**Graphical Abstract**

**Highlights**
- High-resolution bidirectional control of cell activity in the intact mouse brain
- High-resolution manipulation is effective across cortical cell types and layers
- Simultaneous all-optical imaging and bidirectional manipulation
- Minimal crosstalk between imaging and opsin activation

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**In Brief**
Forli et al. developed an all-optical method to image and bidirectionally manipulate brain networks with high spatial resolution and minimal crosstalk in the intact mammalian brain. They validate the method across cell types and layers in the mouse neocortex.

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Two-Photon Bidirectional Control and Imaging of Neuronal Excitability with High Spatial Resolution In Vivo

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SUMMARY

Sensory information is encoded within the brain in distributed spatiotemporal patterns of neuronal activity. Understanding how these patterns influence behavior requires a method to measure and to bidirectionally perturb with high spatial resolution the activity of the multiple neuronal cell types engaged in sensory processing. Here, we combined two-photon holography to stimulate neurons expressing blue light-sensitive opsins (ChR2 and GtACR2) with two-photon imaging of the red-shifted indicator jRCaMP1a in the mouse neocortex in vivo. We demonstrate efficient control of neural excitability across cell types and layers with holographic stimulation and improved spatial resolution by opsins somatic targeting. Moreover, we performed simultaneous two-photon imaging of jRCaMP1a and bidirectional two-photon manipulation of cellular activity with negligible effect of the imaging beam on opsins excitation. This all-optical approach represents a powerful tool to causally dissect how activity patterns in specified ensembles of neurons determine brain function and animal behavior.

INTRODUCTION

Within brain circuits, information about sensory stimuli is encoded in complex spatial and temporal patterns of activity distributed across cells (Kampa et al., 2011; Ohki et al., 2005; Sawinski et al., 2009). For example, population recordings, combined with statistical analysis, showed that specific features of sensory stimuli elicit temporally structured responses in specific ensembles of neurons (Carrillo-Reid et al., 2016; Miller et al., 2014). However, using statistical analysis and correlative evidence to causally test which sensory features are encoded in neural circuits and how this information is used to drive behavior may prove difficult (Panzeri et al., 2017). To achieve this goal, we would ideally need a method to monitor and bidirectionally perturb the activity of multiple neurons maintaining single-cell resolution. With such a technique, it would be possible to study how the concerted activity of identified neurons contributes to network function by activating or inactivating populations of functionally characterized neurons with cellular resolution.

Optical approaches, in particular two-photon microscopy, hold promise to achieve this goal. Moreover, wave-front engineering methods (Emiliani et al., 2005) using digital holography largely extended the potential of two-photon microscopy for imaging (Bovetti et al., 2017; Dal Maschio et al., 2010; Ducros et al., 2013; Moretti et al., 2016; Nikolenko et al., 2008; Quirin et al., 2013; Yang et al., 2015, 2016) and photostimulation applications (Chaigneau et al., 2016; Dal Maschio et al., 2017; Lutz et al., 2008; Packer et al., 2012; Papagiakoumou et al., 2010, 2013; Szabo et al., 2014). In parallel to these improvements in optics, the last decade witnessed the development of a large toolbox of light-sensitive molecules to monitor and manipulate the activity of neurons, including opsins such as channelrhodopsin-2 (ChR2) (Boyden et al., 2005; Nagel et al., 2003), C1V1 (Prakash et al., 2012; Yizhar et al., 2011), and Guillardia theta Anion Channelrhodopsins (GtACRs) (Govorunova et al., 2015) and functional indicators such as GCaMPs (Chen et al., 2013) and RCaMPs (Dana et al., 2016). Combining advanced two-photon approaches with the use of these bioengineered molecules, it became possible to perform simultaneous functional imaging of GCaMP signals and stimulation of various opsins (e.g., C1V1) with high spatial resolution in the rodent brain in vivo (Carrillo-Reid et al., 2016; Packer et al., 2015; Rickgauer et al., 2014; Yang et al., 2018) and in other experimental systems (Dal Maschio et al., 2017; Förster et al., 2017; Hernandez et al., 2016). However, several limitations need to be overcome to efficiently apply these approaches. First, crosstalk between imaging and photostimulation needs to be minimized. For instance, the red-shifted channelrhodopsin C1V1 is maximally activated using 540 nm light (Yizhar et al., 2011), but it is still more than half-maximally activated by 470 nm light. This should toward shorter wavelengths (Yizhar et al., 2011) typical for red-shifted opsins.
scanning rate led to further elevation of C1V1 activation (Figures S1C and S1F). Increasing the raster-photon activation of neurons (Packer et al., 2015; Rickgauer et al., 2014; Ronzitti et al., 2017; Yang et al., 2018). This undesired effect worsens when opsins with slow off kinetics and high-amplitude photocurrents, which are the preferred choice for two-photon activation of neurons with the raster or spiral scanning approach, are used (Chaigneau et al., 2016; Dal Maschio et al., 2017). Second, while published data demonstrated cellular resolution two-photon activation of neurons (Packer et al., 2015; Rickgauer et al., 2014), evidence for efficient patterned two-photon inhibition, as well as all-optical imaging and high-resolution inhibitory manipulation, is still to be provided. Third, whether single-cell two-photon optogenetics can be efficiently applied across the various cell types that are engaged during sensory stimulation and that differ in morphology, biophysical properties, and cortical depth is unclear.

