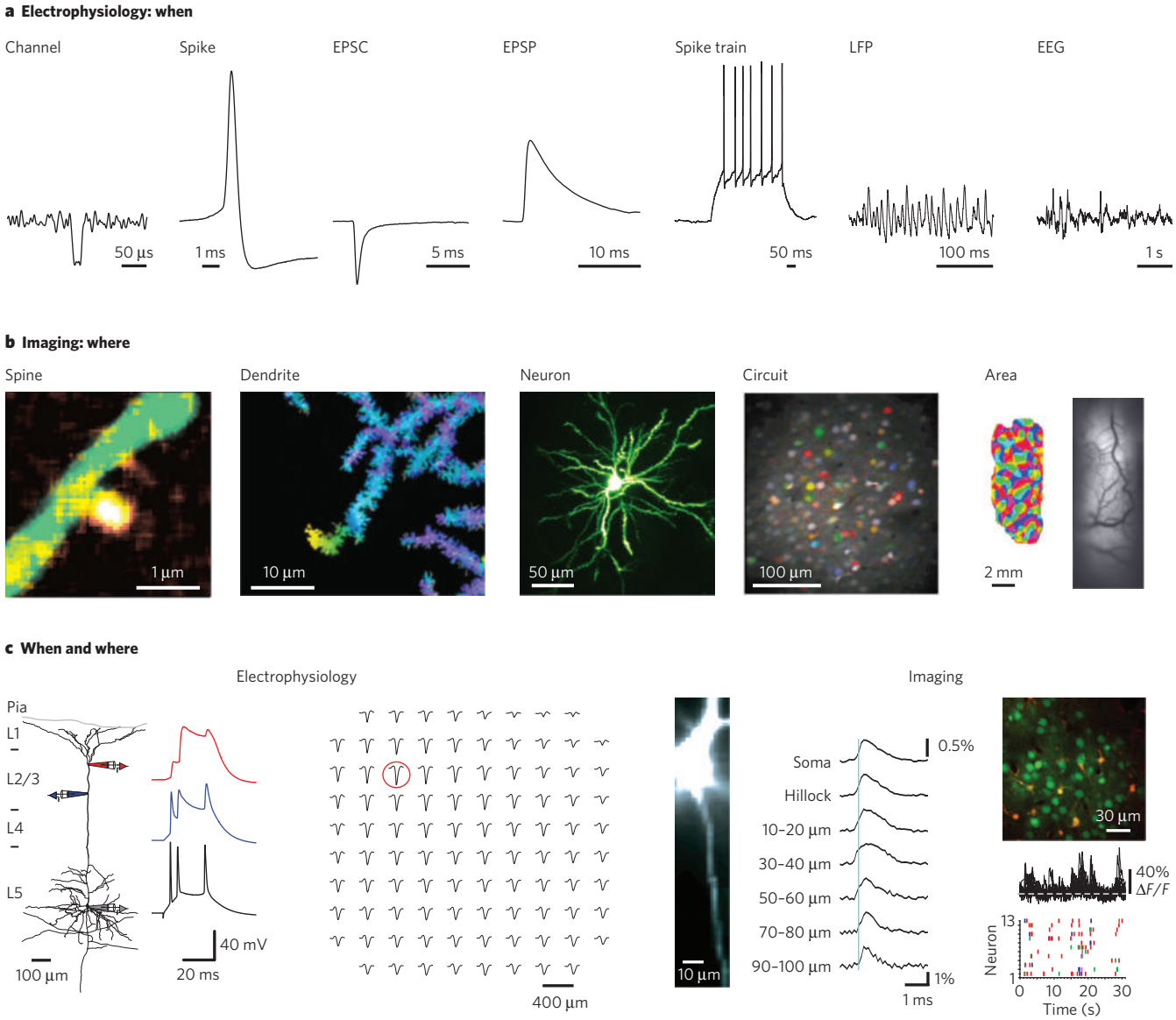


Figure 2 | Measuring the 'when' and 'where' of neuronal signals using electrophysiological and optical approaches. a, The primary strength of electrophysiology is its combination of time resolution and sensitivity, allowing the precise determination of the temporal pattern of neuronal signals over many orders of magnitude, with high signal-to-noise ratio. Representative examples shown are of a single nicotinic acetylcholine channel opening (courtesy of S. Hallerman, D. Ljaschenko and M. Heckmann, University of Würzburg, Germany); an action potential in a cerebellar interneuron *in vivo* (courtesy of P. Chadderton, University College London, UK); an excitatory postsynaptic current (EPSC), an excitatory postsynaptic potential (EPSP; courtesy of I. Duguid, University College London) and a spike train (P. Chadderton) from cerebellar granule cells *in vivo*; gamma oscillations (local field potential, LFP) recorded in the hippocampus of an anaesthetized rat (courtesy of B. Atallah, University of California, San Diego); and an electroencephalogram from an anaesthetized mouse during visual stimulation (B. Atallah). **b**, The primary strength of imaging approaches is their spatial resolution, allowing physiological events to be localized with high spatial precision over a wide range of scales. Representative examples shown are of calcium compartmentalization in a single hippocampal spine measured using a genetically encoded calcium sensor and two-photon microscopy (courtesy of Y.-P. Zhang and T. Oertner, Friedrich Miescher Institute, Basel, Switzerland); local calcium transient triggered by parallel-fibre synaptic input to a fine spiny branchlet in the dendritic tree of a cerebellar Purkinje cell (courtesy of A. Konnerth, Technical University Munich, Germany); a layer-2/3 pyramidal neuron filled with a calcium dye and visualized in mouse visual cortex *in vivo* using two-photon microscopy (courtesy of S. L. Smith, University College London); an orientation map in rat visual cortex measured using two-photon microscopy[8]; and optical imaging of voltage-sensitive-dye fluorescence to record receptive-field properties across cat visual cortex (courtesy of A. Benucci and M. Carandini, University College London). **c**, In some cases, both spatial and temporal information can be obtained using electrophysiological or imaging approaches on their own. Examples include measuring the interaction of backward-propagating action potentials with dendritic calcium spikes recorded in layer-5 (L5) cortical pyramidal neurons using three simultaneous patch-clamp recordings (courtesy of M. Larkum, University of Bern, Switzerland); recording the origin of the LFP signal in cat visual cortex using a 96-electrode array with a spatial resolution of 400 µm (reference spike from the electrode indicated in red; courtesy of I. Nauhaus and M. Carandini, University College London); optical mapping of the initiation site of the action potential in layer-5 pyramidal neurons using voltage-sensitive dyes (courtesy of L. Palmer and G. Stuart, Australian National University, Canberra, Australia); and optical monitoring of activity patterns in rat visual cortex loaded with a calcium dye (green), counterstained with an astrocyte-specific marker (sulforhodamine 101, orange) and imaged using two-photon microscopy, with traces showing calcium transients and detected spiking activity (raster; for ≥2 detected spikes; 2 spikes red, 3 green, 4 dark blue, 5 light blue, 6 pink and 7 brown) simultaneously recorded from 13 neurons in the awake animal (courtesy of D. Greenberg, A. Houweling and J. Kerr, Max Planck Institute for Biological Cybernetics, Tübingen, Germany).

