spontaneous activity. Future work will be important to further refine and test this model, as well as determine how other parameters of neural circuit function, such as intrinsic excitability and inhibitory synaptic strength, respond homeostatically to changing patterns of neural activity and whether this differs from those mechanisms that sense changes in overall firing rate. ❐

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ALL-OPTICAL NEUROPHYSIOLOGY

Sculpting light to reveal brain function

New techniques enable simultaneous optogenetic stimulation and calcium imaging from ensembles of tens of neurons in vivo. Improved opsins are localized to the cell body, minimizing spurious activation of the optically unresolvable neuropil. Two-photon light pulses are sculpted in space, time, and wavelength to efficiently target the desired cells.

Adam E. Cohen and Samouil L. Farhi

Every electrical engineer has on her or his bench a function generator and an oscilloscope: the first to inject test signals into an electrical circuit and the second to record the output at distinct points. Together these tools permit detailed measurement of the input–output properties of subcircuits or individual circuit elements, ultimately revealing the working of the whole. Neuroscientists have used electrodes to attach function generators and oscilloscopes to neural tissue for more than a hundred years, but we still haven't deciphered the input–output properties of most of the brain's circuits. Why? One reason is the immensely more complex wiring of the brain compared to most electrical circuits. Each neuron may connect to thousands of synaptic partners, but stimulating and recording more than a handful of specified cells in vivo is beyond the limits of electrodes.

Optical tools promise to combine the high resolution in space and time needed to interact with many neurons simultaneously, but the optical properties of brain tissue present many challenges. A new study by Mardinly et al.[1] caps off a series of recent advances[2–4] to deliver a powerful optical and molecular toolbox for millisecond resolution stimulation of up to 50 neurons, in vivo. By combining targeted optogenetic actuation with two-photon (2P) Ca^{2+} imaging (Fig. 1), the authors explore how controlling tens of neurons can influence the rest of the circuit—though one must remember that 50 neurons is still a minute fraction of the total population in any functionally distinct brain circuit.

Brain tissue is a Gordian tangle of axons and dendrites, intimately wrapping every cell. Ensuing refractive-index variations cause a photon to deviate gradually from its initial path, so that only a small portion of photons remain on their initial trajectories at depths greater than 50–100 µm. On longer scales, photons ricochet throughout the tissue until they are absorbed, over a path length of a centimeter or so. This mismatch between the short scattering length and the long absorption length is why brain tissue looks whitish. This mismatch also presents a challenge for one-photon fluorescence imaging: the in-focus image is overwhelmed by background from fluorescence photons that have meandered in from elsewhere.

2P scanning microscopy revolutionized functional brain imaging through its ability to form background-free images deep in tissue. 2P excitation occurs at a rate proportional to the square of the intensity, so this effect is localized to a sharp laser focus. The scattered near-infrared input photons never reach intensity sufficient to drive 2P excitation, so there is minimal background. Rapidly scanned 2P laser foci are now routinely used to record Ca^{2+} dynamics from hundreds, and sometimes thousands, of neurons expressing genetically encoded calcium indicators such as GCaMP6.

While 2P Ca^{2+} imaging lacks millisecond time-resolution and lacks sensitivity to subthreshold events, from the perspective of monitoring the overall activity of neuronal ensembles, current tools form a reasonably good 'oscilloscope'.

The function generator problem—modulating specified neurons with high temporal precision—has so far proven far more difficult. Microbial rhodopsin optogenetic actuators—cation channelrhodopsin activators, anion channelrhodopsin inhibitors, and light-powered pumps—in principle provide a means to control the activity of arbitrarily selected subsets of neurons with light. The short scattering length and long absorption length of photons in tissue together present a problem for one-photon targeted actuation: at depths greater than ~100 µm, a substantial portion of photons nominally pointed at a cell will miss their target. Furthermore, even the photons that reach the target may continue meandering through the tissue for up to 1 cm, giving the photon ~1,000-fold more places to interact that are not on the target cell than that are on the target cell. A natural impulse is to use 2P excitation for optogenetic modulation too. Here too, the Gordian tangle of brain tissue conspires to make this challenge harder than it might sound.

