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Optical dissection of brain circuits with patterned illumination through the phase modulation of light

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HIGHLIGHTS

- Optical investigation of brain circuits: challenges and limitations.
- Phase modulation with liquid crystal optical modulators: principles and optical setup.
- Phase modulation for patterned photo-stimulation.
- Phase modulation for scanless imaging.
- Future applications of phase modulation in neuroscience.

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ABSTRACT

Brain function relies on electrical signaling among ensembles of neurons. These signals are encoded in space – neurons are organized in complex three-dimensional networks – and in time—cells generate electrical signals on a millisecond scale. How the spatial and temporal structure of these signals controls higher brain functions is largely unknown. The recent advent of novel molecules that manipulate and monitor electrical activity in genetically identified cells provides, for the first time, the ability to causally test the contribution of specific cell subpopulations in these complex brain phenomena. However, most of the commonly used approaches are limited in their ability to illuminate brain tissue with high spatial and temporal precision. In this review article, we focus on one technique, patterned illumination through the phase modulation of light using liquid crystal spatial light modulators (LC-SLMs), which has the potential to overcome some of the major limitations of current experimental approaches.

Contents

Abbreviations: AM, acetoxymethyl; Arch, archaerhodopsin; AODs, acousto-optic deflectors; ChR2, channelrhodopsin-2; DMD, digital micromirror devices; DOE, diffractive optical element; eMS2PM, encoded multisite two-photon microscopy; GPC, generalized phase contrast; Halo, halorhodopsin; LC-SLM, liquid crystal spatial light modulator; LED, light emitting diode; TF, temporal focusing.

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1. Introduction

Brain function stems from the coordinated activity of neurons, the cellular components of the brain. Neuronal cells are organized in complex three-dimensional circuits that contain tens of different cellular subtypes organized in highly specialized functional subnetworks (Kandel et al., 2012; Bear et al., 2006; Purves et al., 2011). A key question in neuroscience is how activity patterns in these complex cellular circuits control higher brain function and behavior (Mainen and Sejnowski, 1995; Cohen and Maunsell, 2009; Mitchell et al., 2009; Averbeck et al., 2006; Buzsaki, 2006; O'Connor et al., 2009). For example, in primary sensory areas of the rodent brain, the presentation of an external stimulus (*e.g.*, a visual input or the deflection of a single mystacial whisker) generates complex spatial and temporal patterns of activation (Fig. 1) in neurons across different brain regions (*e.g.*, sensory cortices) (Stosiek et al., 2003; Ohki et al., 2005, 2006; Kerr et al., 2007; Grewe et al., 2010; Ko et al., 2011; Froudarakis et al., 2014). How do these patterns generate the perception of a sensory experience? How are different features of the external stimulus encoded in the spatial and temporal domain? Addressing these questions has been traditionally challenging due to difficulties in manipulating the activity of specific neurons on a rapid timescale (neuronal responses to external sensory stimuli can be as short as a few milliseconds). Advances in optics (Emiliani et al., 2005; Reddy and Saggau, 2005; Duemani Reddy et al., 2008; Papagiakoumou et al., 2008, 2009; Grewe et al., 2010, 2011; Katona et al., 2012; Ahrens et al., 2013) and the development of molecules for detecting (Tsien, 1980, 1981; Miyawaki et al., 1997, 1999; Pologruto et al., 2004; Griesbeck et al., 2001; Nakai et al., 2001; Nagai et al., 2001; Heim et al., 2007; Akemann et al., 2012; Chen et al., 2013; Knopfel, 2012) and manipulating neuronal activity (Zemelman et al., 2002; Nagel et al., 2003; Lima and Miesenbock, 2005; Boyden et al., 2005; Volgraf et al., 2006; Zhang et al., 2007a,b, 2010; Szobota et al., 2007; Janovjak et al., 2010; Gradinaru et al., 2009; Sohal et al., 2009; Szobota and Isacoff, 2010; Levitz et al., 2013) has revolutionized the study of the central nervous system and our ability to tackle these types of questions (Knopfel et al., 2010). For example, optogenetics allows the generation or suppression of electrical activity in genetically identified cells in the intact brain (Adamantidis et al., 2007; Gradinaru et al., 2009; Tsai et al., 2009; Kravitz et al., 2010; Witten et al., 2010; Beltramo et al., 2013), and the development of genetically encoded fluorescence indicators permits brain networks to be imaged with subcellular resolution (O'Connor et al., 2010; Harvey et al., 2012; Huber et al., 2012; Akerboom et al., 2012; Chen et al., 2012, 2013; Zariwala et al., 2012; Dombeck et al., 2010; Cui et al., 2013; Dal Maschio et al., 2012a; Bovetti et al., 2014).

Optogenetics is based on the use of light-sensitive molecules that depolarize (excitatory opsins) or hyperpolarize (inhibitory opsins) cells upon illumination with an appropriate wavelength (Miesenbock, 2004, 2011; Miesenbock and Kevrekidis, 2005; Deisseroth et al., 2006; Zhang et al., 2007a, 2010; Fenno et al., 2011). Over the last several years, the toolkit of available molecules for these types of experiments has expanded enormously, and it is now possible to choose opsins based on specific properties including absorption spectra, selectivity for particular ions, conductance, photosensitivity, response kinetics and subcellular localization (Zhang et al., 2008; Berndt et al., 2009, 2014; Gunaydin et al., 2010; Gradinaru et al., 2010; Chow et al., 2010; Mattis et al., 2012; Prigge et al., 2012; Chuong et al., 2014; Klapoetke et al., 2014). The use of these molecules has allowed the role of specific cellular subtypes in controlling network activity and driving behavior under physiological and pathological conditions to be causally tested (Fenno et al., 2011; Tye and Deisseroth, 2012). However, these studies have also highlighted some of the limitations of the current approaches for specific applications (Peron and Svoboda, 2011; Vaziri and Emiliani, 2012; Packer et al., 2013). For example, in most *in vivo* optogenetic studies it is difficult to quantify how many cells are engaged by the optical stimulation and how firing properties are modified during light illumination. The outcome of an optogenetic manipulation, in terms of action potentials, is the integrated effect of how many photons reach the opsin-expressing cells (which is difficult to evaluate in the intact brain due to scattering and absorption), the expression level of the opsin within the cell (which can vary significantly from cell to cell) and the biophysical properties of the neurons under investigation (for example, neurons with high input resistance and low rheobase require less light-induced current to reach the action potential threshold). For individual cells, the pattern of action potentials elicited by optical stimulation can be precisely determined with intracellular electrophysiological recordings (Zemelman et al., 2002; Boyden et al., 2005; Zhang et al., 2007b; Chow et al., 2010). However, this approach cannot be extended to the tens/hundreds of cells that are likely to be recruited in most optogenetic studies. For this goal, an optical approach to monitor the neuronal activity of multiple cells would be ideal (Hausser, 2014), but coupling functional imaging with cellular resolution *during* optogenetic manipulation has proven to be challenging because single-photon light that is used for opsin activation leads to saturation of the fluorescence detector (Wilson et al., 2013). Moreover, opsin activation *in vivo* is mostly performed by placing a light emitting diode (LED) or a fiber optic (Cardin et al., 2010; Zhang et al., 2010) close to the region to be illuminated (wide field illumination, Fig. 2a). This illumination scheme does not allow spatial control, leading to simultaneous stimulation of all opsin-expressing neurons. Thus, current approaches are not optimized to address spike timing across different neurons and cannot replicate the complex spatial and temporal patterns of activation that are observed in neuronal assemblies during, for example, sensory stimulation (Ohki et al., 2005; Kerr et al., 2007; Grewe et al., 2010; Froudarakis et al., 2014).