A number of factors contribute to the relatively low effective signal-to-noise ratio of this approach. First, despite decades of screening experiments, for many of the commonly used dyes the fluorescence change per unit change in membrane potential is relatively small, although there are some exceptions (see, for example, ref. 22). Second, VSDs indiscriminately label internal and plasma membranes; dye that labels internal compartments does not contribute to the signal but does contribute to the resting fluorescence, which decreases the $\Delta F/F_0$ value per unit change in membrane potential and, thus, the signal-to-noise ratio. Finally, VSDs suffer from the dual problems of phototoxicity and bleaching, which limit the available excitation intensity and the duration of the experiments. The use of second-harmonic-generation microscopy can help eliminate the problem of intracellular background fluorescence, as in this approach only dye molecules in the plasma membrane contribute to the signal[21,23]. However, the effective signal-to-noise ratio of second-harmonic-generation microscopy remains limited, owing in part to photodamage associated with the need for high laser intensities; furthermore, given that second-harmonic generation normally relies on the detection of transmitted light, it is unlikely to be suitable for most mammalian *in vivo* preparations.

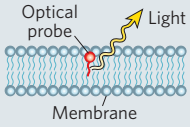
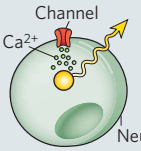
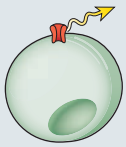
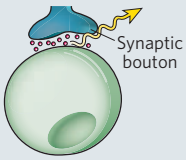
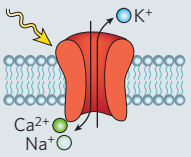
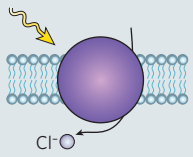
An approach that shows tremendous promise is the design of genetically encoded voltage sensors. Such sensors have an important advantage over conventional VSDs: their expression can be genetically targeted, both to subpopulations of neurons, making them particularly useful for targeted imaging of population activity, and subcellularly, to the plasma membrane and away from intracellular organelles. The design of such sensors has broadly followed one of two strategies. First, fusions between green fluorescent protein (GFP) and a voltage-gated channel, such as the Shaker potassium channel[24], allow voltage-dependent conformational changes in the channel to be translated into GFP fluorescence changes. An alternative strategy is the use of hybrid GFP systems, in which fluorescence resonant energy transfer is used to report the interaction between GFP, anchored to the cytoplasmic face of the membrane, and a dye that undergoes voltage-dependent redistribution between the inner and outer membrane leaflets.

Both strategies have yielded sensors with good voltage sensitivity. The hybrid voltage sensors have $\Delta F/F$ values of up to 34% per 100 mV (ref. 25), and further improvements are expected, particularly for sensors based on GFP–channel fusions, which can exploit the cooperativity of channel gating. However, the favourable voltage sensitivity of these sensors is undermined by their slow kinetics, with on-rates typically of the order of tens of milliseconds (although hybrid voltage sensors have more rapid kinetics), making detection of single action potentials difficult. Other problems include poor targeting to the plasma membrane, increasing the background signal; low brightness, resulting in low photon counts; and the fact that the inclusion into the membrane of mobile molecules (that is, compounds or proteins that translocate from one leaflet of the membrane to the other when the voltage across the membrane changes) leads to the increase of membrane capacitance (so-called capacitive loading). In some cases, this is so severe that it can block action-potential generation[15]. As a consequence of these limitations, the technical milestone of reliably recording single action potentials using a genetically encoded voltage reporter has not yet been achieved in any neuron. Furthermore, as with any genetically encoded probe, possible unanticipated physiological effects resulting from exogenous expression of proteins need to be assessed, in part by using electrophysiological approaches. Nevertheless, there is great optimism in the field that rational probe design, coupled with high-throughput screening methods, will soon allow us to achieve this milestone.

