VSDI: A NEW ERA IN FUNCTIONAL IMAGING OF CORTICAL DYNAMICS

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Abstract | During the last few decades, neuroscientists have benefited from the emergence of many powerful functional imaging techniques that cover broad spatial and temporal scales. We can now image single molecules controlling cell differentiation, growth and death; single cells and their neurites processing electrical inputs and sending outputs; neuronal circuits performing neural computations *in vitro*; and the intact brain. At present, imaging based on voltage-sensitive dyes (VSDI) offers the highest spatial and temporal resolution for imaging neocortical functions in the living brain, and has paved the way for a new era in the functional imaging of cortical dynamics. It has facilitated the exploration of fundamental mechanisms that underlie neocortical development, function and plasticity at the fundmental level of the cortical column.

Sensory processing, motor coordination and higher brain functions are carried out by elaborate networks, made up of millions of neurons. An important question in brain research is: how do the properties of single neurons and their intricate synaptic connections combine to form networks that are capable of such feats? Some properties of neocortical networks are not evident in the activity of single neurons. In some primary sensory areas, such as the primary visual cortex, there is a one-to-one mapping of the external word onto the cortical sheet. However, visual perception also involves global processing of the whole scene, mediated by long-range horizontal connections and top-down influences (see online supplementary information S1 (figure)). How does this global processing occur? To understand the properties and functions of large networks of neurons, it is necessary to study the activity of neuronal populations, rather than that of single neurons. Imaging techniques are ideally suited to this goal.

The processing machinery in the neocortex is organized in cortical columns[1–3]. To follow neuronal computations at this fundamental level requires a spatial resolution of about 200 μm. Furthermore, because communication in these networks occurs over milliseconds, an imaging approach with millisecond time resolution is required. Electrical communication in cortical networks comprises two basic signals: the initial input of subthreshold and suprathreshold synaptic potentials, which shapes the output patterns, and the final output of action potentials. Without an imaging technique that can reveal the spatio-temporal patterns of the subthreshold input, perception and other higher brain functions cannot be fully understood. Finally, to investigate behaviour, plasticity and higher brain functions, it is essential to be able to image the neocortical function of behaving mammals, particularly monkeys.

In vivo voltage-sensitive dye imaging (VSDI)[4] fulfils these technical requirements and should enable us to resolve outstanding fundamental questions relating to distributed processing, ongoing activity, visual processing, perception and plasticity. However, like any technique, VSDI has limitations, which are discussed later.

In this article, we briefly review the technique of VSDI and describe some of its applications to the fundamental issues mentioned above. We conclude by discussing what the future holds, necessary continuing developments to overcome current limitations and additional questions that are likely to be explored. For earlier reviews relating to this topic, see REFS 5–13.

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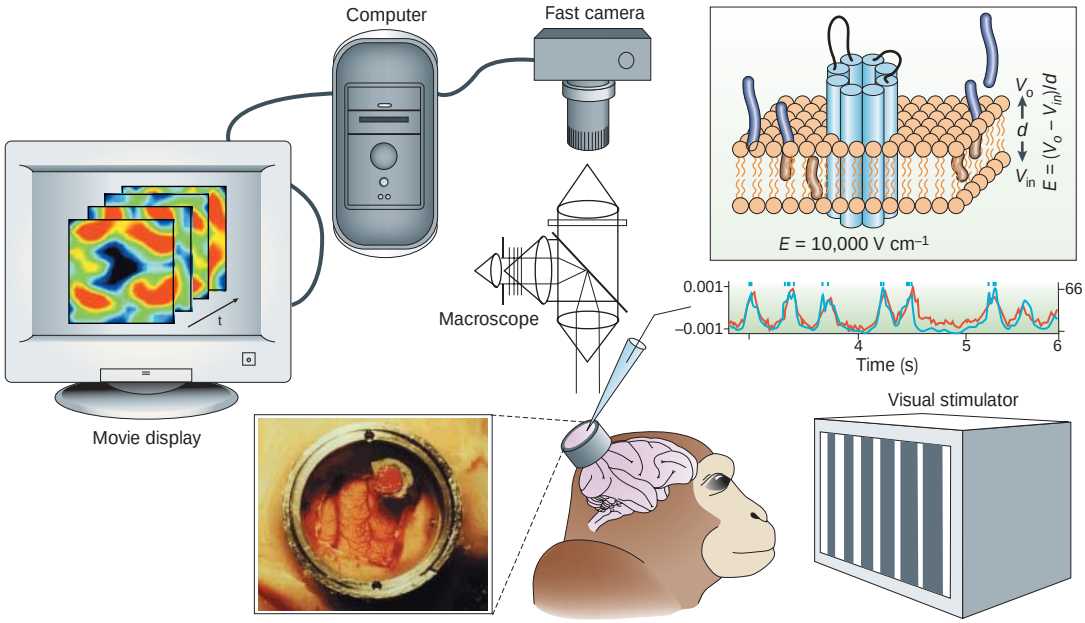

Figure 1 | **Optical imaging of cortical dynamics *in vivo***. The exposed cortex is covered with a sealed chamber (left bottom inset), which acts as if the monkey had a transparent skull. After the cortex is stained with a suitable voltage-sensitive dye (VSD; blue/orange molecules in top right inset), it is illuminated with light at the peak excitation spectra of the fluorescence VSD used (630 nm in this case). A sequence of images of the fluorescing cortex is taken with a fast camera (100–10,000 Hz) using a MACROSCOPE. During image acquisition the monkey is visually stimulated. The acquired images are digitized and transferred to the computer that controls the experiment. Functional maps or movies of the cortical activity are analysed and displayed on a colour monitor. Various types of electrical recording, microstimulation or tracer injection can be carried out simultaneously with the imaging. The similarity between the two traces above the electrode, comparing intracellular activity (blue) and VSD population activity (red), indicate that, *in vivo*, VSD measures mostly synaptic membrane-potential changes (spikes recorded electronically were truncated — blue lines at the top — and were not seen in the optical signal). Modified, with permission, from REF. 50 © (1999) Springer Verlag.

MACROSCOPE
An improvised microscope made of photographic lenses that provides much larger numerical aperture (NA) for low magnification (for example, one or less) than a standard microscope objective. This is crucial for fluorescence imaging of large areas (for example, 10 mm) because illumination intensity and fluorescence collections depend on the square of the NA.

VOLTAGE-SENSITIVE DYES
The ones discussed here are organic molecules with a molecular weight of about 500 Da and a length shorter than 20 Å These molecules usually have a hydrophobic portion that sticks to the membrane and a charged chromophore that prevents a 'flip' to the cell interior. The dyes have a high absorbtion coefficient and, usually, a high quantum efficiency for fluorescence when they bind to the neuronal membranes.

FUCTIONAL OPTICAL IMAGING
A means of recording neural activity by measuring the optical properties of brain tissue, using either voltage-sensitive dyes or intrinsic signals relating to the oxygen saturation of haemoglobin or light scattering.

BARREL
A cylindrical column of neurons found in the rodent somatosensory neocortex. Each barrel receives sensory input from a single whisker follicle, and the topographical organization of the barrels corresponds precisely to the arrangement of whisker follicles on the face.