Various strategies can help restrict expression to a small number of cells and achieve more precise spatial control during optogenetic manipulation. For example, tiny volumes of virus can be locally injected (Stroh et al., 2013; Packer et al., 2013) or small tapered fibers optic can be used (Heiney et al., 2014). Alternatively, genetic strategies can be used to express opsin based on

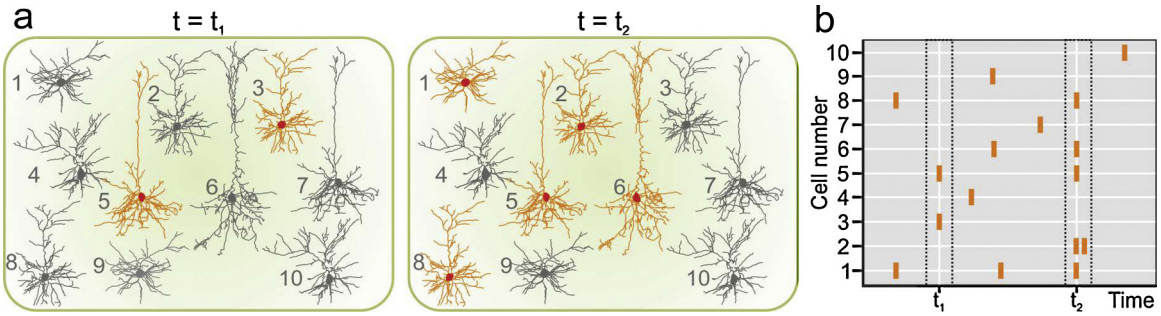

Fig. 1. Neuronal circuits display complex spatial and temporal patterns of activity. (a) A simplified network model composed of ten neurons is shown. Complex patterns of electrical signals that display diverse degrees of synchrony among different cells are generated over time. For example, at $t = t_1$ (left panel) two cells (orange) are concurrently active while most other neurons (gray) remain in an inactive state. At $t = t_2$ (right panel), electrical activity is synchronously generated in a larger number of cells (neurons nos. 1, 2, 5, 6, 8). Modified from Beltramo et al. (2013). (b) A raster plot showing electrical activity (vertical orange bar) in the different cells over time. The times corresponding to $t = t_1$ and $t = t_2$ are highlighted (dotted gray line).

the cell's level of electrical activity. *Fos*-mediated expression has been used to express channelrhodopsin-2 (ChR2) during a memory task (Liu et al., 2012; Ramirez et al., 2013). However, the level of *Fos* expression integrates the neuron's electrical activity on a timescale of hours, and thus it is not easy to deduce the precise relationship between spiking activity and ChR2 expression. A complementary approach would be to control the delivery of light that is used for photo-activation in both in space and time (patterned illumination). This can be achieved by scanning a two-photon laser spot sequentially according to an arbitrary scan path. Importantly, the most used opsins have proved to be excitable with a two-photon process (Mohanty et al., 2008; Rickgauer and Tank, 2009; Prakash et al., 2012). With respect to single-photon stimulation, two-photon excitation grants optical sectioning and small excitation volumes (Denk et al., 1990; Helmchen and Denk, 2005; Svoboda and Yasuda, 2006), however, using two-photon optogenetics is complicated by the low conductance of ChR2 (Feldbauer et al., 2009). The main requirement in ChR2 stimulation is that many ChR2 molecules must be simultaneously excited to produce currents large enough to cause a cell to spike. Opsins with longer deactivating time are more efficient in the scanning two-photon configuration because prolonged deactivation time compensates for the poor temporal summation of two-photo stimulation events (Prakash et al., 2012). However, the scanning modality and the longer deactivation time of the opsin limit the temporal resolution of photo-manipulation. Moreover, this approach cannot generate truly synchronous illumination in multiple neurons.

One appealing solution to these limitations is the use of techniques that allow illumination of multiple cells (Fig. 2b) with enlarged shapes of arbitrary geometry. This excitation scheme permits efficient two-photon optogenetic stimulation of single cells, because cellular regions that are large enough to contain many copies of the optogenetic actuator are simultaneously illuminated. Moreover, this approach allows true simultaneous activation across neurons, because multiple shapes can be simultaneously projected on different cellular targets.

This review focuses on one such technique: phase modulation of laser light with liquid crystal spatial light modulators (LC-SLMs). Originally developed to correct optical aberration induced by atmospheric turbulence for astronomy applications (Tyson, 1991; Hardy, 1998), this technology has been more recently used for optical-tweezers (Eriksen et al., 2002; Melville et al., 2003) and neuroscience applications (Lutz et al., 2008; Nikolenko et al., 2008; Watson et al., 2010; Papagiakoumou et al., 2010). We will first briefly describe the principle underlying patterned illumination through the phase modulation of light with LC-SLMs. Then we will present the main results obtained with phase-modulation approaches, focusing on both photo-activation and imaging applications. We will finally discuss the strengths and weaknesses of patterned illumination through phase modulation for future experimental applications. The discussion of other methods for patterned illumination such as digital micromirror devices (DMD), microLED arrays, and tapered optical fibers is beyond the scope of this article, and we refer to other important contributions for detailed descriptions of these alternative approaches (Knapczyk et al., 2005; Zhu et al., 2012; Degenaar et al., 2009; Grossman et al., 2010; Pisanello et al., 2014).