Here we developed an experimental approach to address all of these challenges in the mouse cortex in vivo. We combined digital holography to stimulate blue light-sensitive opsins with two-photon imaging of a red-shifted functional indicator. We show that holographic illumination of ChR2 (Nagel et al., 2005) with extended shapes can be used to efficiently stimulate various cellular populations, including principal cells and different interneuron types, in cortical layer 2/3 and in layer 4, the main thalamorecipient lamina in sensory cortex (Feidt et al., 2013). We then characterized the two-photon excitability of the chloride-permeable channelrhodopsin GiACR2 in slice preparation and showed that it efficiently decreases neuronal firing with high spatial resolution in vivo upon holographic illumination. Finally, combining soma-targeting of opsins (ChR2 and GiACR2), which improved the spatial resolution of stimulation, with the use of the red-shifted calcium indicator jRCaMP1a, we provide a proof-of-principle demonstration of simultaneous two-photon imaging and bidirectional holographic stimulation of cells with negligible effect of the imaging beam on opsin excitation.

RESULTS

To test whether using a blue light-sensitive channelrhodopsin would lead to a reduction in the undesired cross-activation during activity reporter imaging, we expressed ChR2 and C1V1 in cultured hippocampal neurons and characterized the photocurrent evoked by two-photon scanning at the wavelengths typically used for calcium imaging of green and red calcium indicators (920 and 1,080 nm, respectively) (Figure S1). ChR2-expressing neurons showed lower relative peak current amplitudes when scanned at 1,080 nm than did C1V1-expressing neurons scanned at 920 nm (Figures S1C–S1E). The relative average photocurrent evoked during scanning at 1,080 nm was also higher for C1V1 (Figures S1C and S1F). Increasing the raster-scanning rate led to further elevation of C1V1 activation (Figures S1C and S1F) because of the slower closing kinetics of C1V1 (Yizhar et al., 2011). Conversely, increasing the raster scanning rate when recording from ChR2-expressing cells did not increase photocurrents (Figures S1D and S1F), consistent with its faster off kinetics.

High Spatial Resolution Two-Photon Holographic Stimulation In Vivo

To stimulate neurons with high spatial resolution in vivo, we used a liquid crystal spatial light modulator (SLM)-based holographic module, which was integrated in a commercial laser scanning two-photon microscope (Figure 1A) (Dai Maschio et al., 2010, 2011), and we programmed the holographic module (see Experimental Procedures) to project on the sample plane elliptical shapes that were centered on the cell body of target neurons (Figure 1B; Figure S2). To validate our approach, we performed simultaneous two-photon targeted juxtasomal recordings and photostimulation experiments in anesthetized mice in layer 2/3 principal neurons co-expressing ChR2 and the red fluorescent protein tdTomato, which facilitated targeting neurons under the microscope (Figure 1C). Once a stable electrophysiological recording was achieved from an opsin-positive neuron (see Experimental Procedures for definition), a high-resolution image was acquired and an elliptical shape (ellipse axis: 7–16 μm) was projected on the cell body of the recorded neuron. Significant increase in action potential (AP) firing frequency was observed upon two-photon holographic illumination with extended elliptical shapes (stimulus duration: 500 ms; stimulus power: 30–92 mW/cell; ΔAP = 920 nm) (Figures 1D and 1E). To verify that the observed effect depended on opsin activation, not on membrane depolarization due to direct two-photon stimulation (Hirase et al., 2002), we performed similar experiments in opsin-negative cells (Figure S3). We found that holographic illumination with extended shapes of the same spatial profile and light intensity did not modify the membrane potential or the AP firing rate of recorded opsin-negative neurons in vivo (Figures S3C and S3D).

To evaluate the spatial resolution of our stimulation method, we measured the spiking response to holographic stimulation of opsin-positive neurons while incrementally shifting the stimulation shape in the radial and axial direction (Figure 1F). We found spatial constants (see Experimental Procedures for definition) of ~20, ~32, and ~16 μm in the radial, axial up, and axial down directions, respectively. Targeting ChR2 to the soma (Figure 2) increased the average spiking response in the illuminated neuron (ChR2: ΔAPFreq = 1.2 ± 0.3 Hz, N = 15 cells from 6 mice; soma-targeted ChR2: ΔAPFreq = 4.6 ± 1.0 Hz, p = 2.2E–2, Mann–Whitney test, N = 21 neurons from 6 mice; stimulus power: 30 mW for both ChR2 and soma-targeted ChR2). Somatic targeting of ChR2 improved the spatial resolution of holographic stimulation compared to non-soma-targeted opsins, decreasing the axial up (13 and 32 μm for soma-targeted and non-soma-targeted opsins, respectively; p = 1.4E–2, unpaired Student’s t test) spatial constant (Table S1). Confocal analysis of fixed sections from injected animals confirmed restricted expression in the somatic and perisomatic compartments with the soma-targeted ChR2 compared to the non-soma-targeted ChR2 (Figure S4). Table S1 also shows the density of opsin-expressing cells under our experimental conditions. The values of the radial and axial space constants of photostimulation resolution normalized to the soma diameter of the stimulated cells are shown in Table S2.
Two-Photon Holographic Stimulation across Cortical Cell Types and Layers