The technology and history of VSDI

The principle of VSDI. The first optical recordings of electrical activity using VOLTAGE-SENSITIVE DYES (VSDs) were made by Tasaki and collaborators[14] in the squid giant axon, and by Cohen, Salzberg and colleagues in the squid giant axon[15] and in individual leech neurons[5,16], following the efforts of Waggoner and colleagues in screening and synthesizing dyes[7,8,15]. To OPTICALLY IMAGE electrical activity, the preparation under study is first stained with a suitable VSD. The dye molecules bind to the external surface of cell membranes and act as molecular transducers that transform changes in membrane potential into optical signals — changes in absorption or emitted fluorescence that occur in microseconds. These optical changes are monitored with light-imaging devices, positioned on the target or, more often, in the image plane of the target formed by an optical device such as a microscope (FIG. 1). The amplitudes of the VSD signals are linearly correlated with both changes in membrane potential (rather than changes in current) and the membrane area of the stained neuronal elements under each measuring pixel[15].

VSDI, as opposed to recording[17,18], began with photodiode arrays in invertebrate ganglia[19] and was subsequently used to study the activity of single-cell processes in culture[20]. The first studies of population activity were carried out in mammalian brain slices[21] and in the salamander olfactory bulb[22]. The results of these studies indicated that optical imaging could be a useful tool for studying the mammalian brain *in vivo*. However, preliminary *in vivo* experiments in rat and cat visual cortex revealed several problems, including considerable noise due to respiratory and heartbeat pulsation. Moreover, the relative opacity and packing density of the cortex limited the penetration of the excitation light and the ability of available dyes to stain deep layers of the cortex. Subsequently, other dyes (for example, RH-414)[4,23] were developed that could stain cortical tissue more effectively. The problem of heartbeat noise was overcome by synchronizing data acquisition with an electrocardiogram and subtracting a no-stimulus trial. These improvements facilitated *in vivo* imaging of responses to a natural sensory stimulus: retinotopic responses in the frog optic tectum[4,24], of the whisker BARRELS in rat somatosensory cortex[25] and of the salamander olfactory bulb[26–29]. The development of even better dyes (such as RH-704 and RH-795) improved the quality of the results obtained in cat and monkey visual cortex[30,31].

Until recently, optical imaging based on slow, intrinsic signals, such as haemodynamic or light-scattering signals[31], was easier than VSDI and provided spectacular maps of functional cortical organization[32–37]. However, these signals are not directly related to electrical activity and offer low temporal resolution — in the order of seconds. Spectroscopic studies of the mechanisms that underlie the intrinsic signals[32,34,38] revealed the spectral and dynamic characteristics of the intrinsic signals, which introduce considerable haemodynamic noise during

VSDI. We took advantage of these findings to improve VSDI. The latest generation of voltage-sensitive dyes are excited outside the absorption band of haemoglobin, to minimize pulsation and haemodynamic noise, and offer a 30-fold improvement in signal-to-noise ratio[39]. This advance enables us to reveal the dynamics of cortical information processing, and its underlying functional architecture, at the necessary spatial and temporal resolution in both anaesthetized and behaving animals. Methodological advances relating to implants of transparent artificial dura[40] now enable chronic recordings to be made. Furthermore, VSDI can be combined with simultaneous intracellular or extracellular recordings, microstimulation and tracer injection owing to the development of an electrode assembly attached to a cranial window[41].

Spatial and temporal resolution. The VSD response time is in microseconds and the ultimate spatial resolution of VSDI is about 0.5 μm — it is limited by optics and light scattering. Initially, VSDI used a cumbersome 12 × 12-diode array camera[19]. Work by Kamino[42], Matsumoto[43,44], Ichikawa[44] and their colleagues led to higher spatial resolution, as did the use of stroboscopic light[45,46]. Impressive high-resolution VSDI at slower video rates was accomplished by Kauer and colleagues[27]. Today, commercial voltage-sensitive dye imagers, with up to 1,000,000 detectors (CCD) or 10,000 detectors (CMOS), running at 10,000 Hz[47,48], are available. The spatial resolution of VSDI is limited not by detector technology, but by the properties of the dyes.

What does the dye measure in vivo? In simple preparations, when single cells can be visualized, the dye signal looks just like an intracellular recording[6,15–20,23]. Controversies about what the dye signal reflects during *in vivo* measurements were resolved by direct intracellular recordings *in vivo*, combined with VSDI, first in the anaesthetized cat[49,50] and later in the rat somatosensory cortex[51]. The results established that the dye signal precisely reports changes in membrane potential (red and blue traces in FIG. 1; see also online supplementary information S2 (figure)). These electrical recordings confirmed that previous limitations of the VSDs had been eliminated; in particular, the newer dyes[39] cause minimal PHARMACOLOGICAL SIDE EFFECTS or PHOTOTOXICITY. VSDs can also bind to glial membranes and show slow changes in the membrane potential of activated glia[52,53], but the similarity of the two traces indicates that the contribution of slow glial depolarization to the VSDI signal is minimal.

During *in vivo* imaging of the neocortex, a single pixel contains the blurred images[22,50] of various neuronal compartments — including the dendrites, axons and somata of a population of neurons — rather than a single cell. The VSD signal is linearly related to the stained membrane area, and most of the dye signal originates from cortical dendrites and non-myelinated axons rather than cell bodies, because their membrane areas are orders of magnitude larger than that of neuronal somata. The dendrites of cortical cells are often far more confined than the axons, so most of the signals in a given pixel originate from the dendrites of nearby cortical cells. Therefore, the VSD signal *in vivo* mainly reflects dendritic activity. This dendritic origin of the signal can affect the spatial resolution of VSDI, because the dendrites of cells in a given cortical column might cross cortical areas that correspond to adjacent cortical columns, which have different functional properties[54]. So, the existence of a dye signal in a particular cortical site does not imply that cortical neurons at that site are generating action potentials. Nevertheless, by using threshold approaches or image-processing approaches, such as differential imaging[32], VSDI has provided high-resolution maps, which correspond to cortical columns in which spiking occurs, and offer a spatial resolution better than 50 μm[39,50].

Integrated VSDI and electrode techniques. To facilitate the combination of optical imaging with electrode-based techniques, a skull-mounted cranial window — the 'sliding-top cranial window' — and a removable microdrive-positioned electrode were developed[41]. These devices have been used for studying sensory processing, acutely or chronically, in the cortex of cats and monkeys. The assembly enables optical imaging of intrinsic signals or VSDs, combined with simultaneous microstimulation, extracellular recording (single- and multiple-unit recording and local field potential), or intracellular recording, and the targeted injection of tracers. After the functional architecture has been determined by optical imaging, electrodes can be visually targeted into a selected cortical site. Using such targeted electrode manipulations, rather than random penetration, overcomes the problem of searching for 'a needle in a haystack'.

Despite the extensive use of microstimulation in various brain regions to affect neuronal and behavioural responses, many questions remain about the spatio-temporal pattern of activation that is evoked by microstimulation. Microstimulation and VSDI studies of the frontal and motor cortices of awake monkeys have recently shed light on this issue.

The FRONTAL EYE FIELD and neighbouring area '8Ar' of the primate cortex are involved in the programming and execution of saccades. Electrical microstimulation in these regions elicits short-latency contralateral saccades[55]. A combination of VSDI and microstimulation has been used to determine how the spatio-temporal dynamics of microstimulation-evoked activity are converted into saccade plans. Microstimulation was shown to elicit neural activity with complex spatio-temporal dynamics, both inhibitory and excitatory; these dynamics depend on the stimulated area, and can have important behavioural correlates. The observed spread was large and depended on the amplitude of the microstimulation. Similar results were obtained in the motor cortex[56,57]. These results emphasize the importance of further characterization of microstimulation-evoked activity for the interpretation of its behavioural effects[57] and what it has revealed about the neural basis of cognition[58,59].