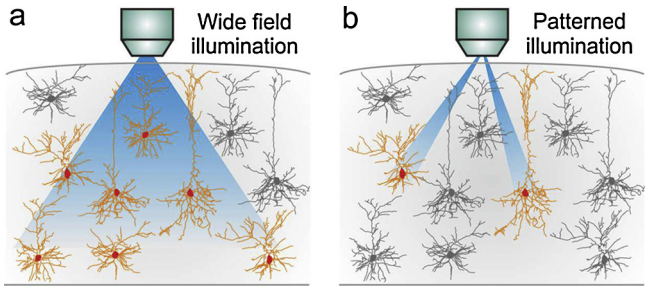

Fig. 2. Patterned illumination for probing brain networks with complex spatial and temporal patterns of neuronal activation. (a) In the wide field configuration, which is most commonly used in current optogenetic studies, light is delivered to the whole field of view of the objective, resulting in poor spatial specificity. If neurons express light-sensitive molecules, all of the illuminated cells are activated (red cells). (b) In the patterned illumination approach, light is targeted to specific cells, allowing the generation of complex spatial patterns of neuronal activation.

1.1. Phase modulation using liquid crystal spatial light modulators

According to the Huygens–Fresnel principle (Fresnel, 1816), the wave-front of a monochromatic wave propagating in the direction of an arrow (A_1B_1 in Fig. 3a) at time instant $t_0 + \Delta t$ is the envelope of hemispherical waves emitted by point sources located on the wave-front of the same wave at time instant t_0 (AB, in Fig. 3). In this context, the complex field distribution at a certain distance z in the direction of light propagation is obtained by integrating a set of hemispherical waves generated by a collection of points in the source plane. Moreover, in the Fraunhofer approximation the complex far field distribution in the observation plane can be expressed as the Fourier transform of the initial complex field distribution in the source plane. The amplitude and phase of the complex field in the observation plane are determined by the input Fourier frequency components in the source plane (Goodman, 2005). This implies that the complex spatial field distribution at the focal plane of an objective lens can be defined by a map of phase delays applied to the corresponding spatial frequency distribution in the

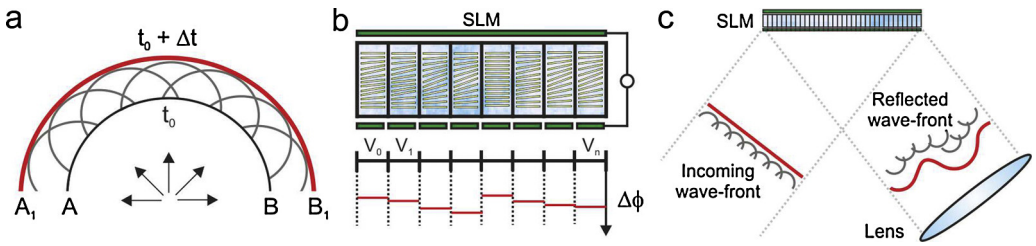

Fig. 3. Liquid crystal spatial light modulators. (a) Schematic representation of the Huygens–Fresnel principle. (b) Each pixel cell of the LC-SLM contains liquid crystals immersed in a dielectric medium (cyan). A voltage difference applied to individual pixel electrodes (green rectangles) changes the orientation of the liquid crystals, modifying the pixel's refractive index and generating different phase delays (bottom panel). (c) The planar wave-front of a laser beam is transformed into a complex wave-front by phase modulation with the LC-SLM. Modified from Difato et al. (2012).

objective back focal plane. These phase maps are generally referred to as diffractive optical elements (DOEs) (Soifer, 2002). By properly changing the distribution of spatial frequencies in the back focal plane, it is thus possible to modulate according to arbitrary geometries the amplitude of the field distribution in the sample plane.

LC-SLMs, which are essentially a dynamically programmable matrix of active pixels (Efron, 1994), are an efficient tool to generate different DOEs at the back focal plane of the objective (Fig. 3b and c). Each pixel contains nematic liquid crystals that typically have a rod-like molecular structure with one unique symmetry axis of anisotropy, called the optic axis or director. This property results in two different refractive indices for different polarization, an extraordinary refractive index for light polarized perpendicularly to the optic axis, and an ordinary refractive index for light polarized parallel to the optic axis. Because the orientation of the liquid crystal within each LC-SLM pixel is controlled by an electrical bias voltage (Fig. 3b), changing the voltage causes the liquid crystal to re-orient with respect to the direction in which the light is propagating (Khoo, 2007; Jung, 2009). This changes the effective refractive index, and thus the phase delay, that light experiences while traveling through the pixel (Vicari, 2003). Using this approach, the planar wave-front of the laser beam is turned into a complex wave-front as it is reflected off the LC-SLM (Fig. 3c).

1.2. Optical setup for patterned illumination with LC-SLMs

The basic setup for using an LC-SLM to generate patterned illumination with phase modulation is shown in Fig. 4a (Lee et al., 2007; Papagiakoumou et al., 2008; Lutz et al., 2008; Golan et al., 2009). The system is composed of a laser (either a continuous-wave visible or pulsed infrared source), an intensity modulation unit (*e.g.*, Pockels cells), the LC-SLM and some relay optics. The first telescope (L_1 and L_2 in Fig. 4a) expands the laser beam to match the dimensions of the LC-SLM window, which is typically $\sim 1 \times 1\ cm^2$. A half-wave plate ($\lambda/2$ in Fig. 4a) is positioned upstream of the LC-SLM to match the polarization of the incident beam with the working orientation of the LC-SLM. The second telescope (L_3 and L_4 in Fig. 4a) matches the beam dimension with the objective back aperture. In this configuration, the LC-SLM is thus optically conjugated with the objective rear pupil. This basic design can be inserted into more complex optical systems and added as a modular system to conventional two-photon scanning microscopes (Dal Maschio et al., 2010). One possibility for integration is to conjugate the LC-SLM plane with the galvanometric mirror plane, which is in turn coupled with the objective back aperture plane (Fig. 4b). The LC-SLM acts as a mirror when it is off, and scanning microscopy can be performed without significant changes in the spatial resolution of the microscope (Dal Maschio et al., 2010, 2011). When phase modulation is required, the LC-SLM is turned on, the galvanometric mirrors are held stationary and different patterns of light can be projected at the sample plane based on the high resolution images acquired in the scanning configuration (Fig. 4c–f). This technology can also be used to generate patterns of light in three dimensions (Fig. 5). A detailed protocol for the implementation of an LC-SLM into a two-photon scanning microscope can be found in (Difato et al., 2012; Nikolenko et al., 2013).