We investigated whether holographic stimulation could be efficiently applied to cell types other than layer 2/3 excitatory neurons. To this end, we first expressed ChR2 in two major subpopulations of cortical interneurons in layer 2/3, the somatostatin-positive (SST⁺) and the parvalbumin-positive (PV⁺) cells. Using simultaneous photostimulation and two-photon targeted juxtasomal recordings in vivo, we found that illumination with an extended shape (stimulus power: 30 mW/cell) increased the firing rate of targeted interneurons (Figures 3A–3C, left and middle). We then expressed ChR2 selectively in sodium channel, non-voltage-gated 1 alpha-positive (Scnn⁺) excitatory neurons of layer 4, the main thalamorecipient cortical population of the sensory cortex. We found that holographic stimulation (stimulus power: 50 mW/cell) increased the spike rate of layer 4 Scnn⁺ neurons (Figures 3A–3C, right). In all excitatory neurons recorded in layer 2/3 (Figures 1 and 2) and layer 4 (Figure 3), the spontaneous firing rates before and after photostimulation were not significantly different (Table S3). In all cell types the response to photostimulation depended upon the illumination power (Figure S5).

High Spatial Resolution Two-Photon Holographic Inhibition In Vivo

Our previous data demonstrate that holographic stimulation with extended shapes can be used for activation of neurons with high spatial resolution in vivo. We determined whether holographic illumination could also be used for efficient two-photon optogenetic inhibition with similar spatial precision. To address this question, we focused on GtACR2, a chloride-permeable channelrhodopsin (Govorunova et al., 2015). Although the two-photon excitability of GtACR2 has not yet been reported, we reasoned that its large photocurrent, high light sensitivity, and blue light-sensitive,
single-photon absorption spectrum made it a good candidate for two-photon holographic stimulation at $\lambda < 1,000$ nm. In addition, expression of GTACR2 has been reported to be well tolerated by neurons (Govorunova et al., 2015). We first expressed this inhibitory opsin in the cortex and recorded GTACR2-mediated photocurrents in a patch-clamp, voltage-clamp configuration from opsins-positive cells (see Experimental Procedures for definition) in acute brain slices (Figure 4A). We found that holographic illumination of GTACR2-expressing neurons with an elliptical shape targeted to the cell body of the recorded cell ($\lambda_{\text{exc}} = 920$ nm; stimulus power: 30 mW; stimulus duration: 500 ms) triggered clear outward currents (range: 6–112 pA; holding potential: $-50$ mV; chloride equilibrium potential: $-68$ mV). Peak amplitude of photocurrents increased with power (Figure 4B) and showed a nearly power-squared dependence for low power values (Figure 4B, inset). Moreover, while keeping light power density constant, we performed holographic two-photon illumination at different light wavelengths (range: 740–1,040 nm). We found that GTACR2 photocurrents had large peak amplitude at 920 nm and decreased for longer and shorter wavelengths (Figure 4C). We thus concluded that GTACR2 can be efficiently stimulated through a two-photon absorption process, that holographic illumination triggers clear inhibitory photocurrents in opsin-expressing neurons, and that the two-photon absorption spectrum of GTACR2 shows a clear peak near 920 nm.

We then asked whether holographic stimulation of GTACR2 could be used to decrease neural excitability with high spatial resolution in vivo. To this end, we performed whole-cell, current-clamp recordings from layer 2/3 cortical neurons expressing GTACR2 in anesthetized mice (Figures 4D–4I). We found that illumination with an extended shape (stimulus power: 10–80 mW/cell; stimulus duration: 500 ms) while a small depolarizing current was injected (current amplitude: 74 pA) led to a significant hyperpolarization of the cell (average membrane potential before [Pre]: $-46.2 \pm 1.1$ mV, during [Stim]: $-50.1 \pm 1.1$ mV, after [Post]: $-44.4 \pm 1.3$ mV; $p = 2E-15$, ANOVA test with Bonferroni’s correction, $N = 14$ from 7 mice). Moreover, we found that holographic illumination decreased cellular firing induced by a small current injection (Figures 4D and 4E). We measured the spiking response of GTACR2-positive neurons to holographic illumination while incrementally shifting the stimulation shape in the radial and axial directions (Figure 4F). We found spatial constants of $\sim 11$, $\sim 33$, and $\sim 29$ µm in the radial, axialup, and axialdown directions, respectively (Table S1). Prolonged illumination (stimulus duration: 10 s) of GTACR2-expressing neurons decreased the firing rate (Figures 4G and 4H), and it hyperpolarized the membrane potential of the illuminated cell for the duration of the light stimulus (Figure 4I). The values of the cell resting membrane potential Pre- and Post-photostimulation were not significantly different (average resting membrane potential Pre: $-61.2 \pm 2.2$ mV, Post: $-62.2 \pm 1.5$ mV; $p = 0.43$, Student’s t-test, $N = 5$ from 2 mice).