PHARMACOLOGICAL SIDE EFFECTS
Organic molecules may bind to ion channels or other important components of the neuronal machinery, and as a result they have the potential to modify ion conductances, neurotransmitter receptors and other membrane properties. These modifications could change the electrical behaviour of single neurons and/or neuronal networks.

PHOTOTOXICITY
(Photodynamic damage.) Once excited by illumination, certain organic molecules collide with oxygen molecules, which can create singlet oxygen radicals. Singlet oxygen is highly reactive, and can oxidise proteins and other membrane components, causing damage to cell membranes.

FRONTAL EYE FIELD
An area in the frontal lobe that receives visual inputs and controls movements of the eye.

We have discussed the results of simultaneous VSDI and intracellular recordings[49–51], and the combination of VSDI with single-unit and local-field-potential recordings will be discussed in the context of visualization of neuronal assemblies. Electrode-based techniques, including tracer injections, remain a powerful tool, but the simultaneous use of optical imaging can minimize the sampling problem, and add another dimension to electrode-based techniques.

Applications and recent findings

Global long-range processing. Real-time optical imaging has been used to investigate how far across the cortical surface synaptic activation spreads from a sensory point stimulus — the cortical point-spread function. Experiments using 2-deoxyglucose to examine retinotopy showed the representation of a retinal image on the cortical sheet, with little or no spread in layer 4 (REF. 60). By contrast, long-range horizontal connections[54,61–63] indicated that activity would spread in layer 2/3. However, many questions remained: how large are the postsynaptic potentials? Are they inhibitory, excitatory or both? How quickly do they occur? When do they disappear? What is their spatial distribution? As mentioned above, the VSD signal seen in cortical tissue reflects mainly postsynaptic potentials in the fine dendrites of cortical cells rather than action potentials in cell somata, so VSDI is well suited to answering these questions.

Retinotopic imaging experiments in monkey striate cortex[30] showed activity over a cortical area that was much larger than predicted on the basis of standard retinotopic measurements in layer 4, but was consistent with the anatomical finding of long-range horizontal connections in the visual cortex. The results of these imaging experiments were used to calculate the cortical point-spread function, which reflects the extent of cortical postsynaptic activation by retinal point stimuli (FIG. 2). To show the relationship between the observed spread and the individual cortical columns or modules, the spread function was projected onto a histological section of cytochrome-oxidase blobs (representing cortical modules involved in colour and spatial-frequency processing). The stimulus used caused spiking activity only in neurons in the marked small square, which contained just four cortical modules. However, more than 250 cortical modules had access to the information carried by the signal spread, albeit at lower amplitude (greater than $1/e^2$). Such a large spread has been confirmed by intracellular recordings[64]. The apparent 'space constant' for the spread was 1.5 mm along the cortical axis parallel to the ocular-dominance columns, and 3 mm along the perpendicular axis. The spread velocity was 0.1–0.2 ms^{-1} (REF. 30). Similar results were obtained in the frog optic tectum[4] and the rat barrel cortex[25,51,65–67], where both excitatory spread (see online supplementary information S4 (movie)) and a delayed, inhibitory surround[67] (see online supplementary information S5 (movie)) were observed over long distances.

The extensive lateral spread observed in layer 2/3, far beyond the retinotopic borders in layer 4, indicates that the degree of distributed processing in the primary visual cortex might be much larger than is often conceived; the ability of a local stimulus to affect 250 cortical modules, rather than just 4, presents a challenge for theoreticians studying cortical networks. Such modelling would surely shed new light on how the global perception of simple objects is affected by their background.

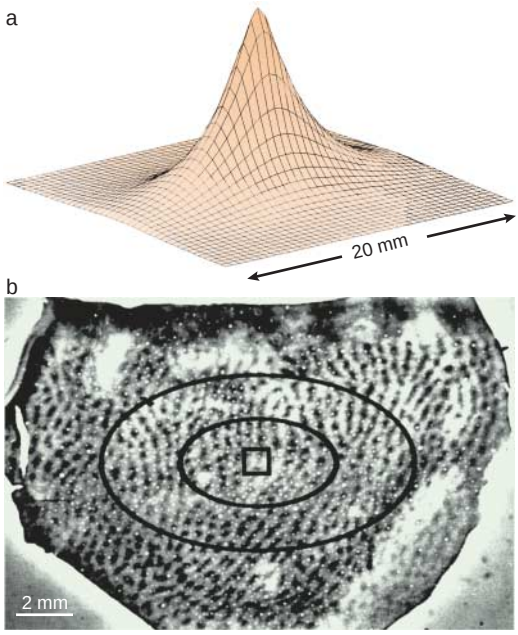

Figure 2 | **Many functional domains are activated during the processing of a small retinal image. a** | Calculation of the activity spread from a small patch in layer 4 (a 1 x 1 mm square) within the upper cortical layers of a macaque. Such cortical activation was produced by a retinal image of approximately 0.50 x 0.25° that was presented to both eyes. The 'space constants' for the exponential activity spread measured with the dye experiments were 1.5 mm and 2.9 mm perpendicular and parallel to the vertical meridian, respectively. **b** | Direct activation in layer 4 (of the area within the square) and the spread in layers 2/3 (elliptical contours) are shown superimposed on a histological section showing the mosaics of cytochrome oxidase blobs, close to the border between cortical areas V1 and V2. The centre ellipse shows the contour at which the amplitude of cortical activity drops to 37% of its peak. The larger ellipse shows the contour at which the spread amplitude drops to 14%. More than 10,000,000 neurons reside in the cortical area bounded by the large ellipse containing a regular mosaic of about 250 blobs. Reproduced, with permission, from REF. 30 © (1994) Society for Neuroscience.

The dynamics of shape processing. Although ORIENTATION SELECTIVITY in the visual cortex[3] has been explored for decades[68], the question of the interplay between feed-forward processes and intracortical connections could not be resolved using traditional techniques. The dynamics of orientation tuning have remained unclear, as have the contributions of intracortical orientation-specific excitatory and inhibitory connections.

An early study of the dynamics of orientation selectivity by VSDI[39] missed the different dynamics of cortical responses to optimal and null stimuli because only differential imaging[35] was used. Imaging the differential responses to orthogonal orientation stimuli provides a better signal-to-noise ratio (see online supplementary

ORIENTATION SELECTIVITY
A property of visual cortex neurons that allows the detection of bars and edges within visual images and the encoding of their orientations. As the cortex is organized in columns, neurons that belong to the same column share the same orientation tuning.

