Precise multimodal optical control of neural ensemble activity

Alan R. Mardinly 1,5, Ian Antón Oldenburg 1,5, Nicolas C. Pégard 1,2,5, Savitha Sridharan 1, Evan H. Lyall 3, Kirill Chesnov 1, Stephen G. Brohawn 1,4, Laura Waller 2 and Hillel Adesnik 1,4*  

Understanding brain function requires technologies that can control the activity of large populations of neurons with high fidelity in space and time. We developed a multiphoton holographic approach to activate or suppress the activity of ensembles of cortical neurons with cellular resolution and sub-millisecond precision. Since existing opsins were inadequate, we engineered new soma-targeted (ST) optogenetic tools, ST-ChroME and IRES-ST-eGtACR1, optimized for multiphoton activation and suppression. Employing a three-dimensional all-optical read-write interface, we demonstrate the ability to simultaneously photostimulate up to 50 neurons distributed in three dimensions in a 550 × 550 × 100-µm³ volume of brain tissue. This approach allows the synthesis and editing of complex neural activity patterns needed to gain insight into the principles of neural codes.

Neural circuits can encode information in the rate 1, timing 2, number 3, and synchrony of action potentials 4, as well as in the identity of active neurons 5. Yet the technical inability to create or edit custom patterns of spatiotemporal neural activity is a key impediment to understanding the logic and syntax of the neural code 6. Experimental approaches that allow high-fidelity temporal control of neural activity 7 from specific groups of neurons would limit the ability of experimenters to assess the necessity of spikes originating from specific, functionally defined neurons.

Optogenetics offers the basis for such a technology, but many neural computations rely on genetically similar yet functionally distinct neurons that are physically intermixed 8,9, and are thus beyond the reach of conventional approaches. Two-photon (2P) optogenetics 10–13 allows experimenters to stimulate neurons based on their precise spatial location as well as their genetic identity. Combined with 2P calcium imaging, this allows activation of specific neurons on the basis of any desired feature 14–18. However, in vivo all-optical approaches in mice have suffered from low temporal precision (>10 ms jitter) 14–16, or could only photostimulate several neurons simultaneously 15,17 and thus could not create precise neural activity patterns. Furthermore, until recently, in vivo multiphoton suppression of neural activity was not previously possible 19,20, critically limiting the ability of experimenters to assess the necessity of spikes originating from specifically defined neurons.

Several optical methods can stimulate neurons using 2P excitation, although all have limitations. A standard 2P optogenetic technique is to scan a small laser spot over neurons that express variants of slow red-shifted opsins like C1V1 17,18. While this approach effectively drives spiking, the slow kinetics of the scanning laser and the opsin preclude precise control of the specific sequence of neural activity, resulting in uncertain trial-to-trial reproducibility 12,14,16. In contrast, scanless multiphoton stimulation using computer-generated holography (CGH) and temporal focusing allows simultaneous illumination of the entire somatic membrane and can provide higher temporal fidelity when used with a fast opsin 12,14–16. However, existing excitatory opsins are too weak or too slow to drive precise neural activity patterns with scanless 2P optogenetics in vivo 12,20. Additionally, only recent innovations have allowed scanless holographic optogenetics to function with high axial resolution in three dimensions (3D) 12.20.

Therefore, to synthesize and edit custom distributed patterns of neural ensemble activity, we engineered powerful new opsins optimized for multiphoton optogenetics. We developed ChroME, an ultrafast and highly potent opsin with 3–5× larger photocurrents than opsins commonly used for multiphoton optogenetics 12,14,15,21. This allows high-fidelity sub-millisecond control of pyramidal neuron spiking. In vivo, we activated ensembles of neurons expressing ST-ChroME using 3D scanless holographic optogenetics with temporal focusing (3D-SHOT) 12, synthesizing precise sequences of neural activity with cellular resolution and millisecond precision. By combining 3D-SHOT with volumetric 2P calcium imaging, we obtained all-optical control of distributed neural ensembles, simultaneously stimulating up to 50 neurons with high temporal precision and cellular resolution. Furthermore, to achieve all-optical suppression, we improved and employed the extremely potent inhibitory anion opsin, GtACR1 12,19,20, which exhibits 80-fold increases in photocurrent over previously employed pump-based opsins for multiphoton optogenetic silencing 19. Critically, we identified a strategy to prevent the cellular toxicity we observed when the transgene was expressed conventionally. Using this new construct, IRES-ST-eGtACR1, with 3D-SHOT, we provide electrophysiological and all-optical demonstration of high fidelity silencing of neural activity from identified neurons in vivo. Together, these data represent a novel technological approach for precise multimodal control of neural ensemble activity with high fidelity.

Results

Requirements for controlling neural activity with millisecond precision. To control neural activity with sub-millisecond preci-
sion, we sought an opsin and a 2P stimulation approach capable of generating large currents with rapid kinetics\textsuperscript{35}. Injecting current into patched neurons (layer L 2/3 pyramidal cells, used throughout this study unless otherwise noted) in brain slices, we could reliably evoke precise spike trains with brief, high-amplitude current steps. In contrast, long current injections, analogous to some spiral scanning approaches\textsuperscript{11,16}, resulted in variable spike number and timing, but required lower current amplitudes (Fig. 1a,b and Supplementary Fig. 1).

To achieve fast currents using 2P excitation, we adopted scanless holographic approaches, CGH and 3D-SHOT, that can simultaneously illuminate the entire soma. We developed an experimental setup that combines a standard 2P imaging system with a custom photostimulation laser path. The photostimulation path features a high-power (20 W or 40 W) 2-MHz, 1,040-nm laser with a spatial light modulator (SLM) placed in Fourier space to simultaneously target neurons in 3D. For targeting neural ensembles in vivo with high spatial resolution, the CGH path was replaced with 3D-SHOT\textsuperscript{28}, a new form of 3D holography with temporal focusing that we recently developed and further improved (Supplementary Fig. 2, Supplementary Tables 1 and 2, and Methods).

We first sought to identify the best opsin for precise temporal control of neural activity using scanless approaches. We rejected channelrhodopsin2 (ChR2), as its 2P excitation peak is centered at 920 nm and would be strongly activated by GCaMP imaging, resulting in undesirable optical crosstalk\textsuperscript{29}. Instead we tested several red-shifted opsins: CIV1\textsubscript{175}\textsuperscript{1}, ChrimsonR, and Chronos\textsuperscript{28} (Supplementary Fig. 3a). CGH stimulation (5 ms, 0.4 mW/µm\textsuperscript{2}) of Chronos+ neurons elicited small photocurrents that were typically unable to generate action potentials (205 ± 50 pA). To improve spatial resolution and photocurrent amplitude, we employed the Kv2.1 sequence tag to synthesize ST-ChroME\textsuperscript{31,34}, variants of Chronos, CIV1\textsubscript{175}, and ChrimsonR that increased photocurrents elicited by CGH stimulation (ST-Chronos: 460 ± 60 pA; Supplementary Fig. 3b). Neurons expressing ST-opsins had photocurrent kinetics similar to those in published reports\textsuperscript{28} and had normal intrinsic properties (Supplementary Fig. 3c–i).

Despite this improvement, photocurrents were insufficient to reliably spike pyramidal neurons (Fig. 1a). We therefore optimized the pulse parameters of the stimulation laser. We tested the effect of peak power, pulse energy, and average power on photocurrents by systematically varying the laser repetition rate (2–40 MHz) and pulse dispersion. We could saturate photocurrents across a range of powers, but high-peak powers (i.e., low rep-rates) saturated more efficiently (Supplementary Fig. 4a–d). Stimulation powers used in experiments did not damage cells\textsuperscript{35} (Supplementary Fig. 4e,f). For all subsequent experiments, we employed 250- to 300-femtosecond laser pulses at a repetition rate of 2 MHz with power varying from 0.1 to 0.4 mW/µm\textsuperscript{2}.

Even after optimization, average maximal photocurrents elicited by CGH stimulation (5 ms, 0.4 mW/µm\textsuperscript{2}) of pyramidal neurons expressing ST-opsins remained relatively weak (ST-CIV1\textsubscript{175}: 380 ± 80 pA, ST-ChrimsonR: 430 ± 60 pA, ST-Chronos: 530 ± 50 pA; Fig. 1c). A simple integrate-and-fire model of typical L2/3 neurons suggested that these photocurrents were unlikely to generate spikes (Supplementary Fig. 5a). Even when we used long light pulses and high light powers, only a minority of neurons expressing these opsins could be activated by CGH stimulation (Fig. 1d and Supplementary Fig. 5).

**ChroME allows high fidelity replay of complex activity patterns.** Since none of these opsins could reliably spike neurons in response to brief holographic stimulation, we engineered a stronger opsin with the goal of holographically stimulating large ensembles of neurons. We focused on mutating ST-Chronos, aiming to develop a variant that would preserve its fast kinetics but would generate sufficiently large photocurrents with brief light pulses. Guided by homology modeling to the crystal structure of CIC2\textsuperscript{36} (Supplementary Fig. 6a), we mutated the pore region of ST-Chronos, identifying a neutral putative pore residue (M140) in Chronos that is negatively charged in other opsins (Supplementary Fig. 6b). We reasoned that mutating this methionine to a negatively charged residue might increase the flux of positive ions through the pore and therefore increase current amplitudes. We tested several mutations via one-photon stimulation in Chinese hamster ovary (CHO) cells against a panel of ST-opsins and identified several mutants with larger photocurrent amplitudes than any other opsin that we tested (Supplementary Fig. 6c,d). One of these mutants, ST-Chronos-M140E, or ‘ChroME’, exhibited rapid decay kinetics while exhibiting photocurrents more than 10×larger than those of ChR2 (Supplementary Fig. 6c–e). Neurons electroporated with ST-ChroME exhibited photocurrent amplitudes 3–5×larger than ST-CIV1\textsubscript{175} or ST-Chronos in response to CGH stimulation (5 ms at 0.4 mW/µm\textsuperscript{2} evoked 1.8 ± 0.2 nA; Fig. 1c). ST-ChroME retained the excitation spectrum and rapid rise time of ST-Chronos (Fig. 1e and Supplementary Fig. 3c), but its decay time constant (3.0 ± 0.4 ms) was slightly slower than ST-Chronos (1.7 ± 0.6 ms; Supplementary Fig. 3d).

In contrast to other ST-opsins (and as predicted by modeling; Supplementary Fig. 5a), 96% of ST-ChroME+ neurons were activated by CGH stimulation (Fig. 1d), requiring lower laser powers and shorter light pulses to evoke spikes than the other opsins (Supplementary Fig. 5b). This was true whether opsins were delivered via in utero electroporation or by viral infection (Supplementary Fig. 6f–k).