**Simultaneous Two-Photon Imaging of Red-Shifted Indicator and Holographic Stimulation of Blue Light-Sensitive Opsins**

Red-shifted channelrhodopsins have been used for two-photon stimulation of single neurons simultaneously with genetically encoded calcium indicators (GECI)-based calcium imaging (Carrillo-Reid et al., 2016; Packer et al., 2015; Rickgauer et al., 2014). One potential drawback of this approach is the remaining absorption by all red-shifted channelrhodopsins in the blue range of their action spectrum (Figure S1) (Mattis et al., 2011). As shown earlier, our data demonstrate that holographic illumination of blue light-sensitive opsins (ChR2 and GTACR2) at $\lambda = 920$ nm can be used to bidirectionally control the excitability of cortical neurons with high spatial resolution in vivo. We therefore asked whether this stimulation approach could be coupled with imaging of red-shifted functional indicators (e.g., jRCaMP), which are typically best excited in the two-photon regime at longer wavelengths ($\lambda \approx 1,100$ nm) (Dana et al., 2016; Dunn et al., 2016), a wavelength at which blue light-sensitive channelrhodopsins show no detectable activity (Figure S1) (Prakash et al., 2012). To test this possibility, we first evaluated whether raster scanning at these long wavelengths caused opsin activation, leading to significant alteration of neuronal spiking activity.
in vivo. We expressed the soma-targeted ChR2 in layer 2/3 cortical neurons and performed juxtasomal electrophysiological recordings from ChR2+ neurons while raster scanning the field of view (FOV) containing the recorded cell at $\lambda_{\text{exc}} = 920 \text{ nm}$. Laser power: 30 mW for SST+ and PV+ cells and 50 mW for Scnn+ neurons. Middle: raster plot showing cell response over consecutive trials for the same neurons displayed in the top panel. Bottom: AP distribution for the trials shown in the middle panel (time bin: 100 ms) for all cell types (SST+, left; PV+, middle; Scnn+, right). (C) Average firing frequency Pre, during (Stim), and Post holographic stimulation of ChR2-expressing layer 2/3 SST+ neurons (left), layer 2/3 PV+ neurons (middle), and layer 4 Scnn+ neurons (right). SST+ cells: $p = 1.3 \times 10^{-9}$, Friedmann test with Dunn’s correction, $N = 31$ cells from 7 mice. PV+ cells: $p = 1.2 \times 10^{-7}$, ANOVA test with Bonferroni’s correction, $N = 22$ cells from 7 mice. Scnn+ cells: $p = 5.2 \times 10^{-7}$, Friedmann test with Dunn’s correction, $N = 19$ cells from 8 mice. Laser power: 30 mW for SST+ and PV+ cells and 50 mW for Scnn+ cells. (D) Firing frequency increase versus displacement in the radial (top) and axial (bottom) directions during holographic illumination for layer 2/3 SST+ neurons (left), layer 2/3 PV+ neurons (middle), and layer 4 Scnn+ neurons (right). SST+ cells: top, $N = 13$ cells from 3 mice; bottom, $N = 12$ cells from 3 mice. PV+ cells: top, $N = 10$ cells from 4 mice; bottom, $N = 11$ cells from 4 mice. Scnn+ cells: top and bottom, $N = 11$ cells from 5 mice. In this figure, the black line represents the average and SEM, individual experiments are depicted in gray. See also Figure S5 and Tables S1–S3.