First, for activation, the submicron laser focus must precisely hit the nanometers-thick cell membrane. This targeting is

difficult in a living, pulsating brain (in contrast, for Ca^{2+} imaging, the laser focus can land anywhere in the cell body). Second, most microbial rhodopsins have low single-molecule conductance, so activation at a single point along a membrane is typically insufficient to modulate activity. To activate many molecules over an extended region, one must paint, quickly, a tortuous two dimensional surface with a sub-wavelength brush. Third, mistargeting of excitation outside the cell can drive spurious activation of neighboring neurons. A neuron soma contains approximately 60% of the cell volume, but only 2–5% of the cell surface area, a consequence of the extraordinarily high surface-to-volume ratio of the thin axons and dendrites[5]. Even if an optical excitation spot targets the membrane of one cell optimally, this spot will invariably overlap with a larger total surface area of axons and dendrites belonging to other cells, possibly leading to off-target excitation. Together, these geometrical effects conspire to impede 2P activation of a target neuron and contribute to spurious activation of nontarget neurons.

Mardinly et al.[1] combined several technical tricks to increase the precision, parallelism, and time-resolution of optogenetic stimulation (Fig. 1). The first trick was to localize the microbial rhodopsin to the soma membrane, excluding it from the axons and distal dendrites. The C-terminal trafficking motif of the soma-localized potassium channel $K_V2.1$ can be transplanted to microbial rhodopsins to achieve comparable soma localization[6]. This trick both enhances the photocurrent from somatic activation by concentrating the protein locally, and decreases spurious activation of nearby neurites. A different soma-localization sequence, derived from the kainate receptor, was also recently used to control trafficking of the microbial rhodopsin CoChR, leading to development of soma-localized soCoChR[7]. A task for future projects will be to study in detail the physiological and biochemical side-effects of these soma localization strategies.

The second trick was to improve the core rhodopsin by identifying a point-mutant of the channelrhodopsin Chronos[8], termed ChroMe, with high photocurrent and fast kinetics. Soma-targeted ChroME (ST-ChroMe) drove precisely timed action potentials with higher fidelity than even the recently developed high-photocurrent soCoChR. Mardinly et al. also made a soma-targeted chloride channel, ST-GtACR1, to enable targeted optogenetic inhibition. A similar result was recently achieved independently by a different group[3].