We next examined the temporal precision of action potentials evoked from ST-ChroME+ neurons and the minority of neurons expressing other ST-opsins that could be activated. At 1 Hz, light-evoked spikes from neurons expressing ST-ChroME or ST-Chronos occurred with short latency and low jitter, whereas the timing of spikes from ST-CIV1\textsubscript{175} or ST-ChrimsonR+ neurons was more variable (Fig. 1f–h). To test temporal precision while eliciting naturalistic sequences of action potentials, we stimulated neurons with Poisson trains of holographic light pulses. Neurons expressing ST-ChroME and ST-Chronos followed these patterns with high fidelity, exhibiting high spike probability and low jitter across a wide range of stimulation frequencies throughout the stimulus train (fidelity index score: ST-ChroME, 0.87 ± 0.03; ST-Chronos, 0.90 ± 0.02; see Methods). However, neurons expressing ST-ChrimsonR or ST-CIV1\textsubscript{175} could not follow complex stimulus patterns (fidelity index score: ST-ChrimsonR, 0.48 ± 0.05; ST-CIV1\textsubscript{175}, 0.25 ± 0.04; Fig. 1i and Supplementary Fig. 5c–g).

Since ST-ChroME allowed fast, reliable responses with brief stimulation, we reasoned that we could employ high-speed SLMs to spike different sets of neurons at high rates. To test the speed at which we could generate spike patterns in two different neurons, we recorded two ChroME+ neurons and used a fast SLM (Supplementary Fig. 7 and Supplementary Video 1) to interleave holographic stimulation of each cell at the maximum SLM rate. We generated a Poisson train of light pulses on each trial and delivered the same sequence to both neurons, separated by 3 ms. This experiment showed the we could generate naturalistic spike trains in multiple neurons offset by brief periods (Fig. 1k).

To test whether ST-ChroME drives reliable spiking under more relevant in vivo conditions, we performed 2P guided loose-patch recordings in anesthetized animals. While only 31% of ST-Chronos+ could be made to spike with 5-ms CGH pulses, over 89% of ST-ChroME+ neurons could be activated in vivo (Fig. 1l). Together, these data demonstrate that ST-ChroME can reliably generate the rapid, large photocurrents necessary to drive the temporally precise, short-latency spikes needed to replicate naturalistic neural activity patterns.
Fig. 1 | ST-ChroME allows precise high-fidelity 2P activation. **a**, Overlay of 25 trials from a representative L2/3 pyramidal neuron showing the V_r response to 5-ms current pulses or sustained current injection (\(I_h\)) near the neuron’s rheobase. **b**, Current needed to induce action potentials as a function of stimulus duration \((n = 8\) L2/3 pyramidal neurons). **c**, Left: grand average photocurrent traces from neurons expressing ST-C1V1\(_{1/1}\) (black, \(n = 19\)), ST-ChromsionR (red, \(n = 11\)), ST-Chronos (green, \(n = 25\)), or ST-ChroME (magenta, \(n = 11\)), via in utero electroporation. Right: photocurrent amplitudes elicited by CGH stimulation. Dashed line represents mean rheobase for 5-ms stimulation of L2/3 pyramidal neurons (ST-ChroME vs. others: \(P < 0.0008\), Kruskal–Wallis test with multiple comparisons correction; all other comparisons: \(P > 0.13\)). **d**, Top: duration of CGH stimulation needed to elicit action potentials in neurons expressing each opsin \((n = 8\) ST-C1V1\(_{1/1}\), \(n = 5\) ST-ChromsionR, \(n = 25\) ST-Chronos, \(n = 8\) ST-ChroME). Bottom: fraction of electroporated L2/3 neurons that could be driven at 1Hz with best CGH stimulation. **e**, Traces shown in **c** scaled to the peak current amplitude for each. **f**, Ten overlaid traces from representative L2/3 neurons expressing ST opsins during 1-Hz CGH stimulation (red line indicates light pulses). **g**, Spike latency for 1-Hz CGH stimulation of L2/3 neurons expressing ST-opsins \((n = 8\) ST-C1V1\(_{1/1}\), \(n = 5\) ST-ChromsionR, \(n = 25\) ST-Chronos, \(n = 8\) ST-ChroME). **h**, Jitter for 1-Hz CGH stimulation of neurons expressing ST-opsins \((n = 8\) ST-C1V1\(_{1/1}\), \(n = 5\) ST-ChromsionR, \(n = 25\) ST-Chronos, \(n = 8\) ST-ChroME). **i**, 2P image of whole cell recording from L2/3 pyramidal neuron expressing ST-ChroME-mRuby2 (image representative of \(n = 10\) ST-ChroME-mRuby2 neurons), **j**, Fidelity index in response to Poisson-like stimulation (ST-ChroME \((n = 7)\) vs. ST-C1V1\(_{1/1}\) \((n = 6)\), \(P = 0.048\); vs. ST-ChromsionR \((n = 4)\), \(P = 0.001\); vs. ST-Chronos \((n = 9)\), \(P = 0.048\); by Kruskal–Wallis test with multiple comparisons correction). **k**, Left: representative traces of two simultaneously recorded ST-ChroME* neurons stimulated with an identical Poisson train for 2.5 ms with a temporal offset of 3 ms. Top middle: example light-evoked spikes in the two neurons. Bottom middle: distribution of the difference in spike times from an example pair of neurons. Right: difference in mean spike times for \(n = 7\) pairs. **l**, Bar graph showing the fraction of neurons expressing ST-Chronos (green) or ST-ChroME (magenta) that could be optogenetically driven in vivo \((P = 0.0089,\) two-sided Fisher’s exact test). All data represent means ± s.e.m.
Fig. 2 | Fast, potent holographic suppression of neural activity. a, Example average traces of whole-cell photocurrents elicited by 500-ms (100 mW, 0.2 mW/µm²) CGH stimulation (pink bar) from CHO cells held at 0 mV expressing inhibitory ST-opsins; color-coded as in b. b, Mean photocurrent elicited during a 500-ms stimulation, as in a, plotted on a log scale (n = 5 cells expressing ST-eNpHR3, n = 8 ST-eArch3, n = 9 ST-ePsuACR, n = 8 ST-eiC++, n = 5 ST-eGtACR1, n = 10 IRES-ST-eGtACR1). c, In vivo firing activity that persists during optogenetic suppression of L2/3 neurons expressing ST-opsins. Each dot represents mean activity for a single neuron (5–60 sweeps per cell; n = 7 no-opsin controls, n = 6 ST-ePsuACR, n = 8 ST-eArch3, n = 10 ST-eGtACR1). d, Example confocal images from juvenile (14–15 d old) and adult (35+ d old) mice expressing ST-eGtACR1-mRuby2 or H2b-mRuby3 IRES-ST-eGtACR1. Imaging conditions are matched within an opsin. Representative image from 3 mice each condition. e, Example whole-cell voltage-clamp recording of a L2/3 neuron expressing IRES-ST-eGtACR1 held at 0 mV and stimulated for 500 ms (pink bar) with varying illumination powers. f, In vivo activity that persists during optogenetic suppression of L2/3 neurons expressing ST-opsins. Each dot represents mean activity for a single neuron (5–60 sweeps per cell; n = 8 ST-eArch3, n = 10 IRES-ST-eGtACR1). g, Overlay of 30 current-clamp traces from a L2/3 pyramidal neuron expressing IRES-ST-eGtACR1 during current injection, aligned to the onset of a 50-ms stimulation at three different power levels. h, Top: as in g, the time for suppression to take effect calculated for a 50-ms light stimulation. Reported as the tau (τ) of a fit to the observed number of action potentials after light onset in 1-ms bins, assuming a Poisson noise model (n = 6 neurons). Bottom: membrane potential of each neuron during the last 10 ms of a 50-ms stimulus as a function of stimulus intensity (n = 6 neurons). i, Duration of suppression, defined as the mean time until the next action potential as a function of stimulus intensity. Grey lines indicate individual replicates, black shows mean and s.e.m. (n = 6 neurons). j, Left: overlay of 30 whole-cell current-clamp traces during light stimulation of different durations. Bottom: schematic of current injection protocol, in which onset of current injection was varied with respect to the light stimulus. Right: quantification of the duration of suppression as a function of stimulus duration. Grey lines indicate individual replicates, black shows mean (n = 6 neurons). All data represent ± s.e.m.

Anion opsins permit rapid and potent silencing of neural activity. We next asked whether we could identify or engineer an optogenetic silencer to suppress neural activity with high efficacy and temporal precision. We synthesized and tested a suite of ST-inhibitory opsins with ER export motifs (‘e’)36,37 including pumps (eNpHR3 and eArch3)38,39 and anion channels (GtACR1, psuACR29,38, and iC++39). ST-eGtACR1 generated the largest outward photocurrents while retaining moderately fast kinetics (rise time, 1.5±0.7 ms; decay time, 12.5±0.7 ms; Fig. 2a,b and Supplementary Fig. 8a,b). GtACR1 photocurrents were nearly saturated in normal conditions and not improved by the ‘e’ signal (Supplementary Fig. 8c–g). Furthermore, ST-eGtACR1 was more sensitive to 1,040-nm light than to 930-nm light (Supplementary Fig. 8h).

Since these silencers function through different biophysical mechanisms, it was possible that the opsins with the largest photocurrent might not be the most effective suppressor of endogenous neural activity. We therefore tested 2P holographic suppression in vivo by performing targeted loose-patch recordings from cells expressing inhibitory opsins. Of the opsins that we tested, ST-eGtACR1 was the most efficient silencer, reducing activity to 8.4±3% of normal firing rate with 0.2 mW/µm² of 2P stimulation. In contrast, at the same laser power, ST-eArch3 only reduced activity to 37±8%, whereas ST-ePsuACR or light alone did not significantly alter firing rates (82±11% and 90±9%, P = 0.31 and P = 0.47, respectively, Wilcoxon signed-rank test vs. no change; Fig. 2c and Supplementary Fig. 8i).

However, unlike all other opsins we expressed in vivo, we had difficulty identifying neurons positive for ST-eGtACR1-mRuby2. This seemed to be a problem only in adult animals, but was partially mitigated by the ‘e’ signal (Fig. 2d and Supplementary Fig. 9a). We suspected this might be related to aggregation of GtACR1 protein when highly expressed, possibility leading to degradation...
or toxicity. To address this problem, we generated a bicistronic construct with a nuclear-localized fluorophore (H2B-mRuby3-IRES-ST-eGtACR1) that lowers expression levels of the opsin and spares the fluorophore from degradation. IRES-ST-eGtACR1 exhibited large photocurrents in CHO cells and neurons (460 ± 200 pA in CHO cells, 920 ± 140 pA in neurons; Fig. 2b,e). Antibody staining to a FLAG epitope on the GtACR1 protein confirmed that it remained soma-targeted (Supplementary Fig. 9b). Notably, unlike ST-eGtACR1, cells expressing IRES-ST-eGtACR1 were easily identified into adulthood (Fig. 2d and Supplementary Fig. 9a). Neurons expressing IRES-ST-eGtACR1 had normal intrinsic properties (Supplementary Fig. 3e–i) and spontaneous in vivo firing rates (Supplementary Fig. 9c) even in older mice. Targeted in vivo loose-patch recording revealed that IRES-ST-eGtACR1 neurons reduced their firing to 6.8 ± 5% of nominal rate in response to CGH stimulation (0.3 mW/µm²), suggesting that lowering expression levels did not affect the efficacy of silencing (Fig. 2f).