Figure 3. Two-Photon Holographic Stimulation across Cell Types and Layers In Vivo
(A) Two-photon image of one layer 2/3 SST+ interneuron (left) and one layer 2/3 PV+ interneuron (middle) expressing ChR2-mCherry (red). One layer 4 Scnn+ neuron expressing ChR2-eYFP (green), together with tdTomato (red), is shown on the right. Neurons were recorded in the juxtasomal configuration with a glass pipette (dotted white line) filled with Alexa Fluor 488 (green) in vivo. (B) Top: electrophysiological traces recorded Pre, during (Stim), and Post holographic stimulation (red bar) for one SST+ cell (left), one PV+ cell (middle), and one Scnn+ cell (right). $\lambda_{\text{exc}} = 920 \text{ nm}$. Laser power: 30 mW for SST+ and PV+ cells and 50 mW for Scnn+ neurons. Middle: raster plot showing cell response over consecutive trials for the same neurons displayed in the top panel. Bottom: AP distribution for the trials shown in the middle panel (time bin: 100 ms) for all cell types (SST+, left; PV+, middle; Scnn+, right). (C) Average firing frequency Pre, during (Stim), and Post holographic stimulation of ChR2-expressing layer 2/3 SST+ neurons (left), layer 2/3 PV+ neurons (middle), and layer 4 Scnn+ neurons (right). SST+ cells: $p = 1.3 \times 10^{-9}$, Friedmann test with Dunn’s correction, $N = 31$ cells from 7 mice. PV+ cells: $p = 1.2 \times 10^{-7}$, ANOVA test with Bonferroni’s correction, $N = 22$ cells from 7 mice. Scnn+ cells: $p = 5.2 \times 10^{-7}$, Friedmann test with Dunn’s correction, $N = 19$ cells from 8 mice. Laser power: 30 mW for SST+ and PV+ cells and 50 mW for Scnn+ cells. (D) Firing frequency increase versus displacement in the radial (top) and axial (bottom) directions during holographic illumination for layer 2/3 SST+ neurons (left), layer 2/3 PV+ neurons (middle), and layer 4 Scnn+ neurons (right). SST+ cells: top, $N = 13$ cells from 3 mice; bottom, $N = 12$ cells from 3 mice. PV+ cells: top, $N = 10$ cells from 4 mice; bottom, $N = 11$ cells from 4 mice. Scnn+ cells: top and bottom, $N = 11$ cells from 5 mice. In this figure, the black line represents the average and SEM, individual experiments are depicted in gray. See also Figure S5 and Tables S1–S3.
controlled the SLM to generate extended shapes covering the cell bodies of a group of four neurons (Figures 6C and 6D). We photostimulated the selected neurons (stimulus power: 56 mW/cell) while simultaneously imaging these and the surrounding neurons at 11 Hz. Targeted neurons that displayed clear ChR2-expression (Figures 6C and 6D, neuron 1–3) showed strong and reliable responses to photostimulation. Neighboring neurons responded weakly to holographic stimulation of target neurons, as expected from previous work (Packer et al., 2015). Moreover, in cells expressing only jRCaMP1a, we controlled for potential artifacts induced by holographic stimulation on jRCaMP1a fluorescence. We recorded jRCaMP1a signals at 1,100 nm while performing repetitive short (Figures S7A and S7B) or prolonged (Figures S7C and S7D) holographic stimulation at 920 nm. We found that stimulation of the imaged cell (stimulation power: 30 mW) generated an artifact in the jRCaMP1a signal that could be removed by background subtraction. Increasing stimulation power from 30 to 50 mW resulted in similar effects (Figure S7E). Repetitive stimulation did not decrease jRCaMP1a baseline, and it did not induce evident signs of jRCaMP1a photobleaching (Figures S7F–S7H). Similarly, in background-subtracted traces, photostimulation did not affect jRCaMP1a fluorescence.
not significantly affect the amplitude and the off kinetics of the responses to whisker deflection (Figures S7I–S7M).

Finally, we performed simultaneous two-photon imaging and patterned photoinhibition in PV+ cells co-expressing jRCaMP1a and the soma-targeted GtACR2 (Mahn et al., 2017) in vivo. These neurons display a high spontaneous firing rate under our experimental conditions (Figures 3A, 3C, 7A, and 7D). We found that patterned illumination decreased the baseline jRCaMP1a signal in the stimulated cell (Figures 7A–7C). Simultaneous electrophysiological recording of the stimulated neuron confirmed that the baseline reduction in jRCaMP1a signal was associated with a decrease in the spike rate of the stimulated neuron (Figures 7A and 7D).

DISCUSSION

Simultaneous two-photon imaging and manipulation is increasingly recognized as a crucial tool for the causal investigation of brain networks (Bovetti and Fellin, 2015; Carrillo-Reid et al., 2017; Emiliani et al., 2015; Grosenick et al., 2015). Such a technique allows perturbing the activity of functionally identified ensembles of neurons and testing the role of specific activity patterns in the regulation of network dynamics and behavior (Carrillo-Reid et al., 2017; Dal Maschio et al., 2017; Panzeri et al., 2017). Here we developed an all-optical approach for simultaneous two-photon imaging of a red-shifted functional indicator and bidirectional perturbation of neural activity using blue light-sensitive opsins in vivo. We validated our approach across different cell types and layers of the mouse neocortex. This is a fundamental step to apply all-optical methods to investigate the role of precise spatiotemporal activity patterns in driving higher cortical functions, because activity patterns are distributed in space and time across cellular subtypes (Carrillo-Reid et al., 2017).