1.3. Controlling neuronal activity with patterned illumination

Using patterned light to illuminate arbitrarily shaped regions of interest within the field of view of an imaging system is proving to be a powerful tool in neuroscience applications. Some of the first experiments using LC-SLMs to perform patterned stimulation of neuronal tissue employed visible light and caged neurotransmitters. Using this approach in brain slice preparations, it is possible to focally release caged glutamate onto defined segments of neuronal dendrites and single dendritic spines; this method can also be used to simultaneously project multi-spot excitation onto different dendritic branches in two or three dimensions (Lutz et al., 2008; Yang et al., 2011, 2014; Anselmi et al., 2011; Kam et al., 2013), thus leading to precise, spatially constrained neuronal activation. By coupling phase modulation through LC-SLMs with remote focusing, it is possible to selectively stimulate the dendrites of hippocampal pyramidal neurons in 3D and record calcium responses in arbitrary planes by tilting the imaging plane with a remote mirror (Anselmi et al., 2011). Combined single-photon light patterning with LC-SLMs and functional calcium imaging has also been applied in brain slices to stimulate simultaneously multiple target neurons and glial cells. These results showed that patterned illumination can be used to not only study dendritic integration and compartmentalization but also to examine neuronal circuit function and the bidirectional communication between neurons and glia (Zahid et al., 2010). More recently, patterned optogenetic stimulation with millisecond temporal precision and cellular resolution was demonstrated in retinal ganglion cells expressing light-sensitive opsins (Reutsky-Gefen et al., 2013). All of these studies were performed with single-photon excitation, which is intrinsically constrained by low optical sectioning and limited penetration depth.

The use of infrared-shifted light and two-photon excitation addresses both of these limitations because longer wavelengths penetrate deeper within brain tissue, and the two-photon excitation process assures small excitation volumes. Patterned two-photon stimulation with an LC-SLM has been used to uncage glutamate in cortical slices, thereby activating several dendritic spines in layer V pyramidal neurons while recording stimulus-evoked potentials with a patch-clamp electrode (Nikolenko et al., 2008; Go et al., 2012, 2013). Activation of up to five dendritic spines (Nikolenko et al., 2008) generates reliable excitatory potentials with rise and decay kinetics similar to those measured in traditional single-spine uncaging experiments without the use of LC-SLMs (Araya et al., 2006). Two-photon patterned activation of neurons with an LC-SLM has been coupled to two-photon calcium imaging to map the functional response of a neuronal network to a given patterned stimulus of multiple cells (Dal Maschio et al.,

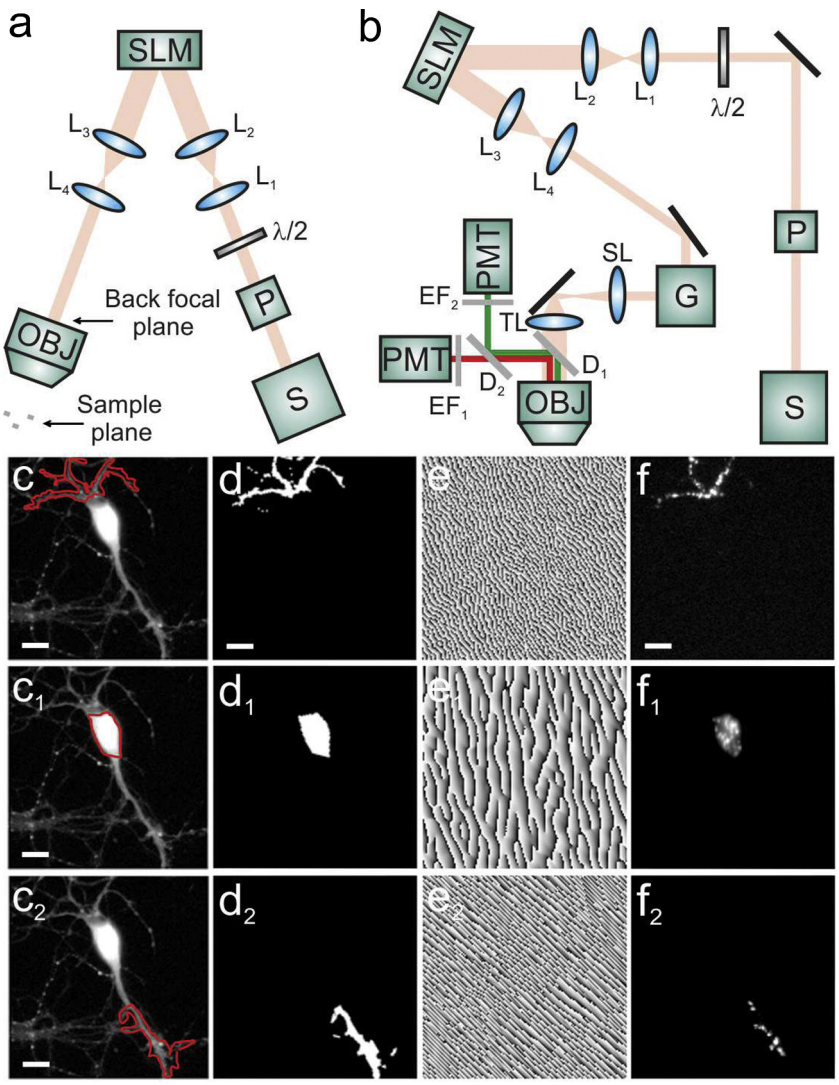

Fig. 4. Patterned illumination with liquid crystal spatial light modulators. (a and b) Schematic of the optical setup for phase modulation (a) and phase modulation with scanning microscopy (b). S, laser source; P, Pockels cell; λ/2, half wave plate, (L_1 and L_2) and (L_3 and L_4), telescope lenses; SLM, spatial light modulator; OBJ, objective; G, galvanometric mirrors; SL, scan lens; TL, tube lens; (D_1) 660 nm long-pass dichroic mirror; (D_2) 575 nm long-pass dichroic mirror; EF_1 and EF_2, emission filters; PMTs, photomultiplier tubes. Modified from Dal Maschio et al. (2012b). (c–c_2) Fluorescence image showing a cultured neuron filled with Fluo-4. The regions of interest (red line) corresponding to the cell body (c_1) or portion of the dendritic tree (c and c_2) are shown. Scale bar: 10 μm. (d) Based on the image showed in (c–c_2), a binary mask is generated corresponding to the selected regions of interest (a portion of the dendritic tree in (d and d_2), or the cell somata in (d_1). (e–e_2) Phase maps corresponding to the binary masks shown in (d–d_2). (f–f_2) Fluorescence images showing patterned two-photon illumination in the regions of interest displayed in (c–c_2). Modified with permission from Dal Maschio et al. (2010).