Calcium as a reporter of activity

Calcium is a major signalling molecule in neurons, and synaptic input and membrane voltage fluctuations often trigger changes in intracellular calcium concentration. Hence, calcium indicators have long been successfully used to infer both subthreshold and suprathreshold activity in neurons. Organic calcium-sensitive dyes, which take advantage of the pronounced calcium concentration gradient across the plasma membrane, represent one of the great success stories of synthetic chemistry[26]. Such dyes are sensitive enough to allow the opening of a single calcium-permeable channel in a spine to be detected using two-photon microscopy[27]. These dyes can therefore be used to monitor the occurrence of both action potentials and synaptic input to spines, allowing even single-vesicle events to be detected reliably, which in turn permits optical quantal analysis[28] and the demonstration that spines can act as individual functional compartments in terms of calcium signalling[6]. Although light scattering, movement problems and the unknown dendritic distribution of active synaptic input have so far prevented the direct detection of synaptic input patterns *in vivo* (with rare exceptions; see ref. 29), membrane-permeant calcium dyes have been used very successfully to monitor network activity in neurons and glia in the intact brain, under both anaesthetized and awake conditions[8,30–32]. However, even with these outstanding indicators, the slow time course of the intracellular calcium signal triggered by action potentials, coupled with the limitations of *in vivo* imaging, have made it challenging to reliably detect single action potentials and reconstruct the action-potential firing pattern[31,33].

Table 1 | Classes of optical probes and reporters

Type of molecule	Illustration	Examples
Measuring		
Voltage sensors		JPW3028* (refs 17, 20) hVOS† (ref. 25) VSFP† (ref. 96)
Calcium sensors		OGB-1* (refs 8, 30) Fluo-4* (ref. 97) D3cpv† (ref. 38) TN-XXL† (ref. 39) GCaMP3† (ref. 98)
Receptor/channel reporters		Fluorescence-labelled Shaker† (ref. 69)
Synaptic release reporters		FM 1-43* (ref. 63) SynaptopHluorin† (ref. 65) FLIPE† (ref. 66) SuperGluSnFR† (ref. 64)
Manipulating		
Optogenetic activators		ChR2† (refs 40, 47, 48, 54) LiGluR† (ref. 49)
Optogenetic inhibitors		Halorhodopsin (NpHR)† (refs 50, 51)

Classes of optical probes used for measuring and manipulating neuronal activity. Representative examples of probes in current use are provided, along with relevant citations. The illustrations show in schematic form the operation of the respective probes. ChR2, channelrhodopsin-2; FLIPE, fluorescent indicator protein for glutamate; hVOS, hybrid voltage sensor; LiGluR, light-gated ionotropic glutamate receptor; NpHR, *Natronomonas pharaonis* halorhodopsin; VSFP, voltage-sensitive fluorescent protein. *Organic probe. †Genetically encodable probe.

The design of genetically encoded calcium indicators has also attracted intense interest in recent years. These indicators are typically based on a calcium-sensitive molecule, such as calmodulin[34] or troponin[35], fused to GFP or other fluorescent proteins, with calcium binding reported by fluorescence changes due to alterations in the efficiency of fluorescence resonance energy transfer or changes to the chromophore environment. Although several generations of sensor development have yielded vastly improved properties, the current range of indicators remain inferior to synthetic indicators in terms of brightness, speed and signal-to-noise ratio when making direct comparisons under identical conditions[36,37]. Furthermore, in practice their use in transgenic animals has often been associated with sensor inactivation, reduced dynamic range or poor protein stability. Thus, despite tantalizing recent advances[38,39], as with genetically encoded voltage reporters, no genetically encoded calcium sensor has yet been shown to reliably report single action potentials in neurons *in vivo*, although the genetically encoded calcium indicators are closer to doing so. Again, rational optimization of sensor properties, aided by structure–function analysis, should soon allow this goal to be achieved.

Replacing the stimulation electrode with a beam of light

Since a method for the direct stimulation of nerves was first reported, over 200 years ago[1], the basic approach, which is based on the use of metal electrodes, has changed surprisingly little; that is, until the advent of optical methods (Fig. 1c, d).