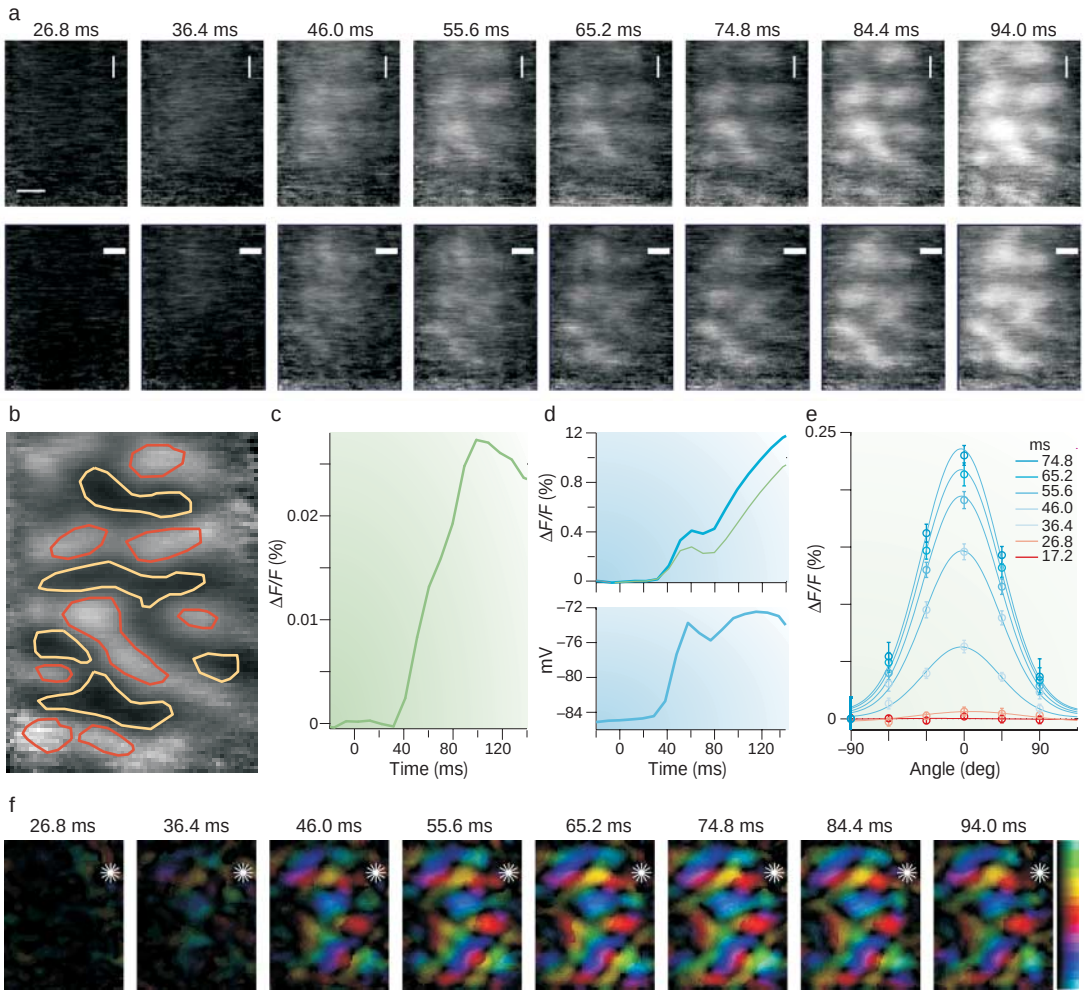

Figure 3 | **Orientation tuning is constant, but dynamic orientation-selective, intracortical suppression shapes the final response**. **a** | Time-series of single-condition orientation maps in the anaesthetized cat produced in response to two orthogonal visual stimuli (top and bottom rows). **b** | Spatial pattern revealed by differential imaging. Red marks cortical regions activated by vertical gratings, yellow by horizontal gratings. **c** | Temporal pattern of differential activity. The evoked DA (deceleration–acceleration) notch is barely visible. **d** | Top, time-course of the evoked response to preferred (blue) and orthogonal (green) orientations. The two responses have the same onset latency but the response to the preferred orientation is larger from the beginning. A striking feature of the evoked responses is the DA notch, confirmed with intracellular recordings (bottom)[49]. **e** | Orientation tuning curves at different times after stimulus onset. The curves after response onset (blue) have the same shape but different amplitudes. The baseline of the curves was shifted to facilitate comparison. **f** | Time-series of orientation-preference 'polar maps': colour represents preferred orientation (top to bottom of colour scale on right is 0–180°), brightness represents the amplitude of the selective response and black spots indicate large but non-selective responses. Preferred orientation is steady from response onset. Modified, with permission, from REF. 71 © (2002) American Association for the Advancement of Science.

information S6 (movie)), but information regarding the dynamics of the individual responses is lost. Therefore, the ultimate goal for functional brain mapping is single-condition mapping[69,70] — acquiring the pattern of activation evoked by a single stimulus, without the need to perform differential imaging of orthogonal stimuli. This is also important because in most cases truly orthogonal stimuli cannot be defined. Technical advances in VSDI have made it possible to obtain time-series of single-condition orientation maps millisecond by millisecond, at sub-columnar resolution[71] (FIG. 3a) and to construct detailed 'polar maps'[33] as depicted in FIG. 3f (see online supplementary information S7 (movie)).

Two types of mechanism have been proposed to contribute to the emergence of orientation-selective responses in cortical visual neurons, a property not shared by their thalamic inputs. In feed-forward models, such responses arise from appropriate alignment of thalamic input, whereas in recurrent models, intracortical interactions are more important. Using VSDI to measure the dynamics of the input to the visual cortex can shed light on this question, because the feed-forward model predicts that orientation selectivity should remain constant with time from stimulus onset, whereas if recurrent interactions are important then selectivity should change as cortical processing takes place.

To investigate the dynamics of orientation selectivity, high-quality single-condition maps were obtained[71]. A time-series of the initial response is shown in FIG. 3a for two orthogonal orientations. As soon as the response

can be observed, the two orthogonal stimuli preferentially activate complementary patches of cortex, as seen in the differential map (FIG. 3b). The amplitude of the difference between the responses to the two stimuli increases at the beginning of the response, and peaks at about 100 ms (FIG. 3c). Single-condition responses (FIG. 3d) were used to calculate orientation tuning. These show the average, over many pixels, of the response to the preferred and orthogonal stimuli. The tuning curves at each timepoint could be calculated directly from the single-condition responses (FIG. 3e). The immediate impression is that of tuning curves with a constant shape but changing amplitude. Indeed, the half-width at half-height of tuning curves was steady from response onset (30–40 ms after stimulus onset). So, sustained intracortical processing does not seem to be necessary — at least for most neurons — to determine the angle of orientation tuning.

Orientation selectivity is also affected by other aspects of the response. The modulation depth of the response (the difference between preferred and orthogonal) changes over time, decreasing after a peak at 100 ms (FIG. 3c). An intriguing phenomenon is nearly undetectable in the differential time-course, but obvious when considering the evoked single-condition responses (FIG. 3d). There is a notch in the evoked response, equivalent to a deceleration followed by acceleration in the rise-time — this is termed the 'evoked DA (deceleration –acceleration) notch'. It is probably caused by a suppressive mechanism that peaks at 50–80 ms, as might be expected with intracortical processing. The evoked DA notch is more pronounced in response to the orthogonal stimulus than the preferred (FIG. 3d).

These results indicate that thalamic input might be the main determinant of orientation-tuning width for most cortical neurons, but that intracortical processing amplifies the orientation-selective component of the response. They also suggest that intracortical suppression contributes to this process by preventing the orthogonal response from increasing as rapidly as the response to the preferred orientation.