2010). Continuous-wave visible and pulsed infrared light has also been recently used for the patterned photo-thermal excitation of cortical cells *in vitro* (Farah et al., 2013).

Two-photon illumination with extended shapes (Andrasfalvy et al., 2010; Papagiakoumou et al., 2010; Packer et al., 2012) has been successfully applied to photostimulate light-sensitive opsins (*e.g.*, ChR2 and C1V1), which have proven to be excitable with a two-photon absorption process (Mohanty et al., 2008; Rickgauer and Tank, 2009). Compared to sequential illumination with a diffraction limited spot, a major advantage of this approach is that ChR2 has low channel conductance, and thus many ChR2 molecules must be simultaneously excited to produce currents large enough to cause a cell to spike. The activation of ChR2-expressing cells in hippocampal brain slices was demonstrated using low numerical aperture Gaussian beam producing excitation spot of 4–14 μm in diameter and temporal focusing (TF) to maintain resolution in the *z* dimension (Andrasfalvy et al., 2010). The author reported maximal two-photon stimulation at λ = 880 nm. The Emiliani group extended this approach by combining LC-SLMs, temporal focusing and generalized phase contrast (GPC) (Papagiakoumou et al., 2010). A major advantage of this approach with respect to the use of low numerical aperture beams is that LC-SLMs allow dynamic control over the lateral dimension of the illuminated region according to arbitrary geometries. Moreover, GPC reduces fluctuations in spatial intensity (also called "speckles") within the volume of excitation, increasing illumination uniformity and light efficiency (Papagiakoumou et al., 2010). Simultaneous illumination of a single cell with differently sized two-photon-extended shapes affects the membrane potential (Fig. 6). Increasing the coverage of the cell somata (Papagiakoumou et al., 2010) with patterned illumination results in augmented depolarization and eventually action potential firing in recorded neurons (Fig. 6). The combination of LC-SLMs with TF and GPC has been used in a cortical slice preparation to simultaneously photo-stimulate up to three pyramidal ChR2-expressing neurons in layer V as well as multiple cellular processes, thus mimicking summed excitation by presynaptic inputs (Papagiakoumou et al., 2010). Importantly, temporally focused two-photon excitation with GPC or phase modulation

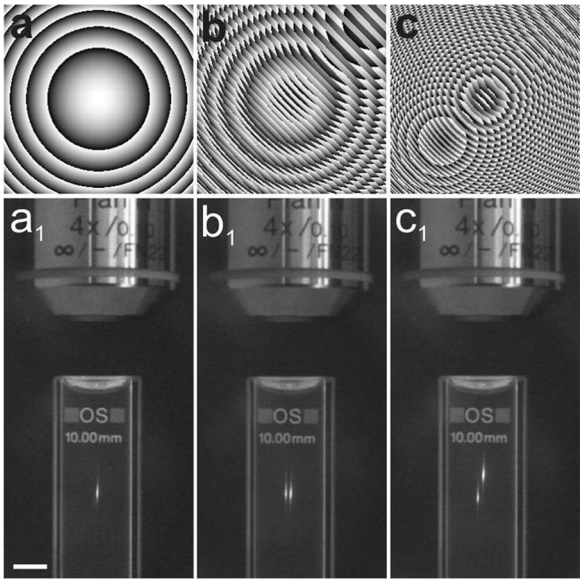

Fig. 5. Patterned illumination in three dimensions. (a and a_1) A cuvette containing fluorescein (a_1) is excited by a two-photon illumination spot generated by the phase hologram shown in (a). Scale bar: 5 mm, λ = 800 nm. (b and c) The same preparation as in (a and a_1) showing the illumination patterns that generate two spots in the same plane (b and b_1) and two simultaneous spots at different axial positions (c and c_1). Reproduced with permission from Dal Maschio et al. (2012b).

with LC-SLMs have also been shown to be particular resistant to scattering, allowing axial confinement up to two scattering lengths deep into brain tissue (Papagiakoumou et al., 2013; Begue et al., 2013). This evidence represents an important and promising result for the application of two-photon optogenetics *in vivo*. The photo-stimulation of two neurons expressing $C1V1_T$ located at different depths in cortical brain slices has been demonstrated using an alternative optical design which couples an LC-SLM with galvanometric mirrors (Packer et al., 2012). In this study, an LC-SLM was used to focus split laser beam onto neurons positioned at different axial planes. Illumination over extended areas within a given focal plane has been performed by sequentially scanning the laser spot over regions of interest with galvanometric mirrors (Packer et al., 2012). The use of C1V1 might be especially helpful when simultaneous two-photon imaging and single cell stimulation is desired (Rickgauer et al., 2014). C1V1 can be preferentially excited at λ = 1064 nm while neuronal activity can be monitored, for example, with GCaMP indicators that are preferentially excited at λ = 920 nm.

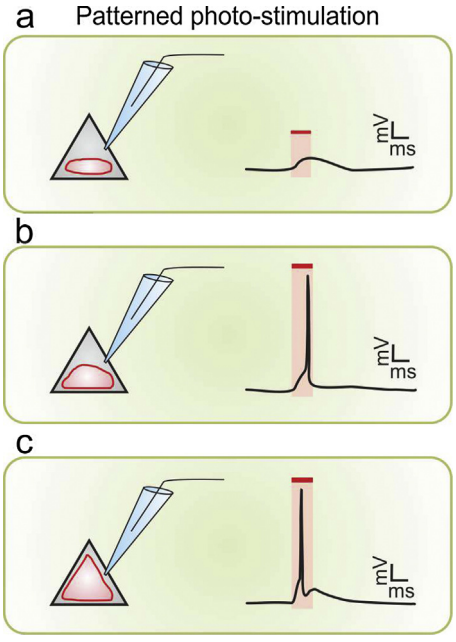

Fig. 6. Photo-activation with patterned illumination. (a) A cell expressing the light-sensitive excitatory opsin ChR2 is recorded with a patch-clamp electrode, and at the same time, photo-stimulated with two-photon light on a small region of its cell somata (red area). The membrane depolarization due to the induced photo-current remains subthreshold (black trace on the right). (b and c) Increasing the area of the illuminated region (red shape), generates a suprathreshold response and action potential firing. Modified from Papagiakoumou et al. (2010).