To measure the timing of suppression, we induced spiking in brain slices through current injection in cells electroporated with IRES-ST-eGtACR1. We varied the onset time of holographic suppression so that spike timing was randomized trial-to-trial, and we varied the stimulation intensity and duration in separate experiments (Fig. 2g–i). We found that onset of suppression was rapid, with spiking eliminated within 1.5 ± 0.3 ms after light onset. Similarly to photocurrent response onsets, the onset time of suppression was power-dependent (Fig. 2g,h and Supplementary Fig. 8e). Despite current injection, cells hyperpolarized to near the reversal potential of GtACR1 when stimulated with <0.1 mW/µm², indicating potent suppression (−54 ± 3 mV at 0.08 mW/µm² stimulation; Fig. 2h). Although the onset of suppression was rapid, suppression of neural activity persisted for 50–250 ms after the cessation of photostimulation, due to the decay kinetics of the GtACR1 channel. This suppression was dependent on both the intensity and duration of the light stimulus (Fig. 2h,i). Together, these data validate IRES-ST-eGtACR1 as a tool for stable, rapid suppression of neural activity using 2P optogenetics.

Creating and editing spatiotemporal sequences of neural activity in vivo. Next, we employed ST-ChroME and IRES-ST-eGtACR1 in the intact brain to create and edit spatiotemporal patterns of neural activity. For this, we employed 3D-SHOT to enable 3D holographic stimulation with high axial resolution in vivo. To validate spatial resolution, we recorded the physiological point-spread function (PPSF) using targeted loose patch recordings from ST-ChroME neurons in anesthetized mice at multiple focal planes (spot diameter: 20 µm; radial full-width at half-max: 11 ± 3 µm, axial full-width at half-max: 28 ± 4 µm; Fig. 3b,c).

The majority of ST-ChroME neurons fired reliably, temporally precise action potentials in response to brief 3D-SHOT stimulation using powers less than 0.2 mW/µm². This was true when electroporated with ST-ChroME-mRuby2 or virally transduced with AAV DIO-ST-ChroME-P2A-H2B-mRuby3 (Fig. 3d and Supplementary Fig. 12). We then stimulated them with naturalistic Poisson patterns, varying the pattern on each trial to generate unique sequences of evoked activity (Fig. 3e). Quantifying these experiments revealed that ST-ChroME neurons reliably spiked with sub-millisecond jitter, allowing the production of spatiotemporal activity patterns with high fidelity (Fig. 3f and Supplementary Fig. 13a).

Conversely, to remove spikes from endogenous neural activity, we recorded from IRES-ST-eGtACR1 neurons. 3D-SHOT stimulation at 0.32 mW/µm² produced at least a 95% reduction in firing in >75% of IRES-ST-eGtACR1 cells (Fig. 3g). The efficacy of holographic suppression increased with stimulation power, allowing us to either completely silence the activity of a neuron during a defined time-window at high power or titrate a neuron’s average firing rate with lower powers (Fig. 3h,i and Supplementary Fig. 10a,b). Suppression appeared constant over the entire stimulation period, consistent with the observation that GtACR1-evoked photocurrents did not substantially desensitize (Fig. 3h,i). Suppression was repeatable over many trials without loss of efficacy or any apparent change in spontaneous firing rates of stimulated neurons (Fig. 3h and Supplementary Fig. 10c). This demonstration of single-neuron suppression using 3D-SHOT represents the second element in a bidirectional toolbox to control spatiotemporal patterns of neural activity.

Holographic spatiotemporal control of cortical inhibitory neurons. Whereas L2/3 neurons typically fire sparsely, cortical inhibitory neurons are heterogeneous and many fire at much higher frequencies. We therefore combined spatial and genetic selectivity by stimulating specific subsets of GABAergic neurons (PV, SOM, or VIP) expressing Cre recombinase transgenically and infected with AAV-DIO-ST-Chronos-mRuby2. Inhibitory neurons are typically more excitable than pyramidal neurons, and ST-Chronos was sufficient to generate reliable action potentials in these cells (Fig. 4a,b). We identified power levels needed to elicit reliable spiking at 1 Hz (<0.3 mW/µm²; Fig. 4c) and performed Poisson 3D-SHOT stimulation (Fig. 4d–f). Stimulation of each GABAergic cell type drove reliable, short-latency spikes with sub-millisecond jitter across many stimulation frequencies, allowing these neurons to follow stimulus trains with high fidelity (Fig. 4g–j and Supplementary Fig. 13b–d). Unlike L2/3 pyramidal neurons and VIP neurons, PV and SOM cells were able to follow stimuli with instantaneous frequencies up to 100 Hz (Fig. 4j). Additionally, we replayed several unique patterns of action potentials with identical mean rates, demonstrating our ability to reliably generate precise activity patterns over many trials (Supplementary Fig. 13e–g).

Addressing multiple forms of optical crosstalk. To edit spatiotemporal activity patterns while simultaneously reading out network activity, we sought to combine our approach with 2P calcium imaging. To accomplish this, we addressed two forms of optical crosstalk that we encountered. First, the photostimulation laser can directly excite GCaMP6, adding severe artifacts to the imaging data. To overcome this problem, we synchronized the stimulation laser pulse gating with the resonant galvo mirrors using a custom thresholded resistor–capacitor circuit (Supplementary Fig. 14a–d). This resulted in a stimulation duty cycle of ~16 kHz, providing stimulation on either side of each imaging line. Because this circuit is tunable, it provides a customizable tool to trade average stimulation power for effective field of view along the x axis. Typically, we sacrifice ~50% of our stimulation power, resulting in light artifacts in <240 µm of imaging area along the x axis (imaging window normally: 550 × 550 µm; with gate synchronization: 310 × 550 µm free of stimulation artifacts; Supplementary Fig. 14d). However, due to the extremely fast duty cycle compared to the kinetics of the opsins, we observed that this loss of stimulation power results in only a 10–20% reduction in photocurrents when using the laser gate (Supplementary Fig. 14e,f). All reported estimates of illumination densities account for losses from use of this circuit.

Second, the imaging laser can excite opsins and thereby modulate neural activity independently of the stimulation laser. To characterize the effect of the imaging laser on photocurrents, we patched opsin-expressing neurons in brain slices and imaged them at different powers, window sizes, and volumes (i.e., frame rates). During 2P imaging of GCaMP6, the imaging laser induced brief photocurrents as the laser contacted the cell. These currents decayed between frames and were substantially smaller than holographic currents (Supplementary Fig. 15a,d). Imaging volumetrically reduced the effective frame rate, decreasing the imaging-induced photocurrents. When reducing the size of the imaging window, thus increasing the
dwell time of the imaging laser on the opsin-expressing neuron, photocurrents increased (Supplementary Fig. 15a–f).

Together, these data indicate that photocurrents caused by the imaging laser under standard widefield volumetric imaging conditions are unlikely to influence firing rates. Nevertheless, to directly test 2P imaging-induced crosstalk in vivo, we performed loose-patch recordings from all four classes of cortical neurons. Pyramidal neurons were electroporated with ST-ChroME or IRES-ST-eGtACR1 or virally transduced with AAV DIO-ST-ChroME, while PV, SOM, and VIP neurons were virally transduced with AAV DIO-ST-Chronos. We did not observe detectable modulation of firing rates by the imaging laser when scanning with 50 mW at 30 Hz over a 400 × 400-μm window, at depths between 100 and 270 μm (Supplementary Fig. 15g,h). However, ChroME+ neurons increased
their firing rates when the size of the imaging window was below 400x400 μm (Supplementary Fig. 15i). These data indicate that widefield volumetric or video-rate 2P imaging is compatible with these optogenetic tools, if care is taken to minimize crosstalk.

Next, to enable 3D all-optical read–write experiments (Fig. 5a), we created a custom suite of software to co-align 3D-SHOT and 3D calcium imaging (Supplementary Fig. 16). This alignment was facilitated by an improved version of 3D-SHOT that employs a rotating diffuser instead of a lens to shape the phase of the temporally focused disc. Using this approach, phase is randomly encoded spatially and temporally rather than shaped into a static spherical pattern. This increases available power through the objective and eliminates the secondary geometric focus27, further enhancing axial confinement (Supplementary Fig. 11).

**All-optical control of neural activity with high spatiotemporal fidelity.** Using these technical advances, we tested our ability to perform all-optical read–write using 3D-SHOT stimulation to generate spikes with high-fidelity, sub-millisecond temporal precision, and cellular resolution in full 3D. Experiments were performed in primary somatosensory cortex (S1) of awake, headfixed mice on a treadmill. Mice expressed both GCaMP6s43 and ST-ChroME in excitatory neurons (see Methods). To avoid failures or extra spikes, we determined the minimum laser power needed for each cell to reliably drive spiking with short pulses (Fig. 5b,c). The all-optical data matched in vivo physiology measurements, as neurons’ optical response function reached 80% of saturation with 0.16±0.02 mW/μm² (Fig. 5d).

We then rapidly activated neurons located throughout a 550-μm×550-μm×100-μm volume (three imaging planes spaced 50μm apart using an electrotunable lens44, as used throughout the study), well within the accessible volume of our stimulation (Supplementary Fig. 11h,i). Neurons were stimulated one by one with a series of ten light pulses (5 ms, 30 Hz), and we read out the effects via GCaMP fluorescence. Generation of action potentials in this manner elicited large increases in GCaMP6s fluorescence. Deconvolution of the calcium signal (see Methods) revealed that the temporal sequence of activation was reliable across many trials and
repeatable in multiple animals (Fig. 5e–g). On average, spatial resolution remained high even in awake in vivo conditions (Fig. 5h), but failures and off-target activation could occur during rare episodes of brain motion (Supplementary Fig. 17a). Such motion was easily identified post hoc, and trials in which motion coincided with photostimulation were excluded from analysis (Supplementary Fig. 17b). Holographic stimulation did not affect the animals’ running behavior (Supplementary Fig. 17c). Fig. 5 | All-optical read–write with high spatiotemporal fidelity. a, Schematic illustrating single-cell 3D all-optical read–write experiments performed in headfixed mice freely running on a circular treadmill. b, Example optical rheobase experiment (ten 5-ms pulses at 30 Hz) with varying light intensity using 3D-SHOT. Top: example ΔF/F calcium trace (black) or deconvolution (red). Bottom: raster plots of z-scored deconvolved calcium traces. c, Left: average deconvolved calcium traces from an optical rheobase experiment (shading indicates 95% confidence intervals (CI); scale bars represent 10% max response and 1x). Right: the example neurons’ all-optical response function. d, Power intensity needed to approach saturation of the all-optical response function (80% of maximum, n = 96 neurons, representative experiment from n = 3 mice; red bars indicate mean ± s.e.m.). e, Five consecutive trials of sequential stimulation of n = 134 neurons from a representative experiment. Each panel corresponds to one trial (separated by dashed lines), and each line shows the trial-wise z-scored deconvolved calcium response for each neuron (see color bar on f). Neurons were stimulated at 2 Hz with ten 5-ms pulses at 30 Hz. f, Mean z-scored deconvolved ΔF/F for each neuron in response to 3D-SHOT stimulation of each holographically targeted neuron. A neuron’s response to its own stimulation is plotted on the diagonal. Data represent the mean z-scored deconvolved calcium response from 12 trials from a representative experiment (n = 3 mice). g, Each point represents the mean change in z-scored calcium response of a stimulated neuron upon stimulation (red) or the mean change in response to stimulation of other cells (gray). Mouse 1: n = 255 neurons, P = 3.76 × 10−51; Mouse 2: n = 115 neurons, P < 4.3 × 10−17; Mouse 3: n = 106 neurons, P < 1.95 × 10−10, two-sided paired t test; red bars indicate mean ± s.e.m.). h, Mean fluorescence of all stimulated neurons, aligned so the targeted neuron is centered (two poststimulation frames per neuron). Image is the mean response of 134 targets. Dashed black lines show the size of the stimulation area, r = 10 µm. Data is from a representative experiment (n = 3 mice). All data represent mean ± s.e.m. unless otherwise noted.