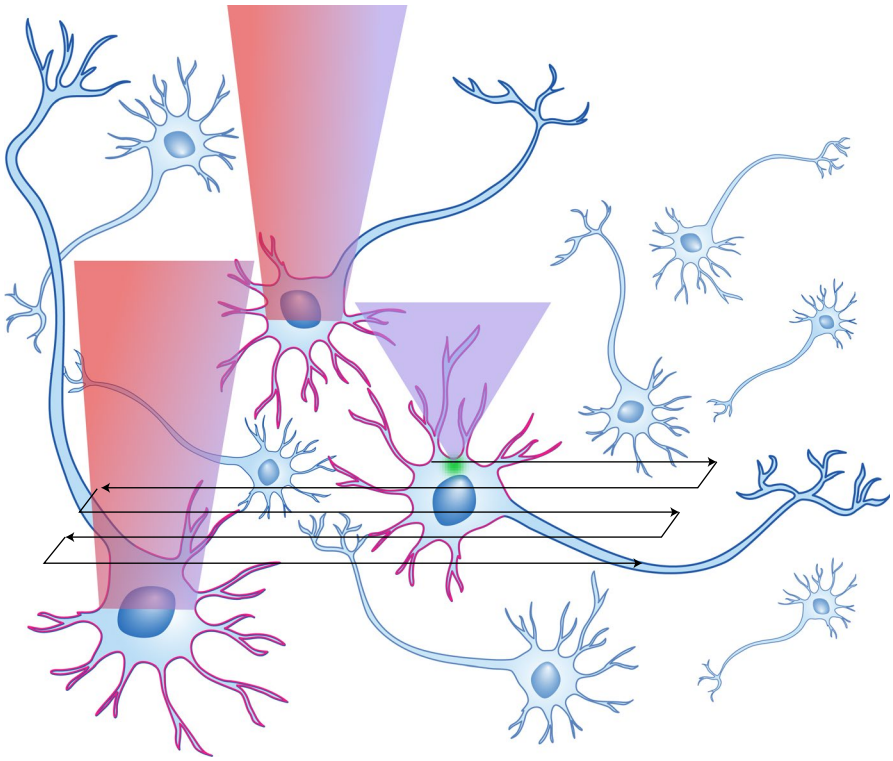

Fig. 1 | Optical control and readout of neural ensembles with 3D-SHOT. Mardinly and colleagues combined protein and optical engineering to develop a system that can optogenetically stimulate up to 50 neurons in parallel, in three dimensions, in vivo (lasers with red to purple gradient). Simultaneous 2P Ca^{2+} imaging reads out the response of the ensemble (purple laser). Magenta outline denotes soma-targeted microbial opsins.

The third, and hardest, trick was to develop the optics to target 2P optogenetic stimulation to arbitrary collections of cells in vivo. Diffractive spatial light modulators can readily spread an illumination beam into complex three-dimensional patterns via a technique called computer generated holography (CGH). However, the nonlinear intensity-dependence of 2P excitation exacts a stiff penalty for splitting up the focus. For a given amount of laser power entering the tissue, it is far more efficient to time-share 2P excitation among multiple sharp points than to split the beam to target multiple points simultaneously. Ultimately, steady-state laser power into the rodent brain is limited to ~200 mW to avoid thermal damage[9].

In the first demonstrations of targeted optogenetic stimulation, a sharp 2P laser focus was rapidly scanned in a spiral pattern on a single cell to sweep as much membrane area as possible[10]. Several groups then used CGH to split the beam into point-like foci targeted to multiple cells. They used galvo mirrors to trace spirals on all cells in parallel. The inability to activate large numbers of channelrhodopsin molecules simultaneously necessitated the use of slowly inactivating channelrhodopsin variants to build up adequate currents. These channelrhodopsins stayed open even after the laser was shut off, however, leading to imprecise control of the number and timing of the spikes[11,12].

In principle CGH could be used to focus multiple points per cell, eliminating the need for galvo scanning. This has been accomplished for <5 cells simultaneously. However, doing so over more cells would dilute the 2P intensity to the point of uselessness. Fortunately, a new laser technology has come to the rescue. Whereas widely used Ti:Sapphire lasers produce laser pulse trains at an 80-MHz repetition rate, Yb-based fiber lasers pack similar time-average power into a much lower repetition rate (1 MHz is typical), with similar pulse width. Thus these fiber lasers pack 80-fold more energy per pulse on a watt-per-watt basis. The lower repetition rate lasers can excite 80-fold more channelrhodopsins in parallel without exceeding the safe power limit, increasing the number of simultaneously stimulated cells from 1 to ~80.

The advent of high peak-energy fiber lasers has spurred a burst of creativity in sculpting light to target multiple cells in parallel. CGH can be used to pack laser foci densely enough to target much of the soma

membrane. However, the overlap of these spots extends the excitation along the axial direction to overflow the cell boundaries. A clever technique, called temporal focusing, broadens the time-course of the laser pulses everywhere but in the focal plane, thereby suppressing out-of-focus 2P excitation. Combinations of CGH and temporal focusing can drop pancake-like excitation spots anywhere in a two dimensional plane[13], but are difficult to achieve in 3D.