1.4. Imaging neuronal activity with patterned illumination through the phase modulation of light

Patterned illumination with an LC-SLM permits the simultaneous generation of multiple excitation points in three dimensions. In combination with fluorescence indicators of cell activity (such as calcium or voltage sensors) and wide-field detectors (such as cameras), this configuration can be used to image neuronal function in a scanless configuration (scanless imaging, Fig. 7). In scanless imaging, acquisition speed is limited by the maximal acquisition frequency of the camera and the signal-to-noise ratio. In brain slices and neuronal cultures loaded with the synthetic calcium indicators Fura-2AM, mag-Indo-1AM (Nikolenko et al., 2008) or Fluo-4 (Dal Maschio et al., 2010), scanless imaging has been shown to efficiently and simultaneously detect calcium signals, evoked either by the injection of current pulses trough a patch pipette or by glutamate uncaging, in multiple neurons at acquisition rates ranging from 15 to 70 Hz (Nikolenko et al., 2008; Dal Maschio et al., 2010). More recently, scanless imaging has been applied to investigate the spatiotemporal organization of cerebellar microcircuits in a slice preparation (Gandolfi et al., 2014). Cerebellar network activity was elicited by stimulating mossy fibers, and the calcium transients, which were recorded in granule and Purkinje cells, correlated with the spike discharge measured by simultaneous whole-cell recording and with the number of incoming input stimuli (Gandolfi et al., 2014). Moreover, scanless imaging has been recently extended to image in three dimensions (Quirin et al., 2014). Using an LC-SLM to simultaneously generate two-photon illumination in multiple focal planes and a phase-only optical mask in the fluorescence detection path (Quirin et al., 2013, 2014), tens of neurons in hippocampal brain slices can be imaged at an acquisition frequency of up to at 125 Hz. Importantly, the image mask increases the depth of field of the objective, allowing fluorescence signals to be collected from out-of-focus planes while maintaining full aperture and collection efficiency. Initial experiments in hippocampal slices have been extended to living larval zebra fish, a preferred model system for optical applications because this animal is highly transparent. In this preparation, scanless imaging was used to record brain-wide calcium transients at a high speed (30 Hz) using the genetically encoded calcium indicator GCaMP5G (Quirin et al., 2014). These important results show that scanless imaging can be used *in vivo*, but the application of this approach to tissues with more scattering, such as the mammalian brain, still awaits experimental demonstration.

In this context, encoded multisite two-photon microscopy (eMS2PM), which uses a single photomultiplier tube for the detection of emitted fluorescence photons (Ducros et al., 2013), is an interesting approach to limit the influence of scattering on fluorescence detection and consequently increase the achievable imaging depth. This method uses an LC-SLM to split the laser beam into beamlets according to the chosen targets, where the intensity of each beamlet is modulated by a DMD with a different sub-millisecond binary code. The fluorescence excited by each beamlet is modulated with the same temporal code imposed on the

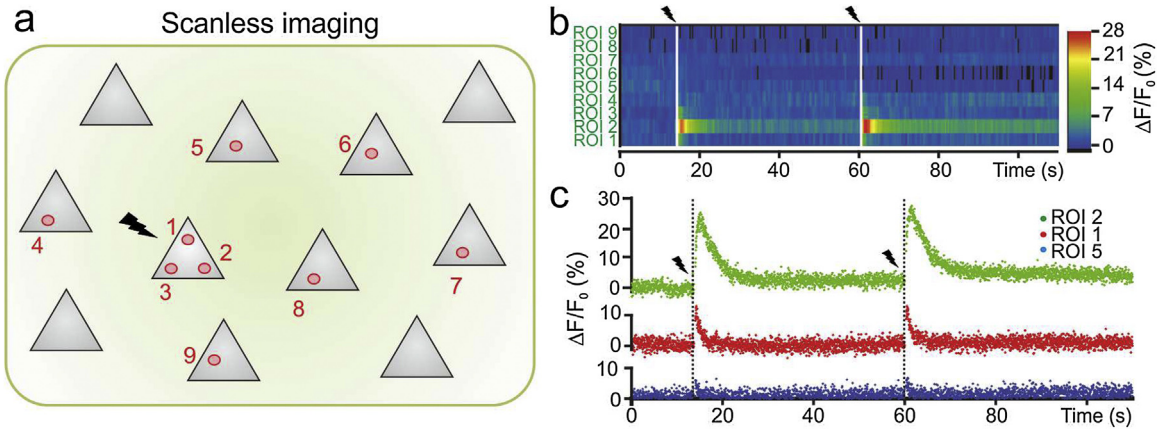

Fig. 7. Scanless imaging of neuronal networks with patterned illumination. (a) A network of neurons expressing a functional fluorescence indicator is illuminated by simultaneously projecting multiple diffraction-limited spots on the sample. Fluorescence is collected with a camera and observed over time following the activation of a single neuron (arrow) by means of glutamate uncaging with a second laser beam. (b and c) Fluorescence values ($\Delta F/F_0$) over time for all of the illuminated regions of interest (ROIs, (b)) and for ROIs nos. 1, 2, 5 (c). Reproduced with permission from Dal Maschio et al. (2010).

exciting beamlet. Fluorescence excited by all beamlets is collected by the photomultiplier tube and demultiplexed *a posteriori* using a decoding algorithm (Ducros et al., 2013). *In vivo* calcium imaging of layer 2/3 pyramidal neurons showed that eMS2PM can resolve fluorescence signals down to 300 μm from cells located less than 10 μm apart.

2. Discussion

As described in the previous sections, patterned illumination through the phase modulation of light has been successfully applied in the photo-stimulation of caged compounds and light sensitive opsins as well as in scanless imaging combined with fluorescent activity indicators. However, most of these studies performed proof-of-principle experiments, and the full utilization of this technology for addressing biological questions in circuits and systems neuroscience is yet to come. Because the application of this approach to neuroscience is relatively recent, several improvements from the optical and molecular point of view are required to fully exploit the technique's potentials. In the following paragraphs we discuss some of the main challenges faced by researchers using the current experimental approaches.