The two main ways to trigger activity in the nervous system are sensory and electrical stimulation. The former is the method preferred by sensory physiologists, because by engaging the nervous system in an ethologically relevant manner it allows the study of the representation of the various features of the physical world through neuronal activity. By contrast, electrical stimulation is used to study mechanisms, such as synaptic transmission, because by bypassing sensory interfaces it can be applied to isolated preparations; furthermore, the activity generated by electrical stimuli is temporally precise and reproducible. Electrical stimulation involves the use of metal or glass electrodes to trigger action potentials in individual neurons or groups of neurons. This approach has three key limitations: it lacks specificity (except when stimulating single neurons or single synapses); inhibiting neurons is difficult; and it is invasive, causing damage at high stimulation intensities. These problems can be overcome by using two complementary optical approaches. The first is the use of caged compounds, which involves a pharmacologically active substance that excites or inhibits neurons, delivered in an inert ('caged') form and activated ('uncaged') by breaking a photolabile bond, generally with ultraviolet radiation. The second is the use of optogenetic tools, a new class of light-sensitive proteins that, when expressed in neurons, allow their activity to be modulated by light. These proteins are either intrinsically coupled to ionic conductances or pumps, or affect neuronal excitability through second-messenger pathways[40–43].

In the brain, neurons of various types and genetic compositions (for example excitatory, inhibitory, projection and local neurons) spatially intermingle with fibres of passage. Consequently, stimulation electrodes are unselective with respect to the structures and cell types that they activate. By contrast, caged compounds and optogenetic tools allow groups of neurons, individual neurons and specific subcellular compartments to be stimulated with extraordinary spatial and genetic specificity. Simply uncaging glutamate (the main excitatory transmitter in the brain) predominantly activates somatodendritic compartments of neurons rather than intermingled axons, consistent with the distribution of glutamate receptors. This approach allows the rapid generation of connectivity maps between cortical layers in cortical slices[44]. With two-photon activation, glutamate uncaging can be used to activate individual synapses along a dendrite[45]. Genetic specificity can be obtained by expressing receptors that are not naturally present in the system. Expression of purinoceptors in genetically defined fly neuronal populations, for example, allows activation of these neurons through ATP uncaging, and the study of the ensuing behavioural outputs[46].

Optogenetic tools represent another valid alternative to the stimulation electrode, and greatly surpass it in specificity and versatility[40–43]. Targeted expression of light-activated molecules such as channelrhodopsin[47,48], light-gated ionotropic glutamate receptor[49] or halorhodopsin[50,51] allows neurons or their subcellular compartments to be stimulated with extraordinary spatial, temporal and genetic specificity. These new optical tools can perform tasks well beyond the capabilities of conventional stimulation electrodes, such as independent stimulation of multiple intermingled populations[52], bistable activation of neurons[53] and stimulation of defined second-messenger pathways (for example to mimic modulatory neurotransmitter pathways[41,43]). These approaches can be harnessed to map functional connectivity[54], to influence the dynamics of neuronal circuits[47,48,50,55] and, ultimately, to control behaviour[49,56,57]. In the long term, complete cellular-level and subcellular-level structural, molecular and genetic maps of the nervous system — the connectome[58] — will be required to target probes to select cell types or subcellular locations to further enhance the precision of these optical probes.

Finally, the inducible expression of optogenetic tools will make it possible to bridge the current gap between sensory and electrical stimulation. One important drawback of electrical stimulation is the uncertainty over whether the ensemble of stimulated inputs actually corresponds to a physiologically relevant pattern, that is, whether a sensory stimulus would ever activate that particular combination of stimulated afferents. The activity-dependent expression of optogenetic tools such that only neurons recruited by a specific sensory stimulus or by a particular task express the protein[59] will represent a first step in solving this problem. Such activity-driven expression will allow the study of the behavioural output generated by the selective activation or inhibition of functionally defined neuronal subpopulations. Similarly, activity-driven expression of optogenetic tools will make it possible to determine connectivity patterns and circuit dynamics of functionally related neuronal ensembles. These recent developments thus afford more specific and much more controlled manipulation of the nervous system, allowing sophisticated tests of causality (see page 923). We can now imagine a time in the near future when activation of neuronal circuits will no longer rely on the centuries-old metal stimulation electrode.

Voltage clamp

A difficult technique to perform using optical tools is voltage clamp, a method that allows the measurement of currents passing through membranes. Electrophysiologists achieve voltage clamp using feedback amplifiers that hold the potential across the membrane at a defined voltage by injecting into the neuron currents equal in amplitude and opposite in sign to those that flow through the membrane. These injected currents provide a measure of the ionic and capacitive currents naturally flowing in and out of neurons. Voltage clamp has led to fundamental discoveries, such as the biophysical mechanisms underlying action potentials[60], the characteristics of synaptic conductances[61] and the functional properties of single ion channels[62]. Let us consider how voltage clamp might be achieved by optical means.

An 'optical voltage clamp' could be realized by expressing two light-gated ion channels, one selective for Na^+, the other for K^+, and each activated by different wavelengths, thus providing the basis for selective light activation of an inward and an outward current. Given sufficient expression of these channels, it should be possible to achieve any arbitrary membrane potential between the reversal potentials of the two channels. The membrane potential would be simultaneously monitored using a voltage probe and coupled to the source of excitation light with a feedback mechanism, with the clamp speed depending on the kinetics of the channels and their density relative to the membrane capacitance. Although this system would permit voltage clamp, quantifying the light-activated currents would be difficult, requiring knowledge of factors including the membrane area, the intensity of the light hitting the membrane, the density of the light-activated channels and the relationship between the light intensity and the single-channel photocurrent. A potential benefit of this optical voltage clamp would be improved 'space clamp', that is, voltage control of all neuronal compartments, which is normally impossible to

achieve with a single electrode. This would allow proper measurement of current sources from all over the dendritic tree.