Cortical correlates of a visual illusion. Visual illusions can reveal fundamental aspects of cortical processing. The illusion of motion that can be produced by non-moving stimuli was described almost a century ago by Gestalt psychologists[72,73]. Although there is psychophysical evidence for top-down involvement of higher-cortical areas in such illusions, others argue for bottom-up (that is, stimulus-induced activity in early visual areas) mechanisms at early processing stages[74–80]. However, none of these studies has directly investigated the underlying neuronal mechanisms. VSDI is highly sensitive to subthreshold activity, and is therefore suitable for exploring the cortical mechanisms that underlie the 'line-motion' illusion[74], in which a stationary square followed immediately by a long, stationary bar produces the illusion that the bar 'sweeps' away from the square (FIG. 4a). VSDI was used to image the responses of the anaesthetized-cat visual cortex to five stimuli[81]: a stationary small square; a stationary long bar; a moving square; a drawn-out bar; and the line-motion illusion, a stationary square briefly preceding a long stationary bar (see online supplementary information S8 (movie)). Flashing the bar alone evoked the expected localized, short-latency and high-amplitude activity patterns, crossing threshold at all of the three regions of interest (ROIs) simultaneously (FIG. 4d, black traces). However, presenting a square 60–100 ms before a bar induced dynamic activity patterns, which resembled those produced by fast movement (blue traces crossing threshold at different times for the three adjacent ROIs). Although it did not move, the preceding small square created gradually propagating subthreshold cortical activity (see online supplementary information S9 (movie)) that correlated with illusory motion because it was indistinguishable from cortical representations of real motion in this area. These findings emphasize the effect of spatio-temporal patterns of subthreshold synaptic potentials on cortical processing and the shaping of perception[81].

It has been reported that when multiple inducers of the illusion (or different stimulus features such as colour, contrast or texture) are used, there is evidence for a second, slower, top-down component. Furthermore, illusory line motion can be modulated by attention, or can be voluntarily induced by nonretinotopic mechanisms, even across sensory modalities. Such controversies in psychophysical studies underscore the importance of physiological measurements.

As the onset of any stimulus will create an intricate spatio-temporal pattern of spreading subthreshold-synaptic activity (FIG. 4d, the bottom two red traces), the conclusion must be that subthreshold-synaptic spread serves a fundamental processing strategy; such 'priming' of activity should strongly affect the spatio-temporal integration of any subsequent sensory input (compare the black and the blue traces in FIG. 4d). Top-down processing and attentional mechanisms remain to be explored in behaving subjects, by bringing together psychophysics and fast functional imaging.

Visualization of neuronal assemblies. Hebb suggested that neocortical neurons operate in assemblies[82] — networks of neurons, local or widespread, that communicate coherently to carry out the computations that are required for various behavioural tasks. This means that the neurons at a given pixel in VSDI might belong to different neuronal assemblies. To explore cortical computations, it is necessary to measure the dynamics of widespread individual neuronal assemblies, rather than the activity originating from heterogeneous populations. A significant step forward in real-time optical imaging has been the visualization of the dynamics of coherent neuronal assemblies by combining VSDI with single-unit or multiple-unit recording. First, a single unit with the desired tuning properties is selected. Second, VSDI and unit recordings are conducted for a long time without any SIGNAL AVERAGING. During off-line analysis, which involves performing spike-triggered averaging on the optical data, the firing of a single neuron serves as a time reference to selectively visualize only the population activity that was synchronized with it. With a sufficient number of spikes, any neuronal activity not time-locked

SIGNAL AVERAGING
A standard procedure used to improve the signal-to-noise ratio. Adding the results of repeated trials means that if a signal is reproducible, it adds up, whereas random noise is averaged out. However, if the signal is variable the true dynamics cannot be explored. It is, therefore, highly significant when VSDI provides large signals in a single trial — as is shown here.

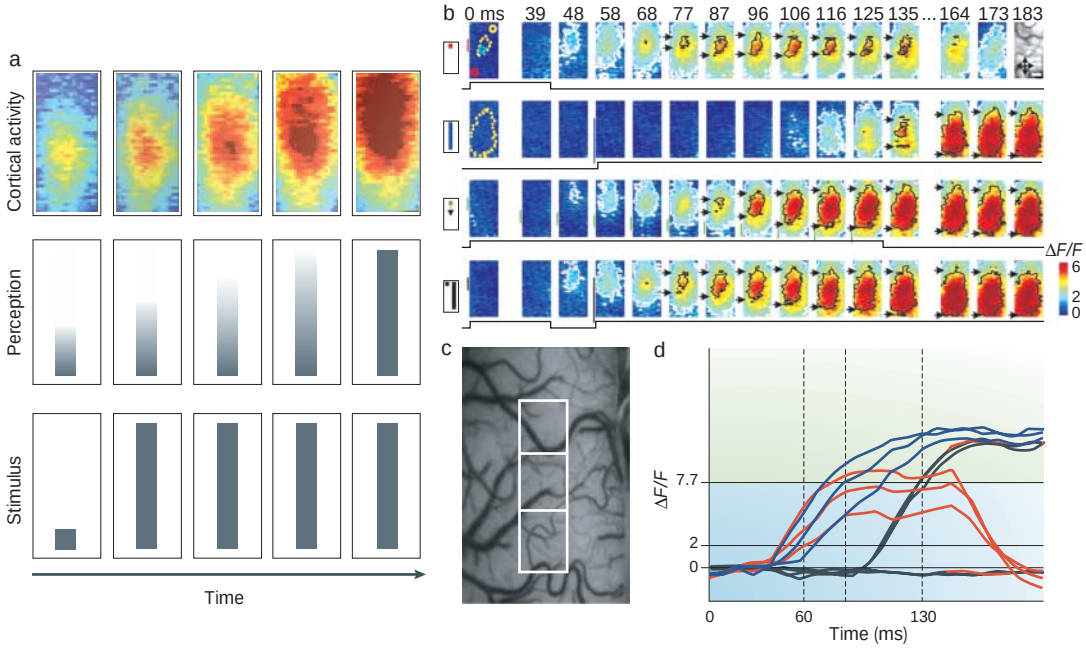

Figure 4 | **Priming by subthreshold spread of synaptic potential can account for the illusion of motion perception.** **a** | Schematic illustration of the two stationary stimuli (square and bar, bottom), the perceived stimulus (middle) and the evoked cortical activity (top) over time. **b** | Time series of the cortical representations of (from top to bottom) the stationary square and bars alone, the moving small square and the illusory stimuli. Yellow dotted contours shown at 0 ms approximate the retinotopic representation of the stimuli; white contours delimit low-amplitude activity ($p < 0.05$). Coloured vertical lines shown to the left of individual panels after stimulus onset indicate estimated sizes (or trajectory) of the stimuli along a posterior–anterior axis. Time in milliseconds after stimulus onset is indicated at the top. The time courses of stimulation are outlined at the bottom of each row. **c** | The cortical area imaged with three contiguous regions of interest (ROIs, white squares) from which the observed time courses of cortical responses were derived. **d** | Responses to the small square (red), the line-motion condition (delayed bar after the square; blue) and identical flashed bar alone (black). Note the large effect of the priming by the square on the latency of the response to the flashed bar (black traces compared with blue traces). Modified, with permission, from REF. 81 © (2004) Macmillan Magazines Ltd.

to the reference neuron is averaged out, enabling the selective visualization of those cortical locations in which activity consistently occurred coherently with the firing of the reference neuron[83,84]. This approach has been used to explore the spatio-temporal characteristics of ongoing cortical activity.

The dynamics of ongoing activity. Even in primary sensory areas, there is a large amount of electrical activity, primarily subthreshold activity, in the absence of sensory input. Some of this activity is coherent over large cortical areas. Is this spontaneous activity just network noise? How large it is relative to evoked activity? Does it affect evoked activity? And does it have an important functional role? VSDI is ideally suited to exploring such questions at the level of neuronal assemblies.