2.1. Patterned illumination for precise, cell-specific optical manipulation of neuronal circuits in the intact brain

The most common strategies for delivering light in optogenetic experiments *in vivo* use LEDs or fiber optics, leading to wide field illumination of the sample and thus a lack of spatial specificity. Patterned two-photon stimulation is a promising approach for targeting photons to specific spatial locations, yet its feasibility has been proved mostly in cultured neurons (Rickgauer and Tank, 2009; Andrasfalvy et al., 2010) and brain slice preparations (Andrasfalvy et al., 2010; Papagiakoumou et al., 2010; Packer et al., 2012). One of the next crucial steps in the field is to extend these findings to the *in vivo* mammalian brain and to develop a robust protocol for the optical stimulation of multiple cells while maintaining single cell resolution (Rickgauer et al., 2014), and several laboratories are currently involved in this task.

Two main strategies can be used to this end: the patterned illumination through a scanning approach, in which a diffraction-limited laser spot is sequentially deflected over the target area (Rickgauer and Tank, 2009; Prakash et al., 2012), the patterned illumination through the phase modulation of light, in which an LC-SLM is used to generated extended illumination areas (Papagiakoumou et al., 2010) or a combination of the two approaches (Packer et al., 2012). As mentioned above (see Section 1), to activate enough channels/pumps to efficiently influence the neuron's firing activity, cell activation/inhibition requires fast illumination of an adequate fraction of the cell. In the scanning approach, resonant scanning mirrors or acousto-optic deflectors (AODs) can be used to rapidly move the laser spot across an area (Wang et al., 2011). However, the number of cells that can be efficiently excited within a given time window depends on the speed of the scanners but also on the risetime of opsin and thus on the dwell time. Thus, faster scanning approaches might not be the ultimate solution for stimulating many cells in short time windows if prolonged dwell times are required to obtain an efficient activation of the opsin. Optogenetic actuators with slower deactivation time (Prakash et al., 2012) might be better suited for stimulation in the scanning mode. However, increasing the deactivation time of the light-sensitive molecule significantly reduces the temporal resolution, making this approach not suitable for addressing questions of spike timing in the millisecond time range. Finally, the scanning mode is intrinsically sequential and thus does not allow for simultaneous activation/inactivation of different cells.

Patterned illumination through the phase modulation of light has the potential to bypass some of these limitations. LC-SLMs modulate two-photon light according to arbitrary shapes, allowing truly simultaneous illumination of multiple neurons. Moreover, by projecting different DOEs over time, patterned illumination with LC-SLMs has the potential to probe neuronal networks with complex spatial and temporal patterns of neuronal activation. However, although two-photon LC-SLM illumination has been successfully carried out in cultures and brain slices, its application for photo-stimulation *in vivo* has not yet been demonstrated. Here we discuss some of the issues that may arise when applying this approach to photo-stimulate cells in the intact mammalian brain.

2.1.1. Total number of stimulated cells

Patterned photo-stimulation of neurons in brain slices requires the delivery of tens of mW per cell (Andrasfalvy et al., 2010; Papagiakoumou et al., 2010). The number of cells that can be simultaneously illuminated is thus limited by the total laser power available in the sample plane. With current laser sources, and considering that the optimal wavelength for opsin stimulation has been reported to be 880–950 nm for ChR2 (Andrasfalvy et al., 2010; Papagiakoumou et al., 2010) and >950 nm for inhibitory opsins (Prakash et al., 2012), the total number of cells that can be stimulated might be on the order of a few dozen. This might already

be enough to correlate activity in single cells with higher brain functions, as several lines of evidence suggest that even single neuron stimulation can have consequences at the behavioral level (Brecht et al., 2004; Houweling and Brecht, 2008; Doron et al., 2014). If larger numbers of neurons are required, one possibility is to use an intermediate approach in which an LC-SLM is used to shape two-photon light into an extended area corresponding to the dimension of a cell body of a neuron, and galvanometric mirrors are used to deflect this shape over multiple cells. Alternatively, an LC-SLM can be used to project small excitation spots centered on multiple cells and the galvanometric mirrors are used to scan the spots on the extended area corresponding to the cell body. Another potential alternative to boost the number of stimulated cells is to increase the available laser intensity at the sample by improving light transmission through the optical path. However, the application of high energy on biological samples may cause tissue heating and cellular damage. Future improvements in the molecular engineering of opsins might also help increase the number of stimulated cells. For example, opsins with higher two-photon absorption cross-sections would require lower light intensity for excitation. In the case of excitatory opsins, improving the photo-current would enable efficient light-induced depolarization through the activation of a smaller number of channels (Klapoetke et al., 2014). Increasing the membrane trafficking and/or the expression of opsins through the insertion of specific sequences would also decrease the laser intensity requirements in the sample (Gradinaru et al., 2010; Prakash et al., 2012; Lin et al., 2013). However, it must be considered that the overexpression of an exogenous protein could be toxic and may affect cell function.

2.1.2. Spatial and temporal precision

When investigating relative spiking among different neurons, the effect of the optical manipulation must be precisely confined to single cells. Genetics allows for the restricted expression of optogenetic actuators into specific cellular subpopulations and patterned two-photon excitation with TF restricts excitation within confined volumes (Papagiakoumou et al., 2009, 2010). However, in a three-dimensional brain circuit where hundreds of cellular processes are highly interwoven, the probability of stimulating unwanted fibers of passage should be minimized. The development of opsins targeted to specific subcellular regions, *e.g.*, the cell somata, axon initial segment, or cellular processes (Grubb and Burrone, 2010; Greenberg et al., 2011), might greatly overcome this concern. Moreover, temporal focusing permits exceptional light confinement in the z direction, and the combination of TF with GPC and with phase modulation through LC-SLMs has been shown to be particular resistant to tissue scattering (Papagiakoumou et al., 2013; Begue et al., 2013). This suggests that LC-SLMs combined with TF and GPC should reach the necessary spatial resolution in the intact mammalian brain. However, its *in vivo* application is yet to be demonstrated. Current LC-SLMs have refresh rates on the order of 50–200 Hz, precluding the application of different DOEs with millisecond intervals. Ferroelectric SLMs (Golan et al., 2009; Reutsky-Gefen et al., 2013) have much faster refresh rates (>1 kHz), but they also suffer from limited diffraction efficiency and modulation capabilities. The development of a new generation of SLMs with high efficiency and refresh rates would certainly represent a major step forward for the application of patterned illumination in neuroscience.

2.1.3. Depth

Patterned photo-stimulation will eventually be limited to optically accessible brain regions. Two-photon excitation with near infrared light reaches a maximal depth of ~1 mm (Theer et al., 2003; Helmchen and Denk, 2005), which approximately corresponds to the thickness of the mouse neocortex. Experiments in deeper brain regions currently require the removal of overlying brain regions or the insertion of an endoscopic probe (Dombeck et al., 2010; Barretto et al., 2009). The use of longer wavelengths (Hong et al., 2014), multi- (>2) photon absorption processes (Horton et al., 2013) and light-sensitive molecules with infrared-shifted absorption spectra (Prakash et al., 2012) may significantly extend the maximal achievable depth of current optical approaches.