Our method expands the potential of simultaneous imaging and perturbation for the functional dissection of brain circuits. Previous work in the mammalian brain in vivo (Carrillo-Reid et al., 2017; Packer et al., 2015; Rickgauer et al., 2014) demonstrated that the blue light-sensitive calcium indicator GCaMP (Chen et al., 2013; Tian et al., 2009) can be coupled to the red-shifted excitatory opsin C1V1 (Yizhar et al., 2011) for simultaneous two-photon imaging and perturbation (see also Supplementary Information). However, red-shifted opsins generally display a blue-shifted tail in their absorption spectrum that may complicate spectral separation and lead to crosstalk between GCaMP imaging and opsin activation under certain conditions (Packer et al., 2015). This is especially true for red-shifted excitatory opsins with long off kinetics that are often the preferred choice for two-photon activation using scanning approaches (Prakash et al., 2012). We showed (Figures 5, 6, and 7) that the use of jRCaMP1a, a red-shifted functional indicator that is excited using two-photon stimulation between 1,050 and 1,150 nm, in combination with blue light-sensitive opsins (e.g., ChR2) that display maximal two-photon excitability around 920 nm, minimizes this form of crosstalk. Using combined imaging and electrophysiological recordings (Figure 5), we found that the activity of ChR2-expressing cells was not changed by raster scanning in vivo ($\lambda_{\text{imaging}} = 1,100$ nm; laser intensity: 30–50 mW; frame rate: 11 Hz; scan resolution: 0.58 μm/pixel; FOV dimension: 58 $\times$ 58 μm²), in agreement with what observed in cultured neurons (Figure S1). The absence of crosstalk likely stems not only from the spectral separation of the two light-sensitive molecules that we have used (i.e., jRCaMP1a and ChR2) but also from the fast closing kinetics of ChR2 (Lin et al., 2009) and the low power required for imaging. The experimental configuration that we presented provides other advantages. For example, the use of red-shifted indicators may facilitate deep imaging by using longer wavelengths for fluorescence excitation and emission, which are less sensitive to tissue scattering (Helmchen and Denk, 2005). In addition, because of their stability for long-term expression (Dana et al., 2016), they can be efficiently used for chronic experiments. Moreover, the stimulation of blue
light-sensitive opsins at 920 nm may decrease tissue heating that is higher at the longer wavelengths ($\lambda = 1,040$ nm) (Podgorski and Ranganathan, 2016) used to stimulate red-shifted opsins (e.g., C1V1) (Carrillo-Reid et al., 2017; Packer et al., 2012, 2015; Prakash et al., 2012; Rickgauer et al., 2014). Although photocurrents generated by ChR2 are generally smaller than those generated by C1V1 (Klapoetke et al., 2014; Yizhar et al., 2011), the two-photon cross section of ChR2 is high (Rickgauer and Tank, 2009), and cells responded efficiently to stimulation (Figures 1, 2, and 3).

Previous work in vivo demonstrated high spatial resolution two-photon activation of excitatory opsins (Carrillo-Reid et al., 2017; Packer et al., 2015; Rickgauer et al., 2014). Here we show that holographic two-photon illumination can be used for efficient suppression of neural activity with high spatial resolution and can be coupled with functional imaging for all-optical readout and inhibitory optogenetic manipulation in vivo. Although previous evidence in vitro showed that some light-sensitive proton pumps are excitable with a two-photon process (Prakash et al., 2012), here we focused on the use of chloride-permeable anion channelrhodopsins. We reasoned that the increased flow of ions per photocycle that characterizes light-sensitive channels would allow generation of larger photocurrents and more efficient hyperpolarization of neurons in vivo compared to the use of light-sensitive pumps. Among the various chloride-permeable opsins (Berndt et al., 2014; Wiegert

Figure 6. Simultaneous Two-Photon Imaging of Red-Shifted Indicator and Two-Photon Holographic Stimulation of Blue-Shifted Excitatory Opsin In Vivo

(A) Schematic of the optical setup for simultaneous two-photon imaging ($\lambda_{exc} = 1,100$ nm) and two-photon holographic illumination ($\lambda_{exc} = 920$ nm). S1, stimulation laser source; S2, imaging laser source; P1–2, Pockels cells; G1–2, galvanometric mirrors; SL, scan lens; TL, tube lens; D1–3, dichroic mirrors; PMT, photomultiplier tube; OBJ, objective; Hol. Module, holographic module (comprising the SLM, the $L_{1/2}$, and $L_{1–4}$ displayed in Figure 1).

(B) Calcium transients in a SST$^+$ interneuron during simultaneous two-photon imaging ($\lambda = 1,100$ nm; laser power: 25 mW; frame rate: 11 Hz; scanned area: $\sim 90 \times 90 \mu m^2$) and holographic stimulation ($\lambda = 920$ nm; laser power: 50 mW). $\Delta F/F_0$ (gray trace) was smoothed with a moving average filter (black trace). The inset shows one layer 2/3 SST$^+$ interneuron co-expressing ChR2-eYFP (green) and jRCaMP1a (red).

(C) Two-photon image showing layer 2/3 neurons expressing soma-targeted ChR2-eYFP (green) and jRCaMP1a (red) in vivo.