All-optical suppression of neurons. Next, we performed all-optical suppression of activity in awake mice. As L2/3 pyramidal neurons fire sparsely, we focused on PV+ interneurons, which have high tonic firing rates. To accomplish this, we created a Cre-dependent viral version of IRES-ST-eGtACR1 (AAV9 DIO-NLS-mRuby3-IRES-ST-eGtACR1), and confirmed its efficacy in vitro via whole-cell recordings and in PV cells in vivo via cell-attached recordings (Supplementary Fig. 18a–c). We co-infected PV-Cre mice with viral DIO-IRES-ST-eGtACR1 and DIO-GCaMP6f. As in Fig. 5, we imaged calcium activity while the animal was awake and headfixed on a treadmill (Fig. 6a). We sequentially suppressed individual PV cells (1-s illumination, 0.16 mW/µm²; Fig. 6b and Supplementary Fig. 18d,e); most (90.6%) cells exhibited reduced fluorescence when targeted but showed no consistent change when other neurons were targeted (Fig. 6b–d). We observed no correlation between the direction or magnitude of a response and its distance from the targeted cell (Supplementary Fig. 18f).

Next, we suppressed groups of four randomly selected PV cells while simultaneously imaging (6–12 groups per experiment, 1-s illumination at 0.08 mW/µm²; Fig. 6e–h). Suppression of ensembles was also selective, as the laser caused suppression in only the cells targeted by the holographic pattern (Fig. 6h). These data demonstrate all-optical suppression of neural activity in multiple neurons across a large working volume.

All-optical spatiotemporal control of neural ensembles. We next employed ST-ChroME to manipulate larger ensembles. When testing spatial resolution of ensemble stimulation in brain slices, we found that use of the ST-opsin, which increases stimulation resolution for one target41, was essential when stimulating groups of neurons with many holograms (Supplementary Fig. 19a–f), something not employed in previous manipulations of neural ensembles13,16,45.

We tested multiphoton spatial resolution in vivo with cell-attached recordings of ST-ChroME+ cells. The spiking PPSF was measured for each cell with holograms targeting 1–50 spots simultaneously...
Throughout a large volume (400×400×200 μm). These experiments showed that 3D-SHOT stimulation in vivo remained spatially precise when targeting up to 50 locations simultaneously (Supplementary 19g–i).

To manipulate large groups of cells all-optically, we prepared mice as in Fig. 5 and selected 150 ST-ChroME+ neurons across three planes (Fig. 7a). We randomly assigned them to unique neural ensembles containing overlapping sets of 10, 25, or 50 neurons and stimulated them with 10 pulses each at 10, 20, or 30 Hz (Fig. 7b, Supplementary Fig. 20a, and Supplementary Video 2). We did not stimulate more than 50 neurons simultaneously due to limitations in available laser power (4.1 Watts available from the objective, resulting in approximately 0.13 mW/μm² or 40 mW per target, accounting for losses from the imaging gate, but not for decreased diffraction efficiency of phase masks encoding 50 spots across the accessible volume). For control trials, we either did not photo-stimulate the brain at all or directed the laser to 50 random spots (Supplementary Fig. 20a). Neurons responded reliably to stimulation when targeted as a member of an ensemble, regardless of the identity of the other ensemble members. These neurons retained normal calcium dynamics when not being stimulated (Fig. 7b and Supplementary Fig. 20b). Stimulation of ensembles was selective and successful when targeting ensembles of different sizes, at different frequencies, and either within or across axial planes (Fig. 7c–h and Supplementary Fig. 20a–c).

During ensemble stimulation, we observed infrequent activation of neurons that were not holographically targeted. Cells located within the PPSF (0–11 μm from the nearest target neuron) showed evidence of the expected facilitation from the stimulation laser (z-score, 0.87 ± 1.3 (mean and s.d.); Supplementary Fig. 21a–c). However, neurons just outside the PPSF were not modulated by the photogenetic stimulus (11.5–25 μm away from nearest target: z-score, 0.07 ± 0.86, mean and s.d.). On average, neurons distal to the nearest target exhibited a small but significant suppression (P = 1.94 × 10⁻⁶; Supplementary Fig. 21a–d) suggesting that photogenetic stimulation engaged cortical circuits for lateral inhibition (30 μm).
Fig. 7 | Manipulating neural ensembles with high temporal and spatial precision. a, Top: schematic of all-optical ensemble experiments, as in Fig. 5. We stimulated 33 ensembles of 10, 25, or 50 neurons with 10 pulses at 10–30 Hz. Bottom: representative images of three-plane field of view (550 × 550 × 100 µm), with depth from pial surface noted. Inset: enlargement showing example calcium source expressing ST-ChroME. b, A representative neuron stimulated as part of five different ensembles composed of varying numbers of cells. Top: normalized mean fluorescence only when they were stimulated (***P < 0.001; Mouse 1, n = 33 ensembles, P = 3.5 × 10−10; Mouse 2, n = 22 ensembles, P = 2.8 × 10−4; Mouse 3, n = 24 ensembles, P = 5.5 × 10−4, paired two-sided t test). c, Normalized z-scored calcium response of the neurons that compose each stimulated ensemble upon stimulation of each ensemble. Color codes show the size of the ensembles (green, 10; brown, 25; blue, 50 neurons). d, Responses of each neuron in each ensemble to ensemble stimulation, grouped by ensemble identity and separated by size. Data represent the mean z-scored deconvoluted calcium response for each neuron. e–g, Maps showing the mean response of all calcium sources to stimulation of four unique ensembles composed of 50 cells across three depths. Green asterisks indicate neurons that were targeted for stimulation. Note: ensembles can be distributed in 3D (ensemble 1) or confined to one depth (ensembles 2–4). Data calculated from 0–300 ms after stimulation.
Fig. 8 | Altering population correlational structure with 2P ensemble stimulation. a, Left: pairwise Pearson’s correlations for nontargeted neurons, calculated based on firing during control trials (n = 365 neurons). Right: pairwise correlations of target neurons during control trials (n = 150 neurons). b, Pairwise correlations between nontargeted neurons (left) or target neurons (right) during trials in which ensembles were stimulated at 30 Hz (ten 5-ms pulses; see color bar on right). c, Cumulative distributions of all pairwise correlations between nontargeted neurons during control trials (black) or during trials in which ensemble stimulation occurred at 10–30 Hz (red). All stimulation conditions decorrelated population activity relative to control trials (P < 0.01), but were not significantly different from each other (P > 0.425; Friedman test with Tukey–Kramer correction for multiple comparisons). Cumulative distributions are from a representative experiment (n = 3 mice; stimulation vs. control trials, nontarget cells: Mouse 1, P = 0.007; Mouse 2, P = 1.3 × 10^{-4}; Mouse 3, P = 0.004, Friedman test with Tukey–Kramer correction for multiple comparisons). d, Cumulative distributions of all pairwise correlations between target neurons during control trials (black) or during trials in which ensembles were stimulated at 10–30 Hz (red). All stimulation conditions increased correlations between target neurons relative to control trials (P < 0.01), but were not significantly different from each other (P > 0.186, Friedman test with Tukey–Kramer correction for multiple comparisons). Cumulative distributions are from a representative experiment (n = 3 mice; stimulation vs. control trials, target cells; Mouse 1, P = 0.003; Mouse 2, P = 5.4 × 10^{-4}; Mouse 3, P = 0.02, Friedman test with Tukey–Kramer correction for multiple comparisons).

Discussion

We developed an integrated experimental approach for multimodal control of neural ensemble activity with cellular resolution and millisecond precision in vivo. This system achieves the simultaneous temporal precision, spatial resolution, reliability, and scale needed to generate or edit custom spatiotemporal activity patterns. It builds on previous 2P optogenetics manipulations of neural activity14–16, but offers the critical advances needed to achieve the faithful reproduction of naturalistic or artificial sequences of neural activity that could help parse temporal and spatial information in neural codes. The generation of ST-ChroME and IRES-ST-eGATCR1, the application and improvement of 3D-SHOT, and the integration and optimization of these systems with fast volumetric calcium imaging provides the increase in performance needed to address many fundamental yet unanswered questions in neuroscience.

Several in vivo mouse studies have previously employed 2P optogenetics with calcium imaging and/or electrophysiology to activate identified neurons13–16. Since these studies used C1V1-YFP, a slow opsin exhibiting 2P photocurrents <500 pA12,13, they had comparatively poor control over the onset, timing, and, perhaps most importantly, the absolute number (and pattern) of evoked spikes over any given time window. Thus, spiral scanning of C1V1-YFP neurons allows all-optical manipulation of neural ensembles (most recently in 3D14), but not generation of spatiotemporally precise patterns of neural activity. However, recent work suggests that spiral scanning may be more power-efficient than scanless holography15.
which should be considered when seeking to maximize the number of neurons simultaneously addressed if precise control of the underlying spike train is not required.

Although scanless 2P optogenetics holds the promise of eliciting short-latency, low jitter action potentials, it was initially limited by the strength of available opsins (photocurrents < 500 pA)\(^{19,49}\). However, two recent papers demonstrated sub-millisecond temporal resolution using scanless holographic optogenetics in brain slices\(^{24,25}\). One employed the ultrafast opsin Chronos fused with GFP to demonstrate sub-millisecond control of firing at high rates in inhibitory interneurons\(^{64}\). This study reported average photocurrents of ~400 pA, agreeing with our observations that Chronos is not powerful enough to reliably elicit spiking in most L2/3 pyramidal neurons, which require larger photocurrents due to their low intrinsic excitability. Here we extend these results by using ST-Chronos-mRuby2 to drive naturalistic spatiotemporal activity patterns in three genetically defined inhibitory neuron subtypes in vivo (Fig. 4).

Another study presented soCoChR\(^{25}\), a combination of a novel soma-restriction sequence (‘so’ versus ST\(^{31,32}\)) and CoChR, a previously characterized potent opsin with slow-off kinetics\(^{29}\) (Supplementary Fig. 6). Direct comparison of the ‘so’ tag and the Kv2.1 tag in brain slices and in vivo are needed to address which tag is preferable. ST-ChroME exhibited larger 2P photocurrent amplitudes than those exhibited by soCoChR, suggesting that ChroME may be preferable for many applications.

Recently, multiple groups have reported that opsins with slow kinetics can generate a temporally precise spike upon 2P stimulation\(^{24,25}\). We elicited spikes with sub-millisecond jitter in 1 of 11 ST-C1V1\(^{+}\) and 4 of 14 ST-ChrinomR\(^{+}\) neurons, though the population averages (8.6 ± 2 and 12 ± 5 ms, respectively; Fig. 1h) agreed with previous reports\(^{11}\). Our data indicate that opsins with slow decay constants are at an inherent disadvantage in reproducing precise spike trains at physiological spike rates\(^{50}\). If more than one action potential per trial is required, faster opsins have a clear advantage. Almost every neuron expressing ST-ChroME exhibited sub-millisecond jitter, even in response to naturalistic Poisson stimulation in vivo.