Mardinly and colleagues reversed the order of the temporal focusing and CGH optics, leading to a technique they called three-dimensional scanless holographic optogenetics with temporal focusing (3D-SHOT)[14]. The result is that all excitation spots have roughly the same shape, but the spots can be dropped anywhere within a 3D imaging volume. Due to the soma localization of the actuators in the most recent work, a little bit of spillover of the excitation beyond the cell does not produce much crosstalk. Thus, the combination of 3D-SHOT with high-sensitivity soma-localized optogenetic actuators together form a potent tool for targeted optogenetic stimulation.

The last trick from Mardinly et al. is to make it all work together, performing the optogenetic stimulation or inhibition alongside GCaMP6 imaging. The authors carefully avoided spurious optogenetic stimulation with their imaging laser by keeping residence time on any pixel short. They avoided spurious contamination of their GCaMP6 images by the stimulation pulses by interleaving imaging and excitation. The net result was experiments in which up to 50 neurons were stimulated simultaneously and the Ca^{2+} response was monitored throughout a 550 × 550 × 100-µm volume. The authors showed that simultaneous activation of multiple neurons could alter the correlational structure of the dynamics of many hundreds of cells, pointing to future explorations of coding dynamics in large-scale cortical function.

The current efforts in 2P optogenetics in vivo are largely focused on technical demonstrations. The next question is: how will these tools help neuroscience? We suggest three interesting routes to pursue. First, as Mardinly et al. suggest, the tool could be used to explore how different temporal patterns of activation of the same set of neurons can affect overall network dynamics and ultimately behavior. The influence of the correlational structure of neural activity has been a topic of longstanding interest, largely inaccessible to prior techniques. Second, the tool could be used to explore the rules of spike timing-dependent plasticity. By evoking repetitive paired activation of distinct neural ensembles, one could explore how the network dynamics evolve as a function of the relative timing of the activation. Third, one could use the technique to map functional connectivity, to see how activation (or inhibition) of a given cell affects the behavior of the neighbors. Unfortunately, Ca^{2+} imaging is an imperfect tool for this goal because it is largely insensitive to subthreshold dynamics. Furthermore, the time resolution of Ca^{2+} imaging is typically insufficient to distinguish monosynaptic from network-level connections. One way forward might be through the use of genetically encoded voltage indicators.

A recent demonstration of simultaneous optogenetic stimulation and voltage imaging in vivo opens the possibility of probing functional connections directly, though the sub-millivolt precision needed to detect monosynaptic connections remains a challenge[15]. Other fluorescent reporters may also reveal subthreshold couplings, such as recently reported glutamate reporters or Ca^{2+} indicators targeted to postsynaptic spines. These tools would provide broadly distributed maps of synaptic strength, though tracing those signals back to specific postsynaptic neurons might be difficult unless the expression is very sparse.

Despite the impressive increases in 2P excitation efficiency reported by Mardinly et al., 2P stimulation remains a horrendously inefficient use of optical power. Typical 2P excitation powers were in the range of 20–60 mW per cell, corresponding to a mean light intensity of 20–60 kW/cm^2. To stimulate 50 cells in parallel requires ~3 W of laser power, well beyond the 200 mW steady-state limit. Mardinly et al. performed all their measurements with a low duty cycle to avoid overheating the brain. For comparison, one-photon excitation of most channelrhodopsins saturates at less than 1 W/cm^2. Thus while it is easy to imagine simultaneous one-photon stimulation of hundreds of thousands of cells, extending 2P stimulation beyond ~50 simultaneous cells will require conceptual advances in optical design or improved understanding of microbial rhodopsin spectroscopy (or both!).

By combining perturbation and readout, function generators and oscilloscopes can establish causal connections in a circuit, where these connections would be difficult or impossible to discern from passive observation alone. With the new generation of molecular and optical tools, neuroscientists are gaining access to an early and rudimentary workbench. Through combined molecular and optical efforts, this toolkit will undoubtedly expand in the years ahead. ❐

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Competing interests

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