Given that implementing such a system will be very challenging, it is worth asking whether voltage clamp itself can be replaced. Indeed, our knowledge of the molecular mechanisms of excitability and synaptic transmission may allow us to investigate channel and synaptic function without current measurements, thus making voltage-clamp recordings unnecessary. For example, synaptic function can be assessed by probing the status of the underlying components, such as by monitoring neurotransmitter release[63–66] or the activation of transmitter-gated channels[6,28]. In turn, activation of distinct subtypes of voltage-gated channel can be probed by directly monitoring conformational changes rather than conductance, for example by using subunit-specific resonance energy transfer as a spectroscopic molecular ruler[67,68]. Preliminary work has shown that even single-channel resolution can be achieved using such an all-optical approach[69], revealing information about channel conformations not obtainable through electrophysiological recordings. However, such a targeted approach can only be used to identify known, and well-characterized, channels; and biophysical characterization of channels demands voltage control. Thus, voltage clamp is likely to remain an indispensable tool for neuroscientists in the future.

Promises and challenges

To replace or complement electrophysiological approaches, optical probes must be implemented *in vivo,* ideally in a behaving organism. This will provide a range of unprecedented opportunities, but it also highlights several hurdles that must be overcome.

Recording from populations

One of the key arenas in which imaging can already surpass the use of electrodes is simultaneous recording from many neurons. Sensory stimuli and motor output are represented by the activity of ensembles of neurons. Within these ensembles, each neuron may respond to a particular feature, or a specific combination of features, of the stimulus, such as position in space, frequency or intensity. The same is true for motor output, where a particular movement in space is specified by the activity of select ensembles within a population of neurons in the motor cortex. Thus, the experimental ideal would be to record every spike from every neuron in response to a sensory input and during the ensuing behavioural output, and use this information as a first step in working out the neural processes underlying this transformation. This is an enormous challenge, because computations in the mammalian nervous system engage thousands to millions of neurons. Electrophysiologists have increased the sampling density in their experiments by using microarrays featuring multiple electrodes[70], but such approaches are limited by the electrodes' invasiveness and finite spacing, and by the difficulty of identifying the origin of blindly recorded neuronal signals.

Optical probes may ultimately make this dream experiment possible. Early steps have involved using multicellular loading of membrane-permeant calcium dyes[8,30–32], as indicators of spiking activity in networks (Fig. 2). In combination with transgenic mouse lines expressing genetically encoded fluorescent markers in specific neuronal subtypes, this approach allows identification of the imaged cells[71]. Unfortunately, as discussed above, the imaging of calcium transients does not offer the same signal-to-noise ratio as electrophysiological recordings, and the slow kinetics of the recorded signal often prevent resolution of individual spikes in a train, unless sophisticated analysis techniques are used[31,33]. These problems, coupled with limitations in scanning speed, make it difficult to define the temporal relationship of spiking between different neurons. Finally, calcium transients are best correlated with suprathreshold neuronal activity, and provide little information about subthreshold membrane-potential fluctuations. Despite all of these drawbacks, however, no electrophysiological approach allows the activity and spatial location of so many active neurons to be monitored simultaneously[8].

Recording the activity of a population of neurons using voltage-sensitive dyes[14] is a complementary approach to calcium imaging: although single-cell resolution is usually not achieved, the kinetics of the signal can follow fast, subthreshold fluctuations in membrane potential[72]. This approach has been used to discover important functional organizing principles in the brain, such as how ongoing activity interacts with visually driven activity[19], and the existence of spontaneously emerging maps of activity[18]. Thus, VSD imaging offers not only the advantages of electrophysiological local field potential recordings, but also the ability to monitor large brain areas simultaneously.

Recording at depth

Light scatters and is attenuated as it passes through tissue. This is detrimental to the high spatial resolution possible with optical approaches — a major advantage over electrophysiology — and to the intensity of the signal that can be delivered to and collected from deeper brain areas. Given the unalterable physical properties of brain tissue, this represents an absolute technical barrier that is difficult to overcome. As a consequence, high-resolution one-photon imaging has been limited to thin preparations or only the most superficial regions (depths <50 μm) of intact tissue. Nonlinear microscopy, including two-photon and second-harmonic-generation microscopy, has been shown to increase penetration depth substantially, but these approaches are currently still limited to the superficial regions of the brain (depths of ~1,000 μm; ref. 73). One way to overcome the fundamental limitations imposed by scattering is simply to remove the overlying tissue to image deeper regions[74]. A less damaging alternative is to insert a probe, such as an optical fibre or a gradient-index lens, into the brain region of interest. Such microendoscopy probes can be used for both one-photon and two-photon imaging in deep regions[4]. The localization of the probes themselves limits the spatial extent of the optical sampling possible. Furthermore, their size, which is typically considerably greater than that of a recording electrode, also means that optical probes may be more invasive than using classical electrophysiological approaches.