We further optimized the extremely potent GtACR1\(^{29}\), specifically for the purpose of multiphoton suppression, achieving fast, reliable, and potent silencing of neurons. Using IRES-ST-eGtACR1, we observe a substantially higher photocurrent than a previous report of 2P suppression\(^{15}\), which employed a different stimulation method and different targeting sequences. Direct comparison of IRES-ST-eGtACR1 to ST-eArch3 yielded an 80-fold increase in photocurrent. Therefore, the approach presented here represents an important advance in optogenetic technology for the editing and synthesis of neural activity patterns that can be used to probe the fundamental logic of sensation, cognition, and behavior at the cellular scale.

Limiting optical cross talk between the read and write channels is a critical aspect of any all-optical approach. Our data show that we can minimize undesired activation of opsin-expressing neurons with the imaging laser. Since even red-shifted opsins absorb at the blue end of the 2P spectrum, an alternative approach is to employ a blue-shifted opsin and a red-shifted calcium indicator\(^{50}\). However, the lower efficacy of these red indicators compared to GCaMPs (at least at present), and the lack of high-pulse-energy lasers at 920 nm restricts the scale of this approach. Nevertheless, for applications seeking to minimize optical crosstalk, this color-flipped scheme may be preferable.

The utility of multiphoton optogenetics for biological applications depends on the number of neurons that can be photostimulated simultaneously and per unit time. The number of simultaneous targets is constrained by the available average power from the stimulation laser and the diffraction efficiency of the SLM phase mask. To estimate the maximum number of light-evoked spikes under various conditions, we built a model based on our empirical data and our hardware specifications. The model indicates that our system could produce thousands of light-evoked spikes in 1 s (Supplementary Fig. 23 and Methods), though detecting these responses using calcium imaging would be technically challenging. We note that laser-induced brain damage or heating will place an upper bound on the maximum duration and power of light that can be directed into brain tissue\(^{61}\) and may substantially constrain the maximum numbers of neurons that can be stimulated in practice.

This combination of temporally focused 3D holography with calcium imaging and fast, potent actuators and suppressors allows new experimental applications. This approach should enable experiments where specific statistical features of neural activity can be varied optogenetically to probe functional connectivity, perception, or behavior. For example, controlling the number, rate, and timing of action potentials written to specific ensembles will allow neuroscientists to test models of cortical dynamics by probing the boundary conditions for the initiation of ‘winner take all’ ensemble activity. Perhaps most notably, our approach provides the tight stimulus control needed to rigorously test how parameterized manipulation of specific neurons can create or alter behavior. Various reports suggest that animals can engage behavioral responses based on the activity of a number of neurons compatible with our approach\(^{50}\). By recording neural activity and simultaneously writing or suppressing custom spatiotemporal sequences of neural activity, investigators can use this new technology to probe how specific, unique patterns of neural activity influence neural circuits and behavior.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-018-0139-8.

Received: 13 October 2017; Accepted: 22 March 2018; Published online: 30 April 2018.

References

1. London, M., Roth, A., Beeren, L., Häusser, M. & Latham, P. E. Sensitivity to perturbations in vivo implies high noise and suggests rate coding in cortex. *Nature* **466**, 123–127 (2010).
2. Gollisch, T. & Meister, M. Rapid neural coding in the retina with relative spike latencies. *Science* **319**, 1108–1111 (2008).
3. Histed, M. H. & Maunsell, J. H. R. Cortical neural populations can guide behavior by integrating inputs linearly, independent of synchrony. *Proc. Natl Acad. Sci. USA* **111**, E178–E187 (2014).
4. Bruno, R. M. & Sakmann, B. Cortex is driven by weak but synchronously active thalamocortical synapses. *Science* **312**, 1622–1627 (2006).
5. Harris, K. D. & Mrsic-Flogel, T. D. Cortical connectivity and sensory coding. *Nature* **503**, 51–58 (2013).
6. Panzeri, S., Harvey, C. D., Piasini, E., Latham, P. E. & Fellin, T. Cracking the neural code for sensory perception by combining statistics, intervention, and behavior. *Neuron* **93**, 491–507 (2017).
7. Jepson, L. H. et al. High-fidelity reproduction of spatiotemporal visual signals for retinal prosthesis. *Neuron* **83**, 87–92 (2014).
8. Clancy, K. B., Schnepel, P., Rao, A. T. & Feldman, D. E. Structure of a single whisker representation in layer 2 of mouse somatosensory cortex. *J. Neurosci.* **35**, 3946–3958 (2015).
9. Ohki, K., Chung, S., Chng, Y. H., Kara, P. & Reid, R. C. Functional imaging with cellular resolution reveals precise micro-architecture in visual cortex. *Nature* **433**, 597–603 (2005).
10. Rickgauer, J. P. & Tank, D. W. Two-photon excitation of channelrhodopsin-2 at saturation. *Proc. Natl Acad. Sci. USA* **106**, 15025–15030 (2009).
11. Vaziri, A. & Emiliani, V. Re-shaping the optical dimension in optogenetics. *Curr. Opin. Neurobiol.* **22**, 128–137 (2012).
12. Packer, A. M. et al. Two-photon optogenetics of dendritic spines and neural circuits. *Nat. Methods* **9**, 1202–1205 (2012).
13. Prakash, R. et al. Two-photon optogenetic toolbox for fast inhibition, excitation and bistable modulation. *Nat. Methods* **9**, 1171–1179 (2012).
14. Packer, A. M., Russell, L. E., Dalgleish, H. W. P. & Häusser, M. Simultaneous all-optical manipulation and recording of neural circuit activity with cellular resolution in vivo. *Nat. Methods* **12**, 140–146 (2015).
15. Rickgauer, J. P., Deisseroth, K. & Tank, D. W. Simultaneous cellular-resolution optical perturbation and imaging of place cell firing fields. Nature. Neurosci. 17, 1816–1824 (2014).
16. Carrillo-Reid, L., Yang, W., Bando, Y., Peterka, D. S. & Yuste, R. Imprinting and recalling cortical ensembles. Science 353, 691–694 (2016).
17. Emilian, V., Cohen, A. E., Deisseroth, K. & Häusser, M. Optical interrogation of neural circuits. J. Neurosci. 35, 13917–13926 (2015).
18. Dal Maschio, M., Donovan, J. C., Helmbrecht, T. O. & Baier, H. Linking neurons to network function and behavior by two-photon holographic optogenetics and volumetric imaging. Nature 94, 774–789.e5 (2017).
19. Papagiakoumou, E. et al. Scanless two-photon excitation of channelrhodopsin-2. Nat. Methods 7, 848–854 (2010).
20. Forli, A. et al. Two-photon bidirectional control and imaging of neuronal excitability with high spatial resolution in vivo. Cell Rep. 22, 3087–3098 (2018).
21. Nikolenko, V. et al. SLM microscopy: scanless two-photon imaging and photostimulation with spatial light modulators. Front. Neural Circuits 2, 5 (2008).
22. Papagiakoumou, E., de Sars, V., Oron, D. & Emiliani, V. Patterned two-photon illumination by spatiotemporal shaping of ultrashort pulses. Opt. Express 16, 22039–22047 (2008).
23. Emilian, V. et al. Wave front engineering for microscopy of living cells. Opt. Express 13, 1395–1405 (2005).
24. Ronzitti, E. et al. Submillisecond optogenetic control of neuronal firing by two-photon holographic photoactivation of Chronos. J. Neurosci. 37, 10679–10697 (2017).
25. Shemesh, O. et al. Temporally precise single-cell-resolution optogenetics. Nat. Neurosci. 20, 1796–1806 (2017).
26. Hernandez, O. et al. Three-dimensional spatiotemporal focusing of holographic patterns. Nat. Commun. 7, 11928 (2016).
27. Pogard, N. C. et al. Three-dimensional scanless holographic optogenetics with temporal focusing (3D-SHOT). Nat. Commun. 8, 1228 (2017).
28. Klapezete, N. C. et al. Independent optical excitation of distinct neural populations. Nat. Methods 11, 338–346 (2014).
29. Govorunova, E. G., Sineshchekov, O. A., Jasz, R., Liu, X. & Spudich, J. L. Natural light-gated anion channels: a family of microbial rhodopsins for advanced optogenetics. Science 349, 647–650 (2015).
30. Ahmadian, Y., Packer, A. M., Yuste, R. & Paninski, L. Designing optimal stimuli to control neuronal spike timing. J. Neurophysiol. 106, 1038–1053 (2011).
31. Baker, C. A., Elyada, Y. M., Parra, A. & Bolton, M. M. L. Cellular resolution circuit mapping with temporal-focused excitation of soma-targeted channelrhodopsin. eLife 5, 1–15 (2016).
32. Wu, C., Ivanova, E., Zhang, Y. & Pan, Z. H. RAAV-mediated subcellular targeting of optogenetic tools in retinal ganglion cells in vivo. PLoS One 8, e66332 (2013).
33. Podgorski, K. & Ranganathan, G. Brain heating induced by near-infrared lasers during multiphoton microscopy. J. Neurophysiol. 116, 1012–1023 (2016).
34. Kato, H. et al. Crystal structure of the channelrhodopsin light-gated cation channel. Nature 482, 369–374 (2012).
35. Gradinaru, V. et al. Molecular and cellular approaches for diversifying and extending optogenetics. Cell 141, 154–165 (2010).
36. Chow, B. Y. et al. High-performance genetically targetable optical neural silencing by light-driven proton pumps. Nature 463, 98–102 (2010).
37. Chuong, A. S. et al. Noninvasive optical inhibition with a red-shifted microbial rhodopsin. Nat. Neurosci. 17, 1123–1129 (2014).
38. Govorunova, E. G., Sineshchekov, O. A. & Spudich, J. L. Proteomus sulcata ACR1: a fast anion channelrhodopsin. Photochem. Photobiol. 92, 257–263 (2016).
39. Berndt, A. et al. Structural foundations of optogenetics: determinants of channelrhodopsin ion selectivity. Proc. Natl Acad. Sci. USA 113, 822–829 (2016).
40. Markram, H. et al. Interneurons of the neocortical inhibitory system. Nat. Rev. Neurosci. 5, 793–807 (2004).
41. Taniguchi, H. et al. A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. Neuron 79, 995–1013 (2011).
42. Hippenmeyer, S. et al. A developmental switch in the response of DRG neurons to ETS transcription factor signaling. PLoS Biol. 3, e159 (2005).
43. Wessellblatt, J. B., Elster, F. D., Piscopo, D. M. & Niel, C. M. Large-scale imaging of cortical dynamics during sensory perception and behavior. J. Neurophysiol. 115, 2852–2866 (2016).
44. Grewe, B. F., Voigt, F. F., van ’t Hoff, M. & Helmchen, F. Fast two-layer two-photon imaging of neuronal cell populations using an electrically tunable lens. Biomed. Opt. Express 2, 2035–2046 (2011).
45. Yang, W., Carrillo-Reid, L., Bando, Y., Peterka, D. S. & Yuste, R. Simultaneous two-photon imaging and two-photon optogenetics of cortical circuits in three dimensions. eLife 7, e32671 (2018).
46. Adesnik, H. & Scanziani, M. Lateral competition for cortical space by LWS-specific horizontal circuits. Nature 464, 1155–1160 (2010).
47. Harris, K. D. & Thiele, A. Cortical state and attention. Nat. Rev. Neurosci. 12, 509–523 (2011).
48. Churchland, A. K. et al. Variance as a signature of neural computations during decision making. Neuron 69, 818–831 (2011).
49. Andrasfalvy, B. K., Zemelman, B. V., Tang, J. & Razeli, A. Two-photon single-cell optogenetic control of neuronal activity by sculpted light. Proc. Natl Acad. Sci. USA 107, 11981–11986 (2010).
50. Huber, D. et al. Sparse optical microstimulation in barrel cortex drives learned behaviour in freely moving mice. Nature 451, 61–64 (2008).