(D) Calcium transients recorded from jRCaMP1a-positive cells (imaging power: 30 mW; frame rate: 11 Hz). The numbers on the left refer to the neurons indicated in (B). Neurons 1–4 (red arrows) were simultaneously stimulated with four elliptical shapes covering the cell somata. Each stimulation episode is indicated by a red bar (stimulation power per cell: $\sim 50$ mW). Periods of stimulation are blanked (see Experimental Procedures). See also Figures S6 and S7.
et al., 2017; Wietek et al., 2014), we focused on GtACR2 because of its higher single-channel conductance and its blue light-sensitive, single-photon absorption spectrum (Govorunova et al., 2015). We first demonstrated that GtACR2 was efficiently stimulated through an absorption process that is compatible with two-photon excitation. Significant photocurrents were generated through holographic illumination of the cell body of GtACR2-expressing neurons in brain slice preparation (Figures 4A and 4B). The photocurrent had maximal peak amplitude for \( \lambda = 920 \text{nm} \), similar to ChR2 (Mohanty et al., 2008; Rickgauer and Tank, 2009). Moreover, holographic stimulation of GtACR2 significantly hyperpolarized principal neurons \( \text{in vivo} \) and efficiently reduced their firing rate while maintaining high spatial resolution of the optogenetic perturbation (Figures 4D–4F). Most importantly, holographic stimulation of GtACR2 could be efficiently coupled with JRCaMP1a imaging for simultaneous functional imaging and optogenetic inhibitory manipulation with high spatial resolution (Figure 7). We observed a decrease in the baseline JRCaMP1a fluorescence in \( \text{PV}^+ \) cells expressing GtACR2 upon patterned illumination at 920 nm (Figures 7A and 7B). Activation of GtACRs may change the intracellular chloride concentration and may lead to \( \text{pH} \) variations. These modifications might interfere with the fluorescence activity reporter. However, two lines of evidence suggest that the decrease in JRCaMP1a baseline activity upon patterned stimulation of GtACR2 is mainly due to a decrease in the cell’s firing rate. First, in simultaneous imaging and electrophysiological recordings, the baseline fluorescence decrease of JRCaMP1a was always associated with the decrease in the cell’s spiking rate (Figures 7A and 7D). Second, our observation is consistent with the high spontaneous firing rate of \( \text{PV}^+ \) cells (Figure 3) being integrated by the slow activity reporter JRCaMP1a and with previous reports (Kato et al., 2015) showing decreased baseline of the fluorescence reporter upon sensory stimulation in \( \text{PV}^+ \) interneurons corresponding to inhibited activity of these cells. A long recovery tail toward baseline level of the fluorescence reporter similar to the one observed in our experiments (Figures 7A and 7B) was also reported in that study (Kato et al., 2015). These results demonstrate that patterned two-photon optogenetics can be applied for high spatial precision optical inhibition of brain networks \( \text{in vivo} \), making it possible to silence endogenous activity patterns triggered by sensory stimulation with very high cellular specificity.

Although our method efficiently decreased crosstalk between the imaging laser and the opsin activation, stimulation with extended shapes induced artifacts in the fluorescence detection, as observed by previous investigators (Baker et al., 2016). This artifactual signal may be due to unwanted stimulation by holographic illumination of the fluorescence protein that is tagged to the opsin (e.g., eGFP), the fluorescence of which may leak into the red fluorescence detection channel despite the barrier filter positioned in front of the photomultiplier tube (PMT). Alternatively, because JRCaMP1a has low, but not negligible, absorption at 920 nm (i.e., the wavelength used for stimulation), the artifactual signal may originate from direct
activation of jRCaMP1a by holographic stimulation (Figure S7). If jRCaMP1a is expressed at high levels and the area covered by stimulated neuronal somata represents a significant portion of the FOV (e.g., when many neurons are stimulated at the same time), the integrated emission of dim jRCaMP1a fluorescence generated by holographic stimulation at 920 nm may generate significant artifacts in the red detection channel, as suggested by our experiments (Figure S7). This artificial signal could be removed using background subtraction (Figure 6B; Figure S7) or required a blanking period (Figure 6D). A solution to this problem could be to synchronize photostimulation with imaging so that stimulation is performed when the portions of the FOV that are of no interest are being scanned (Baker et al., 2016). For stimulus duration longer than frame duration, optimization of protein expression levels or further developments in red-shifted indicators with reduced absorption at the wavelength used for patterned illumination will be needed.

In conclusion, we provide an experimental approach to image and bidirectionally manipulate brain networks with high spatial resolution in living animals. This all-optical approach will likely represent a powerful tool to dissect how activity patterns in specified ensembles of neurons determine brain function and animal behavior.