Independent control of multiple cells

Understanding how the dynamics of neural networks underpin behaviour requires not only the faithful recording of activity across populations in large, three-dimensional volumes *in vivo*, but also the ability to manipulate the activity of the active neurons (see page 923). Because the spatiotemporal dynamics of activity typically are asynchronous, with millisecond timing differences among different cells, this requires an approach that allows targeted manipulation of individual neurons with millisecond precision. When systematic timing differences exist between different genetically defined populations of neurons, such as interneurons and principal cells[75,76], optogenetic tools with different wavelength spectra could be used to regulate the activity of different populations individually[52]. However, ultimately it is necessary to achieve single-cell resolution across the population, which requires spatial restriction of activation to single neurons. This can in principle be achieved using two-photon excitation, which has recently been demonstrated for channelrhodopsin[77]. Protein engineering is likely to yield a new generation of optogenetic probes with large two-photon cross-sections and a wide range of wavelength selectivities. This must be paired with further development of rapid three-dimensional scanning approaches to targeting two-photon excitation at the appropriate cells with millisecond precision[78,79].

Recording activity during natural behaviours

Great progress has recently been made in adapting behavioural assays to head-fixed rodents, thereby allowing high-resolution imaging of the nervous system to be performed during behaviour[32] (see also page 923). However, natural expression of many behaviours requires animals to move freely in their environment. There exists a long tradition of using implanted multi-unit electrodes in freely moving animals to correlate neural activity with behaviour[70,80], and this was recently complemented with whole-cell patch-clamp recording[81] (Fig. 3a), providing a temporal resolution that has not yet been matched by optical approaches. The recent development of head-mounted miniaturized two-photon microscopes[82], together with fibre-optic two-photon microendoscopy for imaging deep