2.1.4. Photo-inhibition

Although a number of studies have addressed the importance of generating spatial patterns of light to photo-stimulate excitatory opsins, less attention has been given to selective optical photo-inhibition. Engineered proton and chloride pumps are largely used to silence the activity of neurons through one-photon wide-field illumination (Zhang et al., 2007b; Chow et al., 2010, 2012). Two-photon raster-scanning excitation ($\lambda > 920$ nm) has been proven to inhibit eArch3.0-expressing neurons in neuronal culture and brain slices (Prakash et al., 2012) at power comparable to that used for two-photon activation of ChR2 (Andrasfalvy et al., 2010; Papagiakoumou et al., 2010) or C1V1 (Prakash et al., 2012; Packer et al., 2012). Moreover, the recent development of red-shifted opsins that mediate strong neuronal inhibition (Chuong et al., 2014) allows control of neuronal activity in deeper brain regions, representing a promising tool for future *in vivo* experiments in which neuronal networks are silenced with patterned illumination.

2.2. Scanning and scanless imaging of neuronal networks

Multiple approaches have been developed in recent years to monitor functional fluorescence signals with cellular resolution in the intact brain (Wilt et al., 2009). The ultimate goal is to perform fast (millisecond) population imaging with single cell resolution in a volume of scattering tissue, a challenging task to be achieved with an all-in-one technique. In the following section, we will discuss the strengths and weaknesses of the scanless imaging approach compared to other techniques for fast functional imaging in brain tissue.

Two-photon raster scanning microscopy with galvanometric (Helmchen et al., 1999; Svoboda et al., 1999) or resonant (Jia et al., 2010) mirrors is probably the most widespread approach for the functional investigation of brain circuits with cellular resolution. In terms of temporal resolution, scanning microscopes based on galvanometric mirrors reach a maximal full-frame acquisition speed of a few Hz (Helmchen and Denk, 2005), and resonant scanning-based systems achieve ~30 Hz (Jia et al., 2010). Smart movements of the mirrors over specific trajectories can significantly increase the acquisition speed up to 100 Hz (Lillis et al., 2008). Multibeam illumination is another means of increasing the temporal resolution of scanning microscopes (Kurtz et al., 2006; Niesner et al., 2007). In this configuration, the acquisition speed is increased by a factor equal to the number of illuminating beam lines (typically 16–64). Thus, when this approach is coupled to galvanometric mirrors, the full frame acquisition speed can be on the order of ~150–200 Hz. More recently, random access microscopy using AODs (Reddy and Saggau, 2005) is being increasingly recognized as a preferred solution to increase the acquisition frequency in functional fluorescence imaging *in vivo*. Grewe et al. (2010) demonstrated that with this approach, 56 cells could be imaged at 298 Hz in the intact mouse brain in 2D (five consecutive points per cell) and that 16 cells could be imaged at 1 kHz. This approach also allows fast volumetric *in vivo* imaging of hundreds of cells (Katona et al., 2012; Froudarakis et al., 2014). However, all of these approaches are based on the sequential deflection of one laser spot. Thus, the acquisition speed in these systems inversely depends on the number of points to be illuminated which increases with number of cells that are being imaged.

Scanless imaging presents some advantages compared to the previously described techniques. First, in contrast with the sequential illumination scheme, acquisition speed is independent of the dimension of the illuminated area in the scanless approach. Second, at equal acquisition speed the dwell time is longer and consequently the fluorescence emitted signal stronger in the scanless compared to the sequential illumination configuration. Third, the use of an SLM does not introduce substantial broadening of the excitation pulse (Difato et al., 2012). Fourth, the point-spread-function of the microscope is not significantly affected by the SLM (Dal Maschio et al., 2010), and it is constant across the field of view. Fifth, given that the beam is continuously illuminating the sample, the scanless modality utilizes smaller values of laser peak intensities, thus potentially decreasing photobleaching (Ji et al., 2008). Sixth, SLMs lead to limited power loss, and they are relatively easy to insert in the optical path of a commercial two-photon microscope (Difato et al., 2012).

However, camera-based detection in scanless imaging also presents some limitations. This optical configuration is sensitive to the scattering of emitted photons (Cheng et al., 2011), which will likely limit the applicability of this technique to more superficial regions of thick and highly scattering tissues. Moreover, the total number of cells imaged in the scanless technique is constrained by the maximal laser power delivered to the sample. If one point is used to illuminate one cell, then the maximal number of neurons that can be imaged in currently used configurations is <100 (Nikolenko et al., 2008). One solution to increase the number of cells that can be imaged is to augment the laser power available at the sample plane (see also the previous section on photo-stimulation). However, extreme caution needs to be used because increasing the power delivered to the sample may result in tissue heating and phototoxic effects. An alternative strategy to increase the number of cells that can be recorded is to augment the detection efficiency. The use of new camera detectors with improved characteristics may help achieve this goal. Detectors that combine high frame rate, high sensitivity and large pixel number would be desirable. The SNR of the system, and thus the total number of cells that can be imaged, may also be improved by implementing adaptive optics (Ji et al., 2010, 2012; Wang et al., 2014). This solution would compensate for distortions of the excitation beam introduced along the optical path and within the biological sample, thus improving the efficiency of fluorescence excitation.

3. Conclusions

The introduction of molecular tools to image and manipulate genetically identified neuronal populations has led to tremendous advances in our understanding of the brain. However, the growing interest in understanding how coordinated activity among individual neurons determines circuit function and animal behavior highlights the limitations of current experimental approaches. The development of new generations of molecular sensors/actuators and more refined optical techniques to precisely control cell function in space and time are required to successfully tackle these issues. Patterned illumination through the phase modulation of light will likely represent an important tool for this objective. We expect major advances in the use of patterned illumination with LC-SLMs in the near future, including cell-specific optogenetic manipulation over large number of cells in the living mammalian brain of anesthetized or head-restrained animals, single cell activation/inhibition in freely moving animals with flexible fiber optic bundles, and simultaneous all-optical imaging and manipulation of neuronal activity in combination with light-sensitive opsins and fluorescent activity indicators. Once these tools are available and robust experimental protocols are established, exciting questions about the importance of spike patterns in controlling network computation, brain plasticity and behavior will finally be within reach.

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