Acknowledgements
We thank M. Feller, A. Naka, and J. Brown for critical feedback on the manuscript and discussions. We thank C. Baker and M. Bolton for soma-targeted ChR2 AAVs. We thank M. Li and the UC Berkeley Vision Science Core, Gene Delivery Module, for preparation of AAVs (supported by NIH Core Grant P30 EY003176). We deeply appreciate the efforts of D. Chiu, C. Douglas, and R. Hakim for important technical assistance. We thank D. Taylor for help with mouse work and histology. H.A. is a New York Stem Cell Foundation Robertson Investigator. This work was supported by The New York Stem Cell Foundation and by grants from the Arnold and Mabel Beckman Foundation, NINDS grant DP2NS087725-01, the McKnight Foundation, NINDS award F32NS095690-01 to A.R.M., the Simon’s Foundation Collaboration for the Global Brain award 415569 to I.A.O., and a fellowship from the David and Lucille Packard Foundation to I.A.O. This research was developed with funding from the Defense Advanced Research Projects Agency (DARPA), Contract No. N660011-17-C-4015.

Author contributions
A.R.M., I.A.O., N.C.P. and H.A. conceived the project and built the system. A.R.M. designed and performed all experiments involving excitatory opsins. I.A.O. designed and performed all experiments involving inhibitory opsins. N.C.P. designed and assembled the light paths and wrote custom holography software. S.S. performed cloning, mutagenesis, cell culture, and one-photon recordings in CHO cells. A.R.M., I.A.O., N.C.P. and E.H.L. wrote code and developed software for experimental control. K.C. performed histology. S.G.B. performed imaging of the Chronos pore region. L.W. contributed expertise on holographic design. A.R.M., I.A.O., N.C.P., and H.A. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41593-018-0139-8.
Reprints and permissions information is available at www.nature.com/reprints.
Correspondence and requests for materials should be addressed to H.A.
Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
**METHODS**

**Ethics.** All experiments were performed in accordance with the guidelines and regulations of the ACUC of the University of California, Berkeley. Protocol # AUP-20140106-6832. Every experiment was conducted in at least three mice unless otherwise stated.

**Transgenic mice.** The following mouse lines were used for this study: ICR (Crl:CD1), the PV-IRE-cre line (B6.129P2-Pvltm1(cre)Arbr); JAX stock #008069), the SOM-IRE-cre line (JAX stock #013044), the VIP-IRE-cre line (JAX stock #010908), the Emx-IRE-cre line (JAX stock #005628), the Drd3-cre line (JAX stock #002958) the tetO-GCaM266 line (JAX stock #024742), and the CaMKII-TTA line (JAX stock #003010). Mice were housed in cohorts of five or fewer with a light-dark cycle of 12:12h, and were used for experimentation during their subjective night.

**Plasmid construction and mutagenesis.** Chronos, eArch3.0, and iC++ were generated by gene synthesis (Chronos and eArch3.0, Genewiz, South Plainfield, NJ; and iC++, Integrated DNA Technologies, Coralville, IA) and anion opsins PsuACR and GtACR1 were provided by J. Spudich (University of Texas Health Science Center, Houston, TX). C1V1;Ch,2i, Chrinomon, and eNhPR3.0 were obtained from Addgene. All opsins were fused to mRuby2 and their C terminus was fused to a Not site (Chronos and eArch3.0) or an Agel site (GtACR1, iC++, PsuACR, and eNhPR3.0) and subcloned into the pCAGGS expression vector between KpnI and Xhol restriction sites by In-Fusion Cloning (Clontech, Mountain View, CA). To target the opsins to the soma and proximal dendrites of neurons, the sequence encoding the proximal restriction and clustering domain of the Kv2.1 voltage-gated potassium channel consisting of amino acids 536–600 was inserted between the opsins and the KpnI and Xhol restriction sites by In-Fusion Cloning (Clontech, Mountain View, CA). For photostimulation, we used the 2P imaging laser paths with minimal losses.

**For 3D hologram computation.** Here, for precise control of the intensity distribution, as well as spatially dependent diffraction efficiency, requires the ability to control the power distribution precisely in each target. To compensate for spatially dependent diffraction efficiency throughout the optical system, we proceed to a power calibration, as shown in Supplementary Fig. 16g–i.

We computed holograms, each targeting one spot in a 3D grid pattern, and we used our stage camera system (Supplementary Fig. 11a) to quantify the amount of 2P absorption, \( P \), achieved by each hologram while supplying the same amount of laser power, \( I \), to the SLM. Experimental measurements of 2P absorption, \( P(x',y',z') \) (Supplementary Fig. 16g), show how intensity degradation when targeting far away from the zero-order. To digitally compensate for this unwanted effect, we estimate losses with a 3D polynomial interpolation of the power calibration data (Supplementary Fig. 16h). Interpolation error measurements (Supplementary Fig. 16e) show how our model fits experimental measurements within the operating volume, with a few known exception of the blocked zero-order. Several methods have been developed for 3D hologram computation. Here, for precise control of the intensity distribution, we used either an iterative method, global Gerchberg–Saxton, or 2P optimized NOVO-CGH® with a Euclidean cost function to maximize contrast in holograms where precise control of the power distribution is required simultaneously in many targets. Relevant algorithms for 3D alignment of 3D-SHOT with 3D imaging, and for digital power compensation with 3D polynomial interpolation, are provided on our repository (https://github.com/adesniklab/3D-SHOT).

**Synchronization of stimulation window to imaging frames (for example, laser gate).** Since 2P excitation by the photostimulation laser yields stimulation artifacts, we developed an electronic circuit to restrict the photostimulation laser to engage only as the resonant galvo mirror reverses course, on either side of the imaging window. The electronic circuit is shown in Supplementary Fig. 14a, with additional details on our repository (https://github.com/adesniklab/3D-SHOT).

**Stroboscopic imaging for SLM high speed performance testing.** To characterize the illumination pattern during high-speed phase transitions (using the Meadowlark 512L), we considered a test case with four holograms, each targeting several randomized points distributed throughout the volume. We employed stroboscopic imaging to measure the intensity during the phase transition at very high speeds by illuminating a fluorescent calibration slide at specific times during the cycle and with time-averaged imaging with a stage camera (Supplementary Table 2). A repeating sequence was played at 300 Hz using the SLM built-in trigger. To observe holograms at any point of this cycle during SLM frame-to-frame transitions, the SLM trigger clock was synchronized with the laser controller to restrict illumination to a 1-ms pulse placed anywhere within the 13.3ms duration of the cycle sequence.

**CHO cell recording.** Chinese hamster ovary (CHO) cells were cultured and recorded as described1. One-photon photostimulation of cells was performed at 550 nm for C1V1;1, eNhPR3.0, eArch3.0, and PsuACR; at 470 nm for Chromo, Chromo+ eArch3.0, and GtACR1; and at 630 nm for ChrimsionR, at a power of 0.55 mW using a Lumencor light engine (Lumencor). For activating opsins, currents were measured at a holding potential of –40 mV for suppressing opsins, currents were measured at 0 mV. For 2P spectra measurements in CHO cells, currents were evoked by rapidly raster-scanning a diffraction limited spot from a Chameleon Tri-Sapphire Laser (Coherent) and average power was normalized across wavelengths by attenuating the laser beam using a Pockels cell (Conoptics).
Brain slice recording. Acute coronal slices were prepared and recorded from mice (ages P14–P29) as described7. For measuring opsins kinetics, the photocurrent elicited by 0.5- or 1-s CGH stimulation (0.04 mW/µm², 200 mW, disc r = 12.5 µm) was measured in voltage-clamp mode. The time to peak current was measured from averaged traces and the constants were measured by fitting the traces from stimulus offset to 80% of baseline to a single exponential. In some neurons expressing ST-Chronos and ST-ChroME, the decay kinetics were better fit with a two-terminal exponential decay function of $I = ae^{-t/T_1} + ce^{-t/T_2}$. The size of the primary decay tau ($T_1$) was unaffected by the two-term fit. This secondary decay tau ($T_2$) was tested in two groups of mice and was 250 ms, 10 mW, 490 nm for ChroME, and the scale constant ($c$) for the secondary tau was maximally 0.3.

For current injection experiments in Fig. 1 and Supplementary Fig. 1, random white noise (mean 0 pA, range ±60 pA) was generated on each sweep, and rheobase was determined by increasing current injections in a stepwise fashion (25 pA/step) until action potentials were recorded. This procedure was repeated for each stimulus duration. In Supplementary Fig. 1, current injections were performed at the rheobase 5 ms or 1 s. White noise stimulation without additional current injection never resulted in action potentials.

For optogenetics experiments, mice were screened by a handheld 300 mW 594-nm laser and filtered goggles for expression, after decapitation and before slicing. After slicing, recordings were made from the slices with strongest expression from the denese area as judged by handheld laser and filter gogoggles. To be included in subsequent datasets neurons, were required to pass an expression test: experiments were continued if the recorded neuron spiked in response to a brief one-photon stimulus (5 ms, 0.5 mW, 490 nm for Chronos and ChroME; and 5 ms, 0.5 mW, 510 nm for C1V10). Cells were eliminated if spontaneous activity and passed the one-photon test were stimulated repetitively (1–5 Hz) with 5-ms light pulses of increasing laser power until they spiked to each stimulus duration in pyramidal neurons, stimulation was achieved with an air puffer (PicoSpritzer II, General Value) directed toward the contralateral whisker pad. Six 50-ms puffs were applied before during and after each optogenetic stimulation; in general, L2/3 neurons were additionally administered 2 mg/kg of dexamethasone as an anti-inflammatory drug. For 2P imaging crosstalk experiments, neurons were placed in the center of the field of view in the focal plane of the natural 2P focus for volumetric imaging. 2P imaging was performed at the specified window size, speed, and power for 1-s sweeps after a 1-s baseline period.

Shaping 2P stimulation pulse and survival curve. For laser pulse shaping experiments, whole-cell recordings were obtained and pulse features of CGH stimulation (radius = 12.5 µm) were varied online while holding the cell. The Satsuma HP 1,040-nm stimulation laser is switchable between 2 and 40 MHz, allowing online testing of the response to multiple repetition rates. Peak power was measured using the EOM to vary average power, and the relationship between peak power and pulse energy was probed using the Satsuma HP laser's onboard dispersion compensation, which allowed us to control pulse-width online to test pulses of identical pulse energy with variable peak power. The stage positions necessary to chisel the laser pulses were determined before the experiment began using a Mini TPA Compact, Tuning-free Autocorrelator (Angewandte Physik & Elektronik GmbH).