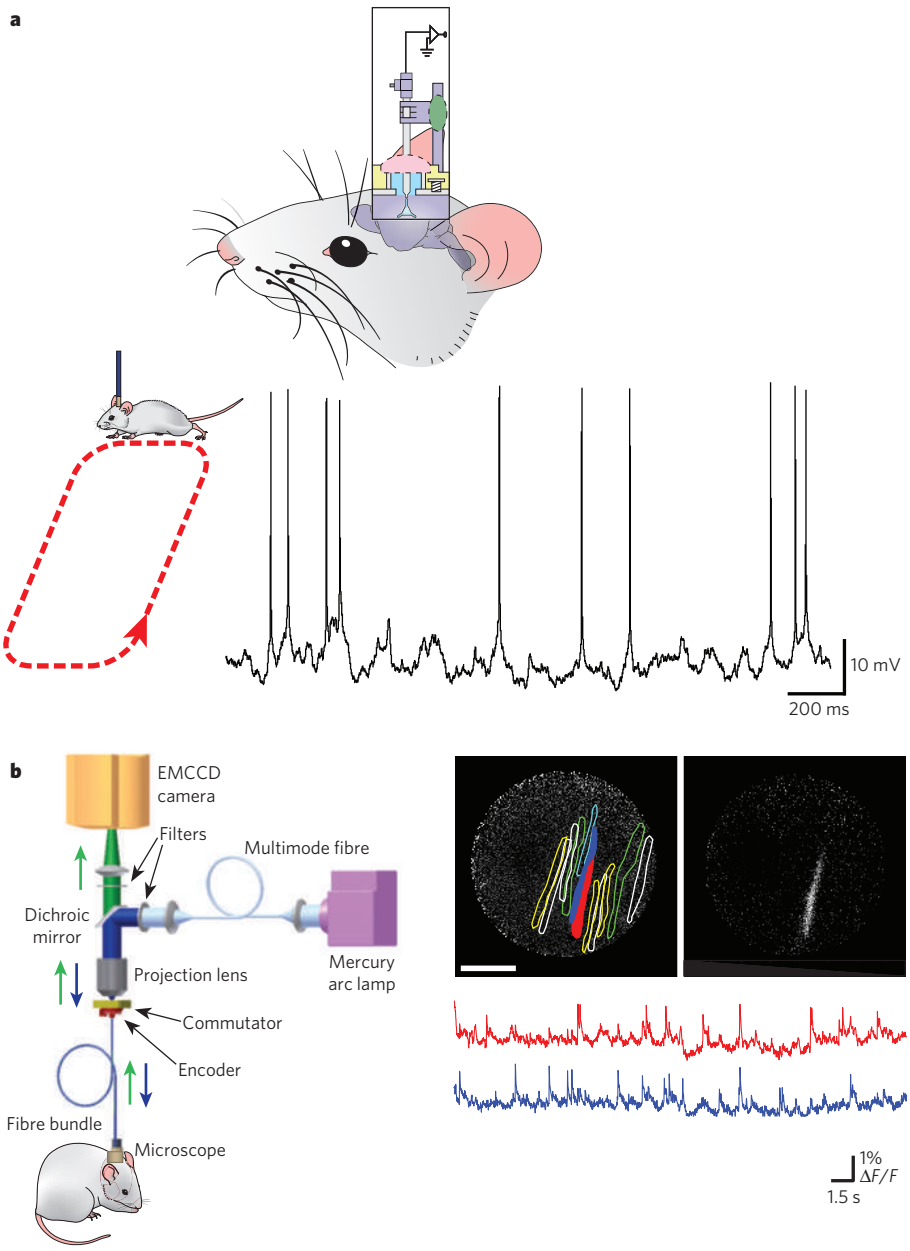


Figure 3 | Recording and imaging on the move. a, Whole-cell patch-clamp recording of a hippocampal neuron in a rat freely moving on a linear track. Top, illustration of the assembly for maintaining patch-clamp recordings in freely moving rats[81]: an electrode holder is mounted on the rat cranium and holds an electrode that is cemented in place at two anchoring sites (shown in green and pink) once a recording is made. Bottom, representative trace of membrane potential recorded from a hippocampal pyramidal neuron during free movement of the rat around a rectangular track (courtesy of J. Tukker and M. Brecht, Humboldt University, Berlin, Germany). **b**, Imaging population activity with single-cell resolution in freely moving animals. Left, illustration of the set-up. Excitation light (blue) propagates in one direction through an optical-fibre bundle. Emitted fluorescence (green) returns along the same path, passes through the dichroic mirror and is imaged using an electron-multiplying charge-coupled-device (EMCCD) camera. Right, viewed from the brain surface, the dendritic arborizations of 12 cerebellar Purkinje cells labelled with calcium-sensitive fluorescent dye are identified as parallel stripes in an unrestrained awake mouse sitting quietly (scale bar, 100 μm); a calcium signal in the red cell is shown in the right-hand image. The traces in the lower panel show the time course of Ca^{2+} signals from the red and blue cells. (Panel **b**, modified, with permission, from ref. 84.)

networks[4] and fibre arrays for one-photon imaging of population activity[83,84] (Fig. 3b), should soon enable us to exploit the many advantages of the optical approach. These imaging strategies, in combination with the development of new generations of genetically encodable optical probes with high signal-to-noise ratios, should allow long-term experiments in freely moving animals in which spatiotemporal patterns of activity in genetically defined networks can be correlated with natural behaviours.

Improving electrophysiology

The optical approaches we have described above are developing very rapidly, but electrophysiology is by no means a static discipline. Even a well-established technique such as patch-clamp recording is still being refined and used in new applications, such as its recent implementation in making whole-cell recordings from single neurons in freely moving animals[81] (Fig. 3a). However, a series of developments are under way that aim to overcome some of the key traditional limitations of electrophysiology. First, there is a widespread effort to produce ever-smaller electrodes — with the target being tip sizes of much less than 1 μm — using nanofabrication techniques such as focused-ion-beam microscopy[85] or by assembling carbon nanotubes[86,87]. Such electrodes could not only be used to record from extremely fine structures such as boutons and spines, but may also improve the signal-to-noise ratio for isolating individual units in extracellular recordings. In addition, owing to their fine calibre, they help to minimize tissue distortion as they are inserted into the brain. Second, to improve spatial sampling in recordings of population activity, multi-electrode arrays are being assembled that contain larger numbers of electrodes with ever-finer spacing[80,88]. These arrays will allow population activity to be sampled, and afferent inputs to be stimulated, with a much higher spatial precision than before. Finally, research into the physical basis for the long-term interactions between silicon-based electrodes and neural tissue[89] should eventually allow invasive brain–machine interfaces to target populations of neurons more precisely, more reliably and over longer timescales.

Perspective

Throughout their history, imaging and electrophysiology have always been closely linked (an example of early work is that in which an optical approach was used to report electrical activity in the electric fish[90]). However, in the context of electrophysiology, imaging was (until recently) primarily used for visualization during electrophysiological recordings. For example, the pioneering experiments of Hodgkin and Huxley[91] relied critically on their visualizing the squid axon at high