To compare the amplitude of coherent, ongoing activity with that of evoked activity (both representing large subthreshold activity, to which VSDI is sensitive), spike-triggered averaging was used during both spontaneous- and evoked-activity imaging sessions with anaesthetized cats. Surprisingly, the amplitude of coherent, spontaneous, ongoing activity in neuronal assemblies was nearly as large as that of evoked activity[84,85]. Large, coherent, ongoing activity was also found in the rat somatosensory cortex[51,86]. The large fluctuations of ongoing activity affect how far cortical neurons are from their firing threshold. Therefore, the amplitude of ongoing activity indicates that it might have an important role in shaping spatio-temporal patterns evoked by sensory stimuli.

In the mammalian visual cortex, evoked responses to repeated presentations of the same stimulus are variable, whether measured by single- or multiple-unit activity, local field potential or VSD signals. Does ongoing activity account for this variability? Arieli *et al.*[87] found that the variability can be accounted for by the spatio-temporal patterns of ongoing activity, reflecting the dynamic states of neocortical networks. The central finding in the initial studies of ongoing activity, using VSDI without signal averaging, was that despite this large variability, the evoked responses in single trials could be predicted precisely by taking into account the preceding ongoing activity. The evoked response in a given single-stimulus presentation is well predicted by summing the evoked response itself, which can be obtained by averaging over many presentations with the preceding ongoing activity (inital state). This prediction is valid providing the ongoing activity pattern, which presumably continues to change during the evoked response, is similar to the initial state[87].

These findings indicate that old notions of 'noise' in brain activity might have to be revised. As ongoing activity is often considerable, it might have an important role

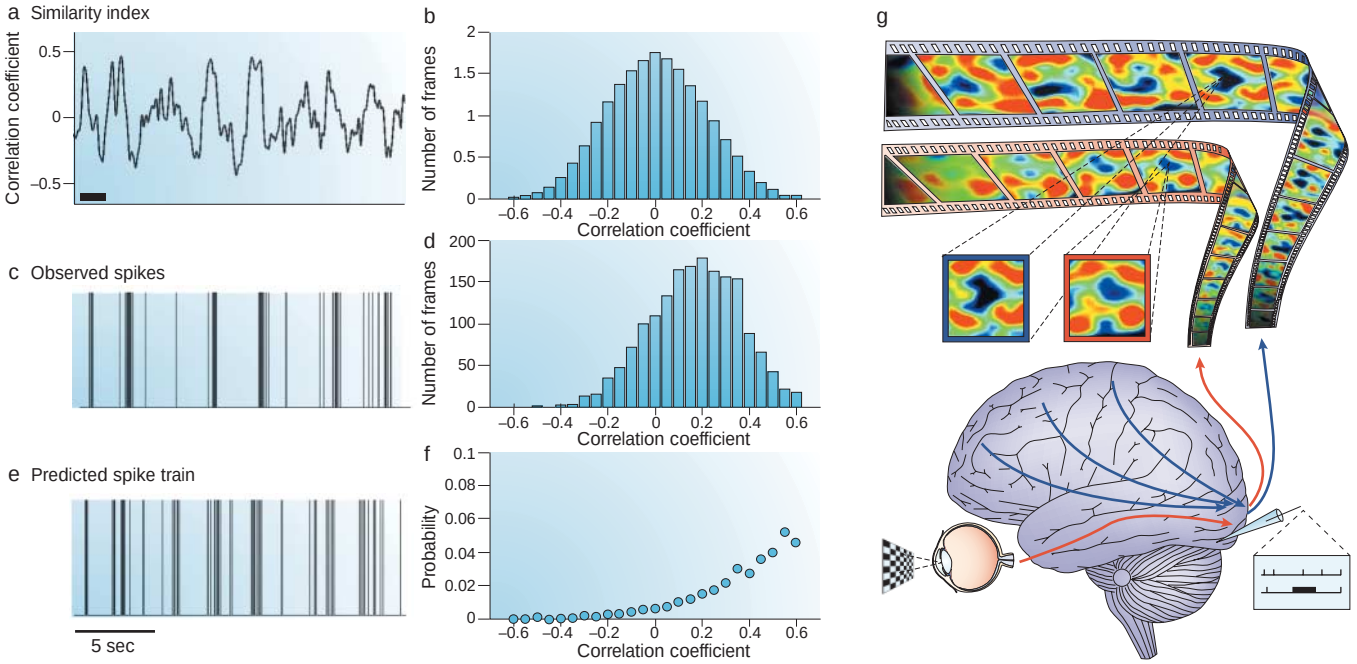

Figure 5 | **A spontaneous spike train of the action potentials of a single neuron can be predicted from the similarity of spatio-temporal patterns of spontaneous activity to the functional architecture. a** | The similarity index (correlation coefficient) for the instantaneous snapshot of population activity and the relevant functional architecture. **b** | Histogram of the correlation coefficient for 20,480 snapshots. **c** | The observed spike train: a strong upswing in the values of the similarity index is seen in panel **a** each time the neuron emits a burst of action potentials. Every strong burst is followed by a marked downswing in the values of the correlation coefficient. **d** | Histogram of the similarity index, calculated only during the occurrence of spikes in 338 snapshots highly skewed towards larger correlation coefficients. **e** | Predicted instantaneous firing rate of a neuron, given a specific similarity index, showing marked similarity to the bursting features in the observed spike train. **f** | Same as **d**, but normalized to the entire distribution shown in panel **b**. **g** | Dynamic brain activity in a given region is a combination of spontaneous internal activity in many cortical areas (blue stream) and the evoked responses (red). The spatial pattern that corresponds to spontaneous action potentials (blue frame) obtained by spike-triggered averaging and the evoked activity (red frame) are nearly identical. Modified, with permission, from REF. 85 © (1999) American Association for the Advancement of Science.

in cortical function. This is because ongoing activity primes the cortex by dynamically varying the distance to threshold and, therefore, affecting the response to any incoming stimulus. So, ongoing activity might provide the neuronal substrate for the dependence of sensory-information processing on context, attention, behavioural and consciousness states, memory retrieval and other aspects of cognitive function. Recent preliminary experiments have shown that behaviour is also affected by ongoing activity[88].

Population predicts single cell spike train. Tsodyks *et al.*[85] found that the instantaneous spatial pattern of population activity and of a single neuron's spikes were very similar during spontaneous activity and evoked activity (see online supplementary information S10 (movie)). Furthermore, the instantaneous firing rate of a spontaneously active single neuron depended strongly on the instantaneous spatial pattern of ongoing population activity in a large cortical area. During spontaneous activity, whenever the instantaneous spatial-population pattern correlated highly with the spatial-population pattern evoked by the preferred stimulus (FIG. 5a) of the recorded neuron, its firing rate increased (FIG. 5c). This discovery made it possible to reconstruct the spontaneous activity of single neurons on the basis of global, spontaneous activity (FIG. 5e). The ability to predict the main features of a spontaneous spike train in a single cell, on the basis of the global-population activity (largely subthreshold) of thousands of neurons, was a striking result[85].

Dynamics of spontaneous cortical states. The idea that network activity can converge to cortical states is a central concept in theoretical brain research. What are the dynamics of intrinsic cortical states? It is now possible to visualize certain aspects of cortical networks and their states in action, at a high spatio-temporal resolution. Such studies can provide insights into the dynamic interplay between activated internal cortical representations and incoming sensory input.