To establish the maximum safe power levels useable before acute cellular damage, GCaMP6s cells (without opsins) were stimulated with increasing power densities until calcium responses ceased (indicating cell death) or cavitation of the membrane was observed. We did not observe any presynaptic mechanisms of cellular damage, but only scored damage when a laser pulse resulted in an acute change in cellular fluorescence. Teto-GCaMP mice were headplated and windowed as if for in vivo imaging (see below) and deeply anesthetized as in cell-attached recordings (see below). Up to 800 mW per target (3D-SHOT disc r = 10 µm) were used.

In vivo patch recording. 2P guided-patch recordings were performed from adult mice (35 d old or older) as described17. For suppression experiments, whisker stimulation was achieved with an air puffer (PicoSpritzer II, General Value) directed toward the contralateral whisker pad. Six 50-ms puffs were applied before during and after each optogenetic stimulation; in general, L2/3 neurons did not respond in a time-locked manner but increased their overall firing rates during stimulation. Glass pipettes (2- to 5-Ω resistance) were filled with HEPES ACSF and Alexa Fluor-488 dye (100 µM) for visualization. Cells were identified by the presence of mRuby2 or mRuby3 fluorescence imaged at 50–100 mW at 930–9,100 nm. Data were acquired using a Multichip 700B Amplifier (Axon Instruments) and digitized at 20kHz (National Instruments). Data was digitally bandpass-filtered at 0.5–2.2 kHz for identification of spikes. All data were acquired using custom written Matlab (MathWorks) software.

Cells were included for analysis if they were spontaneously active and those spikes were sufficiently larger than the noise (>5×d of the noise). Furthermore, cells had to respond to a one-photon LED stimulation (Sola SE, Lumencor), fire action potential(s) if they expressed an excitatory opsin, or temporally cease firing if they expressed a suppressing opsin (5 ms, 0.5 mW, 490 nm for Chronos and ChroME; 250 ms, 10 mW, 490 nm for GtACR).

To determine the fraction of spikeable neurons, cells that exhibited sporadic spontaneous activity and formed a one-photon LED stimulation (Sola SE, Lumencor), fire action potential(s) if they expressed an excitatory opsin, or temporally cease firing if they expressed a suppressing opsin (5 ms, 0.5 mW, 490 nm for Chronos and ChroME; 250 ms, 10 mW, 490 nm for GtACR).

In vivo temperature measurements. To measure brain temperature during holographic photostimulation and imaging, animals were prepared and deeply anesthetized as above for in vivo cell-attached patch recording, except that they were additionally administered 2 mg/kg of dexamethasone as an anti-inflammatory agent. Once the craniotomy was completed, the dura was removed and an 800-µm thick thermocouple coated in DII was slowly lowered at a 45° angle using a micromanipulator (Sutter MP285) until it was 300–500 µm beneath the pial surface. It was then secured in place with Ortho-Jet while the open craniotomy was protected by Gelfoam and ACSF. The thermocouple was attached to a TC-330 thermocouple probe and the 1310-nm diode laser (IntraOptim) was used to control the temperature by monitoring the thermocouple recordings. The temperature-to-voltage conversion was calibrated in a series of water baths. The thermocouple was mounted under 2P illumination based on DII signal, and the objective was placed over the thermocouple for the duration of stimulation. A single phase mask that targeted 50 spots was held static for the duration of the experiment, and duty cycling of the stimulation laser was performed using the EOM.
Chrom-P2A-H2B-mRuby3. Alternatively, triple-transgenic animals expressing teto-GCaMP6s43 were co-injected with AAV-syn-Cre and AAV-CAG-DIO-ST-Chronos-mRuby2 (UC Berkeley Vector core, titer: 4.8 × 10^12). Three different combinations of littermate Emx-Cre or Drd3-Cre animals (P3: three injection sites, two injections per site for experiment 1–2 weeks postinjection. For GCaMP6 injections, mice were used 2–6 weeks postinjection. For opsin injections, 3–4 weeks postinjection was used. 

Software (Olympus), with 488 and 543 nm lasers. Olympus Fluoview system (Fv1000 Olympus Microscope) running the Fluoview software (Olympus), with 488 and 543 nm lasers.

Mice were deeply anesthetized with ketamine/xylazine and Mice were anaesthetized using 2% isoflurane. For viral injection, animals were anesthetized using isoflurane. For opsins injection, mice were anesthetized using 2% isoflurane. The scalp was removed, the fascia (2%) and administered 2 mg/kg of dexamethasone as an anti-inflammatory and

For optical experiments using virus to test ST-Chronos, ST-ChroME, or IRES-ST-eGtACR1, calcium sources were extracted with key parameters diameter 0.05 mg/kg buprenorphine as an analgesic. The scalp was removed, the fascia

For ensemble experiments, neurons were stimulated one by one at a rate of 0.5 Hz for 1 s at 0.16 mW/µm^2 (50 mW per neuron). As the fluorescence response to stimulation lasted more than 2 s, the trial immediately following stimulation was excluded from analysis. For some whole-cell slice experiments using virus to test ST-Chronos, ST-ChroME, or IRES-ST-eGtACR1, viruses were introduced via neonatal injection as described in Desnaklab/3D-SHOT. Volume acquisition occurred at 5.8–6.6 Hz for ST-Chronos -planes each separated by 50 µm. Imaging videos taken at 1,000–1,040 nm, and using custom Matlab software, regions of interest were circled and their centroids were used to compute holographic targets across the accessible volume. To determine the power needed to activate neurons, the relationship between stimulation power and the mean z-scored S vectors were fit with a smoothing interpolant and the power to reach 80% of saturation was reported. To determine the single-neuron response matrix, neurons were sequentially stimulated with ten 5 ms pulses at 30 Hz and the mean z-scored S vector for each neuron was averaged for two frames (~300 ms) after stimulation of each target.

For ensemble stimulation experiments, the S vector was z-scored on a trial-wise basis. For optical rheobase experiments, neurons were stimulated one by one at a rate of 2 Hz with ten 5 ms pulses at 30 Hz with varying laser intensities (corrected for varying diffraction efficiency of holograms targeting areas across the accessible volume). To determine the power needed to activate neurons, the relationship between stimulation power and the mean z-scored S vectors were fit with a smoothing interpolant and the power to reach 80% of saturation was reported. To determine the single-neuron response matrix, neurons were sequentially stimulated with ten 5 ms pulses at 30 Hz and the mean z-scored S vector for each neuron was averaged for two frames (~300 ms) after stimulation of each target.

For ensemble stimulation experiments, the S vector was z-scored on a trial-wise basis. For optical rheobase experiments, neurons were stimulated one by one at a rate of 2 Hz with ten 5 ms pulses at 30 Hz and signal extraction = raw. Calcium sources were then manually examined and accepted

or rejected based on their overlap with morphologically identifiable neurons. Neuronal subtracted fluorescence vectors (F) or the OSVON deconvolution (S) was used for downstream analysis. Calcium signals were acquired continuously, and each z-scored S, fluorescence z-scored S was calculated and compared to the 50% set equal to the tenth percentile of fluorescence observed over the entire experiment. If a trial had motion over threshold (5 µm) during half or more of the stimulation period, the entire trial was excluded, since the results of experiment may not be interpretable, but if motion occurred when not stimulating it was corrected post hoc and the data was included. Holographic targets were aligned to calcium sources by calculating the Euclidean distance between the centroids of all holographic targets and all calcium sources and finding the minimum. Very rarely, targets assigned were aligned to calcium sources with distance >15 µm. These targets were assumed to have failed and were excluded from subsequent analysis.

We found that the OSVON deconvolution signals provided a good estimate for the calcium signal, since local peaks of the S vector aligned with the frame on which stimulation occurred better than ΔF/F. Since deconvolved calcium signals decay much faster than fluorescent signals, this ameliorates analysis problems where slowly declining fluorescence from a recently stimulated neuron may be attributed to holographic stimulation of the next cell in a sequence. Therefore, most subsequent analysis for ST-ChroME expressing neurons was performed based on the S vector.

In GaICR1 experiments with GCaMP6f in PV neurons, reduction of calcium activity was most apparent when analyzing z-scored fluorescence responses, likely due to the relatively high firing rates of PV neurons making true baseline F values hard to determine. For opsin injections, each cell was stimulated once per trial, but instead were repetitively stimulated over the course of each neuron to each unique stimulation by discarding frames in which it was stimulated once per trial, but instead were repetitively stimulated over the course of each trial as part of many different ensembles, we evaluated the response of each neuron to each unique stimulation by discarding frames in which it was directly stimulated as part of a different ensemble. This analysis essentially treated each 73 s sweep as a series of concatenated 2 s single trials, but preserved the baseline information obtained during nonstimulation periods of the long sweep. Responses to ensemble stimulation of ten 5 ms pulses at 10, 20, or 30 Hz are shown as the mean z-scored S vector for each stimulus. Maps showing the response of all neurons to ensemble stimuli show the mean z-scored S vector for all neurons for two frames (~300 ms) after the marked ensemble was stimulated. For ensemble experiments, the S vector was z-scored on a trial-wise basis. For optical rheobase experiments, neurons were stimulated one by one at a rate of 2 Hz with ten 5 ms pulses at 30 Hz with varying laser intensities (corrected for varying diffraction efficiency of holograms targeting areas across the accessible volume). To determine the power needed to activate neurons, the relationship between stimulation power and the mean z-scored S vectors were fit with a smoothing interpolant and the power to reach 80% of saturation was reported. To determine the single-neuron response matrix, neurons were sequentially stimulated with ten 5 ms pulses at 30 Hz and the mean z-scored S vector for each neuron was averaged for two frames (~300 ms) after stimulation of each target. Differences in the distributions of correlation coefficients across trial types were assessed for significance by the Friedman test with the Tukey–Kramer correction for multiple comparisons.

Modeling the speed and scale of photoactivation. A description of the model used to calculate the maximum number of light-evoked spikes per s (Supplementary Fig. 23) is available online in our repository (https://github.com/ adesnaklab/3D-SHOT).

Statistics. All analyses were performed using Matlab (MathWorks). The analyses performed were: paired t test, Mann–Whitney U test, Wilcoxon signed-rank test, Fisher’s exact test, Friedman’s test, and Kruskal–Wallis test. All tests were two-sided unless otherwise noted. For parametric tests, data distribution was assumed to be

Histology. Mice were deeply anesthetized with ketamine/xylazine and transcyanidically perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde. Brains were postfixed for at least 2 h. Brains were embedded in 30% sucrose solution overnight, then sections were washed with PBS, followed by 4% Triton X-100 before being incubated in secondary antibody Alexa Fluor 488 goat antibody, mixed with 0.1% Triton X-100. All sections were mounted on slides and sealed with Vectashield with DAPI (Vector Laboratories). Confocal images were acquired using an Olympus Fluoview system (Fv1000 Olympus Microscope) running the Fluoview software (Olympus), with 488 and 543 nm lasers.