EXPERIMENTAL PROCEDURES

Animal Surgery

All experiments were carried out according to the guidelines of the European Communities Council Directive and approved by the Instituto Italiano di Tecnologia (IIT) Animal Health Regulatory Committee and by the National Council on Animal Care of the Italian Ministry of Health (authorization 29-2011-A, 34/2015-PR). Animals were housed in individually ventilated cages under a 12-hr light:dark cycle. A maximum of 5 animals per cage was allowed. Access to food and water was ad libitum. Experiments were performed on young-adult animals (5–16 weeks old for in vivo experiments, 4–7 weeks old for in vitro experiments, either sex). Details about animal strains and viral injections are described in the Supplemental Experimental Procedures. For in vivo experiments, mice were anesthetized with intraperitoneal urethane (16.5%, 1.65 g/kg). The scalp was removed while infiltrating all incisions with lidocaine. A chamber with a central hole (hole diameter: 4 mm) was attached with dental cement to the animal’s skull for head-fixation. A craniotomy (~700 x 700 µm²) was opened over the somatosensory (or visual cortex, in the case of experiments in Scnn mice) cortex, and the dura was carefully removed (unless otherwise stated). The location of the craniotomy was guided by the intensity of the fluorescence signal of the expressed transgene. The surface of the brain waswise stated). The location of the craniotomy was guided by the intensity of the fluorescence signal of the expressed transgene. The surface of the brain was

Data Analysis and Statistics

For juxtasomal recordings, traces were high-pass filtered (cutoff frequency: 10 Hz) and spikes were detected with a threshold criterion. The threshold value was adjusted for each recorded sweep and set >3 times the SD of the trace. For experiments in Figures 1, 6, and 3 and in Figures S3 and S5, AP firing frequency was calculated in a time window Pre (window duration: 1 s), Stim (duration: 0.5 s) and Post (duration: 1.5 s) holographic stimulation over 15–20 stimulation trials. ΔAPFreq was calculated as the difference between the firing frequencies of the Stim and Pre time windows. Opisin-positive cells (for definition, see In vivo electrophysiological recordings in the Supplemental Experimental Procedures) were considered responsive to holographic stimulation when ΔAPFreq was >1.5 times the firing rate in the Pre period at stimulation power ≤ 92 mW per shape. The fraction of opsin-positive neurons responding to holographic illumination was 14/16 for Ca²⁺/calmodulin-dependent protein kinase II-positive (CaMKII) cells expressing ChR2 (Figures 1C–1E), 17/17 for layer 2/3 cells expressing ChR2 under the human synapsin promoter (Figures 1E and 1F), 31/33 for Sst⁺ cells expressing ChR2 (Figure 3), and 26/26 for PV⁺ cells expressing ChR2 (Figure 3). To compute the spatial resolution, neuronal responses (quantified as ΔAPFreq) were recorded first with the stimulation shape centered on the cell body and then during successive shifts of the excitation volume in the radial (20 µm steps) and in the axial (±25 µm steps) directions. ΔAPFreq as a function of the shift was then plotted for every recorded neuron in the three conditions (radial, axialdown, and axialup) and fitted with a mono-exponential function (ΔAPFreq(x) = A * exp(-l * x)) (Packer et al., 2015). Fitting curves with l < 0 or with values of A that were different by more than 25% compared to ΔAPFreq at position x = 0 were not considered. The spatial resolution, lBest, was defined as the distance at which the evoked response (calculated from fit) was equal to A/2. For the analysis of the recordings from GtACR2 expressing neurons, see Supplemental Experimental Procedures.

Statistical Methods

All values are expressed as mean ± SEM unless otherwise stated. For each experimental group, sample size was chosen based on previous studies (Carillo-Reid et al., 2016; Packer et al., 2015; Rickgauer et al., 2014). No statistical methods were used to predetermine sample size. All recordings with no technical issues were included in the analysis. For N ≥ 10, a Kolmogorov-Smirnov normality test was used to test for normality. For N < 10, a Saphiro-Wilk normality test was adopted. In case of normal distribution, Student’s t test was used to calculate statistical significance when comparing two populations of data. For non-normal distributions, the non-parametric Mann-Whitney test or Wilcoxon signed-rank test (for unpaired or paired comparison, respectively) was used unless otherwise stated. When multiple (>2) populations of data were compared, one-way ANOVA with Bonferroni or Tukey’s honestly
significant difference (HSD) post hoc test was used in case of Gaussian distribution. For non-normal distribution and multiple comparisons, the non-parametric Friedman test with Dunn's post hoc correction was used. All tests were two sided. Statistical analysis was performed using Prism (GraphPad, La Jolla, CA) and OriginPro 9.1 (OriginLab).

**Supplemental information**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.063.

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**AUTHOR CONTRIBUTIONS**

A.F. and N.B. performed in vivo experiments. D.V. performed slice recordings. A.F., D.V., N.B., F.S., and F.N. performed analysis. F.S., N.B., and A.F. performed confocal experiments. A.F., N.B., S.B., and F.S. performed viral injections. M.M. performed experiments on cultured neurons. C.M. and A.F. developed hardware and software. M.M., C.A.B., M.M.B., and O.Y. provided reagents. T.F. conceived and coordinated the project. T.F. and A.F. developed hardware and software. M.M. performed experiments on cultured neurons. C.M. performed analysis. A.F., D.V., N.B., F.S., and F.N. performed analysis. F.S., N.B., and F.N. performed analysis. A.F. and N.B. wrote the manuscript with A.F. and N.B. All authors commented on the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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