Using VSDI, Kenet *et al.* showed[89] that ongoing activity in the visual cortex of the anaesthetized cat comprises dynamically switching intrinsic cortical states, many of which correspond closely to orientation maps (see online supplementary information S3a (figure)). Using VSDI, we can now see both spontaneously emerging and evoked orientation maps, in a single 10-ms snapshot. Cortical states that correspond to all orientations appeared spontaneously and, therefore, it was possible to reconstruct the entire 'angle map' from spontaneous activity (see online

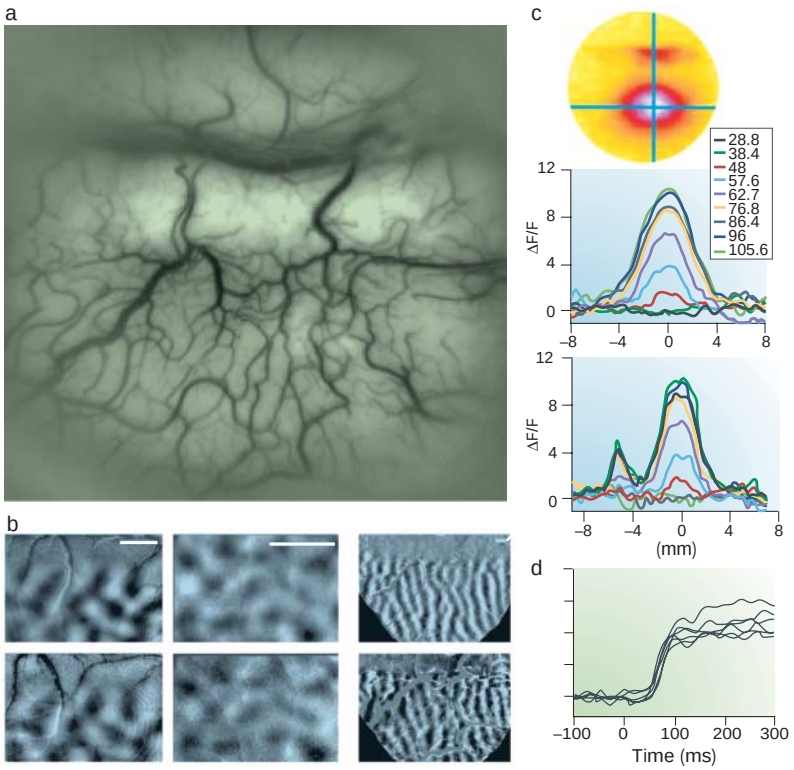

Figure 6 | **Chronic imaging in behaving monkeys. a** | A large area of exposed monkey cortex in perfect condition after almost a year of repeated VSDI, intrinsic imaging and electrical recording. **b** | The functional VSDI maps of ocular dominance (left and right) and orientation (middle), which were recorded from the same patch of cortex in recording sessions separated by up to a year, are virtually identical (compare top and bottom images). **c** | Dynamics of retinotopic activation. Top: retinotopic activation of V1 and V2 (width of cortical area about 14 mm). Bottom: the spread of cortical dynamics along horizontal (upper) and vertical (lower) axes as a function of time — marked by different colours. **d** | Single-trial evoked responses in the awake monkey. As in the anaesthetized animal, the response is variable. Modified, with permission, from REF. 90 © (2002) American Physiological Society.

HYPERCOLUMN
In the visual cortex, an orientation hypercolumn refers to a patch of cortex containing several cortical columns, in which neurons which have similar spatial receptive fields but that cover all possible preferred orientations are found. This concept can be generalized to other visual attributes and to other sensory and motor areas.

BEHAVING MONKEYS
The behaving monkey is often the ideal model for studying higher cognitive functions in relation to human behaviour. Unlike rodents, monkeys can be readily trained to perform complicated tasks that are difficule even for talented students. This model can be used to combine various techniques that are not applicable to human studies, and to give insight into the workings of the primate brain.

supplementary information S3b (figure)). When such an orientation state emerged spontaneously, it spanned several cortical HYPERCOLUMNS and was usually followed by a state that corresponded to a proximal orientation, or dissolved into an unrecognized state or noisy pattern, after a few tens of milliseconds. This dynamic behaviour is depicted in online supplementary information S3c (figure) and online supplementary information S11 (movie). Hypothetically, such dynamically switching cortical states could express the brain's internal context, representing and influencing memory, perception and behaviour.

Testing proposals such as this requires the use of awake, BEHAVING MONKEYS rather than anaesthetized animals. In preliminary experiments on awake monkeys, coherent activity was detected, although the amplitude of ongoing activity was much lower than in anaesthetized monkeys, as expected from the lower amplitude of the electroencephalogram in an awake subject.

Long-term imaging in behaving monkeys. Chronic optical imaging makes it possible to explore the spatial and temporal patterns that underlie higher cognitive functions in the neocortex of behaving monkeys. Slovin *et al.*[90] imaged cortical dynamics with high spatial and temporal resolution from the same patch of cortex for up to 1 year (FIG. 6a).

In the original study, the visual cortices of trained macaques were stained 1–3 times a week. Immediately after each staining session, the monkey started to carry out a simple behavioural task, while areas of visual or motor cortex were imaged using VSDI. The functional maps obtained (FIG. 6b) were confirmed by imaging based on intrinsic signals over a period of up to a year. This confirmation, as well as behavioural performance, indicated that any pharmacological side effects or photodynamic damage were negligible. Online supplementary information S12 (movie) shows the cortical dynamics as the monkey makes a saccade. Additional findings on the visual and motor cortices[56,57] of the behaving monkey will be described elsewhere. Evoked activity could also be detected without signal averaging, as in the above-mentioned experiments in anaesthetized cats (FIG. 6d). This finding indicates that cortical dynamics *per se* can now be explored in awake behaving monkeys.

Cortical development and plasticity. Studies of neocortical development and plasticity in the adult cortex are central topics in neuroscience. Many interesting questions cannot be answered by carrying out acute experiments, and require long-term imaging. Initially, experiments of this type benefited from the advantages of optical imaging based on intrinsic signals. Particularly important were the pioneering findings by Frostig *et al.*[34] that cortical maps could be visualized through the intact dura and a thinned, but closed, skull[91,92]. This proved useful in the pioneering studies carried out by the Bonhoeffer and Stryker groups[93–96] into the development of neocortical maps, and by Frostig and colleagues[97,98], who investigated the plasticity of cortical sensory maps in response to manipulation of the input or experience-dependent modifications.