Viral infection. For viral injection, animals were anaesthetized using 2% isoflurane on a heating pad and headfixed in a stereotactic apparatus (Kopf). After stabilizing the incision site, the skin was opened and a small burr hole was drilled over S1 using a 0.24-mm drill bit (Busch; 3.5 mm lateral, 1.4 mm posterior to bregma). We injected 200–600 nL of virus using a microsyringe pump (Micro) and a two-well glass pipette (Drummond) at a rate of 25–50 nL/s at a depth of 150–350 µm below the pia surface. After the injection was complete, we waited 5 min before retracting the needle and closing the scalp with sutures. On some surgeries, the virus was injected directly after installation of a headplate (see below). For opsins injections, mice were used 2–6 weeks postinjection. For GCaMP6 injections, mice were used 2–6 weeks postinjection. For opsin injections, 3–4 weeks postinjection was used.

We found that the OSVON deconvolution signals provided a good estimate for the calcium signal, since local peaks of the S vector aligned with the frame on which stimulation occurred better than ΔF/F. Since deconvolved calcium signals decay much faster than fluorescent signals, this ameliorates analysis problems where slowly declining fluorescence from a recently stimulated neuron may be attributed to holographic stimulation of the next cell in a sequence. Therefore, most subsequent analysis for ST-ChroME expressing neurons was performed based on the S vector.

In GaICR1 experiments with GCaMP6f in PV neurons, reduction of calcium activity was most apparent when analyzing z-scored fluorescence responses, likely due to the relatively high firing rates of PV neurons making true baseline F values hard to determine. For opsin injections, each cell was stimulated once per trial, but instead were repetitively stimulated over the course of each neuron to each unique stimulation by discarding frames in which it was directly stimulated as part of a different ensemble. This analysis essentially treated each 73 s sweep as a series of concatenated 2 s single trials, but preserved the baseline information obtained during nonstimulation periods of the long sweep. Responses to ensemble stimulation of ten 5 ms pulses at 10, 20, or 30 Hz are shown as the mean z-scored S vector for each stimulus. Maps showing the response of all neurons to ensemble stimuli show the mean z-scored S vector for all neurons for two frames (~300 ms) after the marked ensemble was stimulated. For ensemble experiments, the S vector was z-scored on a trial-wise basis. For optical rheobase experiments, neurons were stimulated one by one at a rate of 2 Hz with ten 5 ms pulses at 30 Hz with varying laser intensities (corrected for varying diffraction efficiency of holograms targeting areas across the accessible volume). To determine the power needed to activate neurons, the relationship between stimulation power and the mean z-scored S vectors were fit with a smoothing interpolant and the power to reach 80% of saturation was reported. To determine the single-neuron response matrix, neurons were sequentially stimulated with ten 5 ms pulses at 30 Hz and the mean z-scored S vector for each neuron was averaged for two frames (~300 ms) after stimulation of each target. Differences in the distributions of correlation coefficients across trial types were assessed for significance by the Friedman test with the Tukey–Kramer correction for multiple comparisons.

Modeling the speed and scale of photoactivation. A description of the model used to calculate the maximum number of light-evoked spikes per s (Supplementary Fig. 23) is available online in our repository (https://github.com/ adesnaklab/3D-SHOT).

Statistics. All analyses were performed using Matlab (MathWorks). The analyses performed were: paired t test, Mann–Whitney U test, Wilcoxon signed-rank test, Fisher’s exact test, Friedman’s test, and Kruskal–Wallis test. All tests were two-sided unless otherwise noted. For parametric tests, data distribution was assumed to be
normal, but this was not formally tested. No statistical methods were used to predetermine sample sizes, but we collected sample sizes that were similar to or exceeded those reported in previous publications. Data collection and analysis were not performed blind to the conditions of the experiments. Randomization was used in all applicable experiments (for experiments with multiple trial types, the order of trials was randomized). Animals and data points were only excluded based on criteria described above.

Accession codes. Addgene: pCAG-ChroME-mRuby2-ST, 108902; pAAV-CAG-DIO-ChroME-P2A-H2B-mRuby3, 108912; pCAG-H2B-mRuby3-IRES-eGtACR1-ST, 108960; pAAV-CAG-DIO-NLS-H2B-mRuby3-IRES-eGtACR1-ST, 109048.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Code availability statement. Code for alignment of 3D holography with 3D imaging, holographic control, hologram computation, and analysis will be hosted online upon publication.

Data availability statement. The datasets generated and analyzed in this study are available from the corresponding author on reasonable request. Additionally, the sequences for constructs created in this study will be made publicly available on Addgene.

References
51. Lim, S. T., Antonucci, D. E., Scannevin, R. H. & Trimmer, J. S. A novel targeting signal for proximal clustering of the Kv2.1 K⁺ channel in hippocampal neurons. Neuron 25, 385–397 (2000).
52. Gerchberg, R. W. & Saxton, W. O. A practical algorithm for the determination of phase from image and diffraction plane pictures. Optik (Stuttg.) 35, 237–246 (1972).
53. Zhang, J., Pégard, N., Zhong, J. & Waller, L. 3D computer generated holograms by nonconvex optimization. Optica 4, 1306–1313 (2017).
54. Pluta, S. et al. A direct translaminar inhibitory circuit tunes cortical output. Nat. Neurosci. 18, 1631–1640 (2015).
55. Pachitariu, M. et al. Suite2p: beyond 10,000 neurons with standard two-photon microscopy. Preprint at bioRxiv https://doi.org/10.1101/061507 (2016).
Corresponding author(s): Hillel Adesnik (NN-T60301D)

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
- Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

- Electrophysiological data was acquired using MATLAB 2014b and 2015a using a custom written data acquisition and experimental control software. Imaging Data was acquired using ScanImage 5.2, running on MATLAB 2015a.

Data analysis

- Data was analyzed using custom code written in MATLAB 2016a and 2016b. Code for experimental control and analysis will be hosted online in a GitHub repository after publication.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The datasets generated and analyzed in this study are available from the corresponding author on reasonable request. Additionally, the sequences for constructs created in this study will be made publicly available on Addgene.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**

A priori sample size estimation was not performed for all individual experiments. However, from experience measurements from whole cell recordings of opsin expressing cells require at least 5-10 observations to resolve a difference of ~25%, as typically observed differences covered an order of magnitude, these 5-10 observations were more than sufficient for our needs. Furthermore, within a cell there was very little trial to trial variability, so only 3-5 repetitions of a given stimulus were needed for an accurate report. Similarly, to compare differences between cells using in vivo cell attached recordings we aimed for 5-15 neurons per condition, as the sources of variability should be similar to whole cell recording. Suppression, during in vivo cell attached recordings did require a power analysis, as it relies on endogenous activity and the variability in spiking during a given window is large. Spike rate was estimate over an initial period of 3-30s and a sufficient number of trials was selected for each cell so that at least 50 action potentials would occur per stimulus type, usually many more. Given the experimental design and assuming a Poisson spiking pattern this will allow for distinguishing changes in firing rate of ~30% in the worst case scenario. Cell attached activation experiments, on the other hand required far fewer trials to confirm success, as endogenous action potentials within a given 5 ms window are rare, 2-5 sweeps or a power curve were sufficient to determine if a cell responded. To account for the variability in poisson-like stimuli, at least 20 trials were repeated for each cell to ensure coverage of many different ISIs. In all cases at least 3 animals were used for each condition unless explicitly stated otherwise, and there was no observed animal dependent effect.

Sample size for all optical experiments was limited by experimental imaging conditions. All resolvable calcium sources corresponding to visually identifiable neurons were included for analysis. For targeting neurons for optogenetic experiments, neurons were pseudo-randomly selected based on expression of opsin or on physical location (biased towards center of field of view in some experiments so that calcium signals could be resolved).

**Data exclusions**

In general very little data was excluded from analysis. Whole cell and cell attached recordings were excluded if a 1 photon illumination failed to elicit a response (depolarization/hyperpolarization or spiking/suppression of spiking). In the case of in vivo suppression 5-10 repetitions of this 1p test were done as cessation of spiking can be difficult to positively identify. Additionally, in vivo cells were excluded if the cell drifted outside of the target of holographic stimulation over the course of the experiment, or if they failed to fire endogenous action potentials within 30s of attachment. Intracellular recordings were additionally excluded in the case that the cells or the seal appeared unhealthy (series resistance unstable or above 30MΩ, membrane potential unstable). Mice were excluded if the expression of opsin was low, or if the IUE was not targeted to the S1 cortex.

**Replication**

All attempts to replicate were successful

**Randomization**

Nearly every experiment performed used a within cell control, comparing conditions with illumination to without, therefore randomization was not applicable. For the all optical stimulation of small groups of neurons (Figures 6-7), all visible and expressing neurons were identified. Groups of eligible candidates of 4-50 were randomly selected using MATLAB. For experiments with multiple trials (different light levels or stimulation frequency), trial order was randomized using MATLAB’s randperm function.

**Blinding**

Investigators were not blinded to group allocation. Nearly every experiment included a within cell control of no stimulus, therefore blinding was not relevant. Between opsin comparison was not possible as many opsins are noticeably different from each other, (nuclear localized, extent of soma targeting, punctate pattern, kinetics, etc...).
### Materials & experimental systems

**Policy information about availability of materials**

**n/a Involved in the study**

- [ ] Unique materials
- [ ] Antibodies
- [ ] Eukaryotic cell lines
- [ ] Research animals
- [ ] Human research participants

**Unique materials**

| Obtaining unique materials | New constructs will be uploaded to Addgene |

**Antibodies**

- **Antibodies used**
  - Monoclonal Anti-FLAG Clone M2 antibody (Catalog # F3165, Lot# SLBQ7119V)

- **Validation**
  - Immunogen sequence DYKDDDDK. The antibody has been validated for use in immunofluorescence studies by the manufacturer and has been used in over 1405 peer-reviewed publications that can be accessed through the manufacturer website. Additionally, primary antibody negative controls were used in this study.

**Eukaryotic cell lines**

**Policy information about cell lines**

- **Cell line source(s)**
  - Chinese hamster ovary (CHO-K1) cells were obtained from the UC Berkeley Cell Culture Facility.

- **Authentication**
  - The CHO-K1 cells were not authenticated during the course of the study independently of standard practices at the UC Berkeley Cell Culture Core Facility.

- **Mycoplasma contamination**
  - Mycoplasma testing was done by the UC Berkeley Cell Culture Facility and cells were routinely replaced with new batches from the facility during the course of the study to maintain low passage numbers. All cell lines tested negative for mycoplasma contamination.

- **Commonly misidentified lines**

  **(See ICLAC register)**

  - No commonly misidentified cell lines were used.

**Research animals**

**Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research**

- **Animals/animal-derived materials**
  - Mice of both sexes were used. All electroporated mice were ICR/CD1 from Jackson Laboratory, cre-lines and CamKII-tTAxTeto-GCaMP6s lines were mixed background between ICR and C57BL6 from Jackson Laboratories. Whole cell recordings were made from mice 14-29 days old and in vivo recordings from 35-100 days old.

### Method-specific reporting

**n/a Involved in the study**

- [ ] ChiP-seq
- [ ] Flow cytometry
- [ ] Magnetic resonance imaging