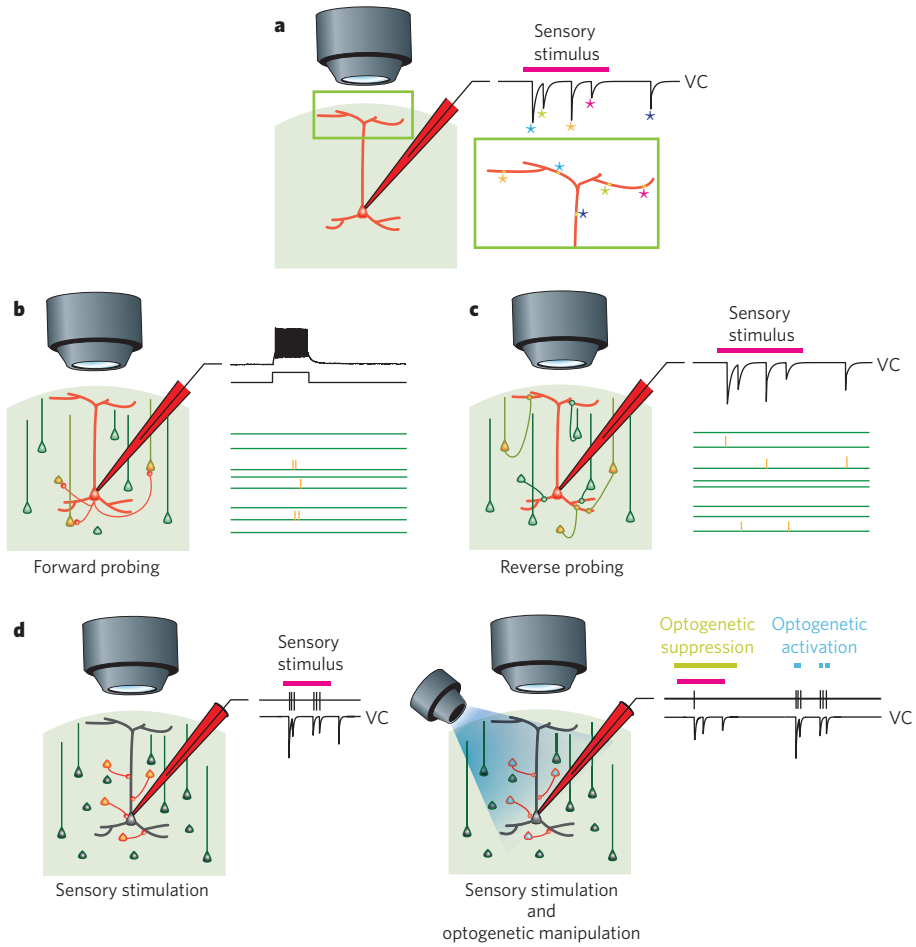


Figure 4 | Synergistic combinations of electrophysiology and imaging. **a**, The superior temporal resolution of electrophysiology can be combined with the spatial resolution offered by imaging to define the spatiotemporal distribution of synaptic inputs to the dendritic tree (red). Patch-clamp recording from the soma of a pyramidal cell *in vivo* is used to measure sensory-evoked synaptic input in voltage clamp (VC), which is then correlated with the location of the input (asterisks) in the dendritic tree by measuring synaptically activated calcium signals using two-photon imaging. **b**, Forward probing of connectivity in networks (see ref. 99). Targeted whole-cell patch-clamp recording from a single principal neuron is accompanied by simultaneous imaging of activity in the local network using a calcium sensor. Triggering of spikes in the principal neuron using an injected current pulse (top traces) allows postsynaptic neurons in the network activated by the recorded neuron (green, silent neurons; yellow, neurons exhibiting spikes) to be identified using two-photon population calcium imaging[8,31,32] with genetically encoded or bulk-loaded[30] calcium indicators. **c**, Reverse probing of presynaptic connectivity in networks (see ref. 100). Recording of sensory-evoked synaptic currents in a postsynaptic neuron using voltage clamp is accompanied by simultaneous imaging of activity in potential presynaptic partners in the local network using two-photon population calcium imaging[8,31,32]. Reverse correlation between presynaptic spikes and synaptic currents can be used to identify functional presynaptic partners activated by sensory stimulation. **d**, Combining targeted patch-clamp recording of receptive-field responses with optogenetic manipulation of defined networks. Sensory responses are recorded from a visually targeted neuron as spiking responses, or the underlying synaptic currents are recorded in voltage clamp (VC). To determine the causal contribution of particular presynaptic cell types to the sensory-evoked response, optogenetic probes (for example channelrhodopsin[47,48] and/or halorhodopsin[50,51]) are expressed in genetically defined cell types (red neurons) in the same network. These probes can then be activated with the appropriate wavelengths of light, either removing the contribution of these neurons to sensory-evoked activity (in the case of halorhodopsin) or activating them (in the case of channelrhodopsin, shown here), to modulate the sensory response or, in the absence of sensory stimulus, to mimic sensory input patterns.

enough resolution to enable them to precisely position the microelectrode inside it (Fig. 1); more recently, two-photon imaging has been used to target patch-clamp recordings to visually or genetically identified cell types *in vivo*[92–94], in turn allowing essential calibrations for a new generation of genetically encoded sensors[38,39].

Optical approaches can now be used to collect functional data and manipulate the nervous system. This provides remarkable opportunities for combining the complementary strengths of electrophysiology and imaging. In particular, the synergism of the exceptional time resolution, signal-to-noise ratio and ability to voltage clamp provided by electrophysiology with the superior spatial sampling and resolution of imaging and optical probing will enable us to link the function of the nervous system to its underlying cellular and molecular structures with unprecedented precision.

Compelling examples of such synergisms between electrophysiology and imaging in linking function to structure include the localization of the functional inputs onto a dendritic tree to constrain the rules for dendritic computations (Fig. 4a); the combination of optical imaging and single-unit[18,19] or multi-unit[95] recording to reveal the role of single neurons in network dynamics; and the combination of patch-clamp recording and two-photon population imaging to map functional connectivity in networks *in vivo* (Fig. 4b, c), which can ultimately reveal the composition of functional ensembles of neurons during behaviour. In addition, combining electrophysiology with optogenetic tools offers the possibility of directly testing causality between structure and function: by selectively and rapidly activating or silencing specific structures, such as neuronal compartments, neuronal types and entire circuits, we will be able to assess their impact on the function of a neuron, on the

dynamics of a circuit and on the behaviour of an organism. For example, such optogenetic tools will be instrumental in establishing the roles of specific dendritic compartments, specific inhibitory neurons or specific layers of neurons in determining the receptive-field properties of cortical sensory responses (Fig. 4d).

This synergism should significantly quicken the pace of discovery as we move towards one of the major goals in neuroscience, namely to link the structure of the brain to its function, and will allow both imaging and electrophysiology to flourish in harmony long into the future. ■

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