Recent advances in VSDI allow similar longitudinal studies of cortical development and plasticity. In preliminary experiments, Petersen and colleagues[99] used VSDI to investigate how a sensory map in the developing rat barrel cortex, and its underlying synaptic connectivity, are altered when the sensory input to the cortex is partially removed by cutting rows of whiskers. The whiskers of rows A, B and C were trimmed every day from postnatal day (P)7 onwards. VSDI was used to image the spatiotemporal dynamics of the sensory response in layer 2/3 evoked by a brief deflection of the D2 whisker in control and partially deprived animals. Whereas in the cortex of the control rats the spread of activity to adjacent rows was symmetrical, in deprived rats the activity spread was asymmetric, spreading more towards the rows of non-deprived whiskers. Sakmann's group is combining chronic VSDI, chronic intrinsic imaging, patch recording and anatomic reconstructions of single cells to determine the nature of the synaptic interactions that mediate neocortical function. Pharmacological manipulations, in which antagonists were added, and then washed[86], have also been used with repeated VSDI sessions to help to determine the receptors involved. This integrated approach underscores the importance of combining

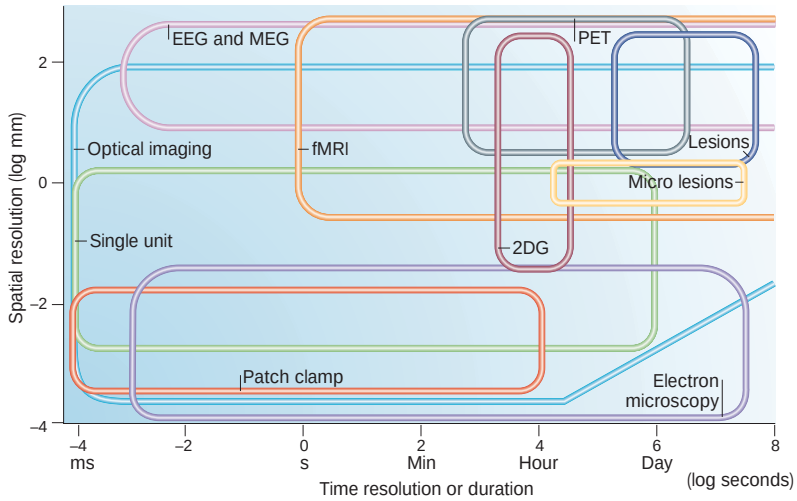

Figure 7 | **Some available tools to meet the challenges of neuroscience.** The spatio-temporal capabilities of each technique are depicted by the coloured rectangles. Optical imaging covers almost the entire area (including VSDI, imaging based on intrinsic signals, ion imaging (such as calcium or sodium), confocal imaging, multi-photon imaging and more). Fortunately, several optical imaging techniques can be combined in a single experiment. Other valuable techniques can also be integrated with optical imaging. EEG, electroencephalography; fMRI, functional MRI; MEG, magnetoencephalography; PET, positron emission tomography; 2DG, 2-deoxyglucose post-mortem histology.

VSDI with other techniques on the same patch of cortex, and of bridging the gap between single cells, their synaptic interactions and population activity.

Combining other technologies with VSDI

As the third millennium begins, neuroscientists are striving for a better understanding of brain development, function and malfunction at several levels: molecular, synaptic, cellular, circuit and whole-brain (FIG. 7). This enormous challenge requires tools that cover 9 orders of magnitude in temporal resolution and 12 orders of magnitude in spatial resolution or experimental duration[100]. Existing techniques have both advantages and limitations. Therefore, it is desirable to combine as many techniques as possible into a single experiment, on the same brain, including powerful approaches that are not discussed here. At present, VSDI offers the highest temporal and spatial resolution of *in vivo* imaging techniques, and is acutely sensitive to subthreshold synaptic potentials of populations of cortical neurons. It also provides an effective way to obtain a more complete description of individual neurons and active neural integration of multiple inputs by their processes, which can be achieved by using voltage imaging with intracellular dyes[101] injected into single neurons *in vivo*. Zecevic and colleagues[102] perfected this approach with submillisecond resolution, and investigated the function of single mitral cells (the principal output neurons in the rat olfactory bulb)[103].

Another advantage of VSDI is that it can be readily combined with other optical-imaging tools such as ion imaging, particularly of sodium and calcium signals[104]; with confocal microscopy or two-photon fast imaging[105]; or with photostimulation[106] of neurons or cortical regions, using either patterned stimulation or selective photostimulation based on specific transmitters or receptors[107]. VSDI can also be used to add the temporal domain to the static images obtained by functional MRI in monkeys[108]. However, VSDI also has limitations: only exposed areas can be explored, and, at present, it is not possible to record from deep brain regions, although miniature 'lightguides', implanted imaging arrays or two-photon microendoscopy[109] might solve this problem. Furthermore, VSDI is not sensitive to spiking, so it can image the input clearly but the output only crudely[81]. Recently, Konnerth and colleagues[110] were able to record spike trains from hundreds of neurons using calcium indicators and two-photon imaging *in vivo*. This promising approach should be combined with VSDI imaging; the former will provide information about the output from hundreds of neurons whereas the latter is most sensitive to the spatio-temporal patterns of the input.

Conclusions and outlook

Several groups, including those of Waggoner[7,8], Loew[11] and Fromherz[111], as well as our own, have made significant progress in developing VSDI during the last three decades by the design and synthesis of new VSDs. Nevertheless, the signal-to-noise ratio that can be obtained with dyes, and the photodynamic damage they can cause, are limiting factors for better VSDI. Furthermore, different preparations often require dyes with different properties[112–114]. Even different cortical areas in the same species can require different dyes (for example, one dye provided a high-quality signal in the rat somatosensory cortex but not in the rat olfactory bulb, although the bulb was well stained[115]). Therefore, continuing efforts to make better dyes and new biophysical and optical innovations are important. Similarly, the development of new genetically engineered *in vivo* probes[116,117] will make experiments easier and improve the results. Of particular importance are probes that will stain only specific cell types and/or specific cellular compartments (axons, somata or dendrites). Additional technological developments using MULTI-PHOTON imaging, adaptive optics or other optical innovations to achieve three-dimensional imaging are desirable (for example, using special lenses that offer a shallow field of view[118]). The combination of existing VSD probes with fluorescence resonance energy transfer (FRET) to detect electrical activity is also promising[119]. Similarly, excitation at the red edge of existing VSDs has been shown to provide up to ten-fold larger fractional change in the VSD signal size[111,120], and imaging at the second harmonic frequency of new VSDs[121,122] might also provide larger signals, enhancing the signal-to-noise ratio. The amount and nature of the data that have been, and will be, accumulated necessitate further methods of analysis, modelling and theoretical research that should lead to new conceptual frameworks regarding cortical function.

The examples presented here show that VSDI already enables neuroscientists to explore the neocortex in ways that were not previously feasible. To increase the scope of meaningful neurophysiological data obtained from the same patch of cortex, VSDI should be combined with targeted-tracer injections, retrograde labelling,

MULTI-PHOTON MICROSCOPY
A form of microscopy in which a fluorochrome that would normally be excited by a single photon is stimulated quasi-simultaneously by several photons of lower energy. Under these conditions, fluorescence increases as a function of the square of the light intensity, and decreases approximately as the square of the distance from the focus. Because of this behaviour, only fluorochrome molecules near the plane of focus are excited, greatly reducing light scattering and photodamage to the sample.

microstimulation and intracellular and extracellular recording. VSDI combined with electrical recordings enables neuroscientists to obtain information about spatio-temporal patterns of activity in coherent neuronal assemblies in the neocortex, at sub-columnar resolution. At present, no alternative imaging technique for visualizing organization and function in the living brain provides a comparable spatial and temporal resolution. This level of resolution allows us to address questions of both where and when processing takes place, providing great promise that this technique, integrated with other approaches, will contribute to the study of how processing occurs. It is likely that every higher cognitive function is represented in highly specific spatio-temporal patterns. The challenge is to reveal the fundamental principles of neural coding, the processing strategies and the spatio-temporal representations of perception, memories and underlying experience-dependent plastic changes, at the levels of both the single cell and the neuronal assembly.

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Competing interests statement
The authors declare competing financial interests: see Web version for details.

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